Regulation of inflammation in uveal melanoma

Souri, Z.

Regulation of Inflammation in Uveal Melanoma

Regulation of Inflammation in Uveal Melanoma

Thesis, Leiden University Medical Center, The Netherlands

Cover design: Stefanie van den Herik

Layout: Zahra Souri

Printed by: ProefschriftMaken

ISBN/EAN: 978-94-6423-342-1

Financial support

This thesis was made possible by the contribution of the Iranian Ministry of Science Research and Technology, Grant UL 2011-4991 from the Dutch Cancer Society (KWF), Stichting Leids Oogheelkundig Ondersteunings Fonds LOOF, a Horizon 2020 grant from the European Community, CURE UM, nr. 667787, Nelly Reef Fund and Ms I. Brouwer.

Regulation of Inflammation in Uveal Melanoma

Proefschrift

Ter verkrijging van
de graad van doctor aan de Universiteit Leiden,
op gezag van rector magnificus prof.dr.ir. H. Bijl,
volgens besluit van het college voor promoties
te verdedigen op dinsdag 14 september 2021
klokke 13:45 uur

door

Zahra Souri

زهرا سوری

geboren te Kermanshah, Iran

Promotor

Prof. dr. M.J. Jager

Co-Promotor

Dr. A.G. Jochemsen

Promotiecommissie

Prof. dr. G.P.M. Luyten

Prof. dr. M. Elas, Department of Biochemistry, Jagellonian University, Krakow, Poland

Prof. dr. B. Esmaeli, Department of Plastic Surgery, M.D. Anderson Cancer Center, Houston, Texas, USA

Dr. E. Kiliç, Department of Ophthalmology, Erasmus Medical Center, Rotterdam

Dr. F. Pakdel, Department of Ophthalmology, Tehran University, Tehran, Iran

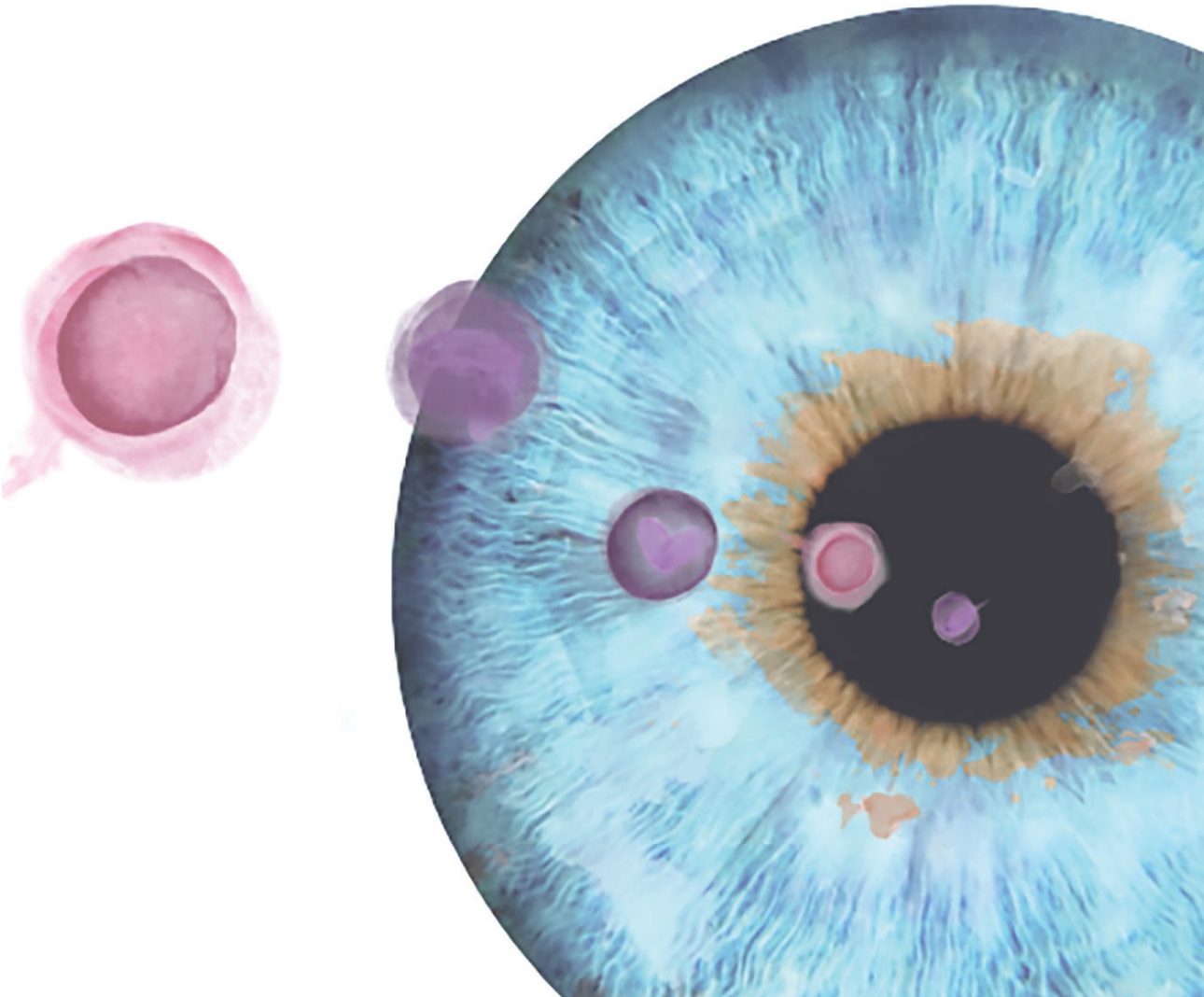
I would like to dedicate my thesis to

my father Manouchehr and my mother Katrin

Table of Contents

Chapter 1

Introduction and outline of thesis



Introduction

Uveal melanoma

Uveal melanoma (UM) is the most common intraocular malignancy in adults. This disease mainly occurs in Caucasians with a light skin, blond hair and blue eyes and has an incidence rate of 7 per million per year (ranges from 4 to 11) in northern Europe and America (Metzelaar-Blok 2001, Virgili 2007, Singh 2003, Singh 2011, Houtzagers 2020). The mean age at diagnosis is 60 years (Weis 2006, Singh 2011). Symptoms include blurred, distorted vision and/or pain in the eye, although approximately one third of patients have been reported as asymptomatic (Damato 2012). The delayed detection causes the patient to carry a more advanced tumour which unfortunately increases the chance of losing the eye (Dogrusoz 2017).

Treatment options for the primary tumour which aim to preserve the eye include plaque brachytherapy, proton beam therapy and stereotactic radiation (Jager 2020), but if the treatment does not show any benefit mostly due to large tumour size, then removal of the globe is advised.

Up to 50% of the patients develop metastases mostly targeting the liver; once detected, the median survival is approximately 4-12 months (Augsburger 2008). As there are no curative treatment options available for the metastases in UM, no improvement in survival has been observed during the last fifty years (Roelofsen 2021). The tumour arises from the uveal tract and involves the pigmented tissues, including the iris, ciliary body and the choroid (Figure 1). 86 percent of the cases arise from the choroid (Mclaughlin 2005). For prognosis, the best location is the iris, mainly because of early detection and small tumour size (Shields 2009) while the worst is known to be the ciliary body, which has the highest chance of metastasis because of a larger tumour size (Jager 2020).

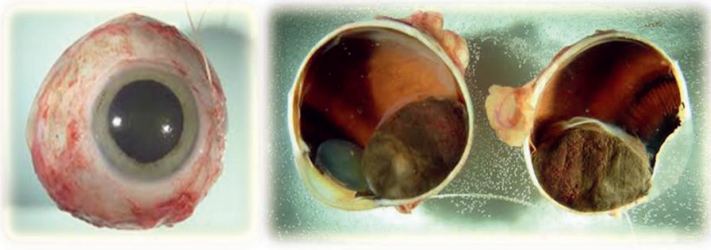


Figure 1: An eye involved with choroidal melanoma

UM are mostly composed of two types of cells, epithelioid and spindle cells. Epithelioid cells are large with large nuclei and round nucleoli located inside a high amount of cytoplasm while spindle cells are small with a tight extracellular space, small nuclei and nucleoli and less cytoplasm (Myamoto 2012). Tumours with an epithelioid cell type are often associated with metastasis (Callender 1931). Tumour basal diameter and thickness are other characteristics known to be associated with a higher melanoma-related mortality (Kujala 2003) and increased risk for metastasis (Shields 2009).

Another way of classification is the Tumour Node Metastasis (TNM) staging, which is based on tumour size, involvement of the ciliary body and extrascleral extension (Edge 2010). This is now known as the American Joint Committee on Cancer (AJCC) classification (Kivela 2013).

Studies have reported that older age at diagnosis is associated with worse prognosis in UM, exerting the highest metastatic risk for ages above 60 years old (Shields 2012). Gender is not highly associated although a study reported that the tendency toward development of melanoma-related metastasis is higher in males compared to females (Zloto 2013).

Uveal melanoma chromosome aberrations

Different genetic abnormalities are involved in the creation of this rare and invasive ocular malignancy. The different chromosomal aberrations which are mostly observed include loss of chromosome 1p, 3, 6q and gain in 6p or 8q (Prescher 1992, White 1998, Kilic 2005, Damato 2009, Dogrosuz 2018, Shields 2017). Loss of one copy of chromosome 3 (M3) is the most reliable biomarker for patient survival. M3 is considered as an early copy number change in UM and has been associated with different histopathological characteristics such as largest basal tumor diameter (Kiliç 2006) and epithelioid cell type (Scholes 2003).

Other chromosome abnormalities enhancing the disease include extra copies of chromosome 8q. Chromosome 8q has been known as an important location responsible for the attraction of macrophages to the eye which could contribute to the development of metastasis in UM (De Lange 2015, Versluis 2015) and often coincides with M3 (Sisley 1997). The combination of loss of chromosome 3 and gain in 8q strengthens the progression of this disease and increases the risk of developing metastasis (Cassoux 2014, Versluis 2015, Gezgin 2017).

Uveal melanoma somatic mutations

Somatic genetic mutations play a major role in the development of uveal melanoma. Two very early somatic mutations give the melanocyte the ability to transform into a uveal nevus: these are GNAQ or GNA11 gene mutations (Vader 2017). These mutations are very common in UM (Van Raamsdonk 2009, van Raamsdonk 2010; de Lange 2015; Piaggio 2019). GNAQ and GNA11 switch on G protein signaling by activating inositol-3-phosphate, diacylglycerol, and cyclic AMP and then activate a set of signaling pathways important for cell growth and proliferation (O'Hayre 2014).

Mutations in the BRCA1 associated protein-1 (BAP1) gene may subsequently occur. The BAP1 gene is located on chromosome 3p21.1 and codes for a ubiquitin carboxy-terminal hydrolase (UCH). Like in other cancers (Jensen 1998), BAP1 acts as a tumour suppressor in UM as well (Harbour 2010), and lack of expression is directly associated with the development of metastasis (Harbour 2010, van Essen 2014, Koopmans 2014).

UM with a high risk of metastases have lower level of BAP1 expression compared to low risk tumours (Harbour 2010, Robertson 2017). Loss of BAP1 expression leads to the attraction of lymphocytes inside the eye, which increases the local inflammation (Gezgin 2017).

Uveal melanoma Immunology

The Immune-privileged eye

Immune-privilege is an adaptive mechanism that occurs in certain organs in order to avoid damages by the immune system. One such organ is the eye, where many cells lack regenerative activity. Suppressing inflammation is important to maintain visual clarity and retinal function. To be able to reach these goals the eye uses a set of different mechanisms: the internal part of the globe is filled with liquids namely the aqueous humor and vitreous fluid, which contain immune suppressive factors such as neuropeptides, αMSH (melanocyte stimulating hormone) (Stein 2007), cytokines such as TGFβ-2, complement inhibitors and macrophage inhibitory factor (MIF) (Apte 1998).

Another mechanism for immunosuppression in the eye is the ability of some local cells such as corneal endothelial cells to transform active T cells into regulatory T cells (Treg cells), thus lowering their cytotoxic activity (Yamada 2010). In the back of the eye, retinal pigment epithelial (RPE) cells form tight junctions and limit the local entrance of leukocytes into the eye

(Sugita 2009). Pigmented epithelial (PE) cells of the iris also inhibit T cell activity through CTLA-4 binding (Sugita 2003) while retinal and ciliary body PE cells suppress T cell activity by producing TGF-β1/2 (Sugita 2006).

Antigens present in the anterior chamber of murine eyes showed ability to escape from the orbit and activate the host immune system; however, the anterior chamber route led to an inhibited immune response to the intraocular antigens, and helped to preserve retinal function; this phenomenon is known as the anterior chamber associated immune deviation (ACAID) (Streilein 1996).

The inflammatory phenotype in UM

Inflammation is known as the seventh hallmark of cancer (Collota 2009). Based on the type of involved immune cells, inflammation can either act as a growth initiator or inhibitor in a malignancy. In cancers such as cutaneous melanoma, breast carcinoma or non-Hodgkin lymphoma, it has been fully accepted that the recruitment of immune cells into the tumour loci is beneficiary for the patient's overall survival (Mlecnik 2011) while this is not the case for UM.

In UM, immune cells are often recruited to the tumour site. This was already found in early studies: infiltrating lymphocytes (CD3, CD4, and CD8) and macrophages (CD11, CD68, and CD163) were described (De la Cruz 1990, Makitie 2001, Meecham 1992, Tobal 1993, De Waard-Siebinga 1996).

Extensive infiltration of lymphocytes and macrophages is associated with poor prognosis in UM (De Lange 2015, Robertson 2017). The accumulation of these cells is positively associated with loss of one copy of chromosome 3 and high levels of expression of HLA Class I and Class II (Maat 2008; de Waard-Siebinga 1996, Jager 2002).

The high number of infiltrating leukocytes and high expression of HLA forms the inflammatory phenotype which is a strong marker for the development of metastasis and negatively impacts survival (Maat 2008, Bronkhorst 2011, and Gezgin 2017).

NK cells in UM

NK cells belong to the innate immune system and are present in UM in a low percentage (Ksander 1991). These cells are recognized by the presence of CD56 marker and absence of CD3. Because of the presence of two strong inhibitors TGF-β and MIF, inside the aqueous humor, NK cells are not able to take any action and are kept inactive in the eye (Apte 1996).

NK-cell mediated lysis is inversely correlated with the level of HLA Class I expression on tumour cells (Ma 1995). Normal eye tissues express low levels of HLA (Niederkorn 2012), making them a target for NK cells. The high level of expression of HLA Class I in most high risk UM provides these tumour cells with an escape mechanism that allows spread through the bloodstream to distant organs such as the liver.

Macrophages in UM

Macrophages are components of the innate immune system involved in host defense mechanisms and are derived from differentiated circulating monocytes. These cells can become activated by Th1 and Th2 cells; based on the type of cytokines they receive, they become polarized into two different subsets of cells, namely M1 and M2 with different programs (Mills 2000). These two types of macrophages act in opposite ways.

The M1 macrophages are also known as classical macrophages and are initiators of anti-tumour responses; they express high levels of HLA Class II. M1 macrophages are mainly involved in antibacterial and anti-angiogenic functions and are able to produce nitric oxide (NO), IL-12 and TNF.

The M2 subtype has a low anti-tumour activity and has a low HLA Class II expression. M2 macrophages are involved in anti-inflammatory and proangiogenic activity and mainly produce IL-10, L-1ra, and type II IL-1 decoy receptor (Mills 2000, Mantovani 2002).

One important underlying mechanism for the recruitment of macrophages towards the tumour site is the presence of inflammatory chemoattractants; CCL2, CXCL12, CXCL8, CXCL1, CXCL13, CCL5 (Nesbit 2002, Azenshtein 2002) attract macrophages and thereby further induce inflammation and angiogenesis at the tumour site. The presence of macrophages has been reported to be positively correlated with the presence of T cells in UM (Bronkhorst 2012, De Lange 2018). The most dominant macrophages in UM are the $CD68^{+}CD163^{+}$ M2 subtype. A high density of these cells is strongly associated with M3 in UM (Bronkhorst 2011). Macrophage density is positively associated to risk factors in uveal melanoma. High numbers of macrophages correlate with more epithelioid cells, high pigmentation and a high vascular density in the tumour area. Moreover, macrophage infiltration correlates with higher rate of metastasis and a shorted survival in UM patients (Makitie 2001).

T cells in UM

T lymphocytes belong to the adaptive immune response. These cells are derived from hematopoietic stem cells in the bone marrow and become mature and differentiate into subtypes inside the thymus: CD8 cytotoxic T cells, CD4 helper cells (Th1, Th2, and Th17) and regulatory T cells. CD8 T cells are the main cells in eliminating invaders with cytotoxic activity. These cells recognize foreign peptides as presented by HLA Class I using their TCR receptor and, following secretion of cytotoxic cytokines, recruit other immune cells in order to kill the pathogens. CD4 helper cells act to mount an immune response indirectly through the secretion of inflammatory factors and are even able to suppress immune responses (Biswas 2010). The regulatory T cells are important in controlling how the immune system acts in response to self-antigens and therefore are often considered as suppressor T cells able to protect self-cells from autoimmune reactions.

The presence of T cells has different roles in different cancers (Yu 2006). For example, in cutaneous melanoma, the presence of T cells improves survival (Mihm 2015); by contrast, T cell infiltration contributes to a worse survival in UM (de la Cruz PO 1990, Whelchel 1993). One reasons behind the aforementioned phenomenon is the presence of Tregs in UM tumours (Lagouros 2009, Bronkhorst 2012). The tumour is able to induce a set of Treg cells (CD4) which may suppress local anti tumoural activity.

HLA Expression in UM

HLA expression is an important inflammatory marker in UM. Based on their structure and type of interaction, HLA molecules are categorized into two types.

HLA Class I molecules consist of classic HLA-A, -B and -C and non-classic HLA-E, -L, -J, -K, -H, and –G subtypes (Goldsby 2003). Classic HLA Class I are expressed on almost all nucleated cells and present antigens to T cell receptors (TCR) of CD8 T cells (Szeto 2021). They are made of one heavy polypeptide chain which is non-covalently associated with β2-microglobulin (β2-m).

The second type, HLA Class II, includes HLA-DP, DM, DO, DQ and –DR (Goldsby 2003) which are only expressed on specific immune cells such as antigen presenting cells (monocytes, macrophages and dendritic cells), thymic epithelium cells and activated T cells. These molecules bind to CD4 T cells and can start immune responses against foreign peptides (Roche 2015).

The natural expectation is that tumour cells decrease their surface HLA expression in order to avoid recognition by cytotoxic T cells (Garrido 1993), whereas the opposite occurs in UM (Jager 2002): as tumour spread from the eye takes place, tumour cells might encounter NK cells present in the blood which target cells with a low HLA Class I expression (Ma 1995). The finding that higher levels of HLA Class I, HLA Class II and β2-microglobulin expression are associated with a lower survival supports this idea (Blom 1997; Erisson 2001).

On the other hand, a high expression of HLA Class I antigens may allow an effective T cell response once metastases have reached the liver. One reason for ineffective local T cell responses might be the loss of expression of specific alleles, thereby decreasing the sensitivity to cytotoxic T cell-mediated lysis, even when high levels of other alleles are present. HLA allotype loss is another strategy which occurs in both HLA-A and HLA-B and might exhibit as haplotype loss or complete loss (Hurks 2000, Anastassiou 2003). An analysis of a series of cell lines showed deficiencies of several alleles. Even after exposure of the cell lines to inflammatory cytokines such as IFNγ and IFNα, some alleles did not show expression (de Waard-Siebinga 1995).

Potential inflammatory regulators in UM

As already mentioned, the presence of an inflammatory phenotype is linked to specific chromosome copy number aberrations, especially with monosomy 3/loss of BAP1 expression (Maat 2008, Bronkhorst 2012, Robertson 2017, Gezgin 2017). The inflammatory phenotype is characterized by an increased density of infiltrating lymphocytes and macrophages, and an increased expression of HLA Class I and II antigens. We set out to study which pathways may play a role in the regulation of this inflammatory phenotype. We looked at the NFkB pathway, epigenetic regulators such as miRNAs and HDACs, and at the potential influence of immune checkpoint molecules.

NFkB pathway as a mediator between inflammation and cancer

An important signaling pathway which regulates the immune system, involved in inflammatory processes is the NFkB pathway. After activation, this pathway ultimately leads to the production of inflammatory cytokines and regulates immune cell activation (Lawrence 2009, Oh 2013). Multiple studies have reported the upregulation of the NFkB pathway in several type of cancers giving it a role in uncontrolled proliferation, angiogenesis, metastasis and resistance to different therapies (Pires 2018). It has been reported that the NFkB pathway is involved in the progression of uveal melanoma (Mier 2007, Dror 2009). Until now no studies have looked at the relationship between this pathway and the inflammatory phenotype of UM.

Epigenetics and regulation of inflammation

Epigenetics is the phenomenon which regulates gene expression without altering the gene sequence. One approach is through methylation of promoters, which represses transcription. Another set of epigenetic enzymes which alter gene expression in malignancies are histone deacetylases (HDAC). These enzymes repress gene expression by the removal of acetyl groups from the histone tails, compacting the chromatin structure and making the promoter unreachable. HDACs are often overexpressed in tumours which potentially enhance the proliferation and development of cancer cells by repressing the expression of cell cycle inhibitors (Halkidou 2004, Song 2005).

MiRNAs as regulators of inflammation

A group of non-translated molecules referred to as miRNAs are involved in the epigenetic regulation of gene expression. These short RNAs are often 17-22 base pairs long and bind to specific mRNA based on complementary sequencing. By binding to 3´UTR regions of complimentary mRNAs, they either stimulate or delay translation of specific transcribed genes (Wahid, 2001). Recent studies have found roles for miRNAs in the invasive behavior of tumour cells. MiRNAs could target oncogenes, tumour suppressors, modulate stem cell characteristics, alter epithelial mesenchymal transition ability, influence cells residing in the tumour microenvironment and therefore play a role in the development of metastasis (as reviewed by Kim 2019).

Microarray studies on different types of tumours indicate that in addition to a tumour suppressive role, miRNAs could also act as oncogenes and halt tumour suppressor genes at a post-transcriptional level; for example the effect of mir-106a on Rb in colon cancer and mir-20a on TGFBR2 in lung cancer (Volinia 2006). In addition, inflammation could also affect miRNA expression in cancers (Wu 2008)

Different studies report different miRNAs to be either up or downregulated in high risk UM tumours (Worley 2008, Smit 2019, Augton 2020) but none of them have studied the involvement of miRNAs in the inflammatory processes of this disease.

Immune checkpoints as Immune modulators

The most abundant immune cells present in the UM tumour niche are T cells; while these cells form extensive colonies around the malignant cells, they are mostly not effective in destroying them and are considered “exhausted” (Niederkorn 2009). The exact reason for this phenomenon is still unknown but suggests a possible role for immune checkpoints.

Well known immune checkpoints are CTLA-4 and PD-L1. CTLA-4 is one of the checkpoints expressed on effector and regulatory T cells which is very similar to CD28 and therefore is able to bind to CD80/86 with higher affinity and hence reduce its stimulatory effect (Algere 2001).

PD-1, which is mainly expressed on T cells and B cells. PD-1 binds to its ligands PD-L1 and PD-L2, which are expressed on the cell surface of tumour cells (Topalian 2012).

Despite several attempts toward the use of checkpoint therapy in UM, most have not been successful enough to serve as an appropriate adjuvant therapeutic approach for the treatment of UM metastasis (Rodrigues 2019); recently, research pointed to another immune checkpoint, Lymphocyte Activation Gene-3 (LAG3) (Durante 2020).

LAG3 is naturally present on the surface of T and NK cells and is relevant for the prevention of auto-immunity (Triebel 1990). The canonical ligand for LAG3 is HLA Class II. HLA Class II is believed to be associated with the development of metastasis in UM (Krishnakumar 2003). In 1988 Jager et al reported that HLA-DQ was associated with infiltration in UM (Jager 1988).

Taken together it seems that the high LAG3 /HLA Class II expression detected in UM tumours might serve as an appropriate mechanism for reviving the cytotoxic anti-tumour ability of CD8 cells.

Outline of thesis

As HLA Class I expression is an important target for cytotoxic T cells but an in inhibitor of NK cells, we were interested in the regulation of its expression. It is furthermore a prognostic marker, associated with loss of one copy of chromosome 3 and loss of BAP1 expression. In **chapter 2,** we review HLA expression in UM, how it is involved in the inflammatory phenotype, how it is regulated and how putative treatments might be effective in its expression. In addition, we report results of experiments with regard to HLA Class I expression in UM cell lines: we show that allelic defects are present in the cell lines even after IFNɤ stimulation. We report how different drugs impact HLA Class I expression both in a negative and positive manner.

In **chapter 3,** we investigate the potential role of the NFkB pathway in the regulation of inflammation in UM and its potential association with HLA Class I expression. We wonder whether the expression of NFkB components are related to the tumours chromosome 3/BAP1 status. We report that members of both canonical and non-canonical NFkB pathway are related to

high amounts of infiltrating T cells and macrophages and also show an association with loss of chromosome3/BAP1 in UM.

In order to increase our understanding for the reason behind the elevated HLA Class I expression in UM tumours, we investigate the involvement of epigenetics in **chapter 4**. We focus on a set of epigenetic enzymes called histone deacetylases and report that these regulators are highly expressed in Monosomy 3 UM. Moreover we show that the chemotherapeutic HDAC inhibitor, Quisinostat, is not only able to inhibit cell growth in-vitro but further increases HLA Class I at both the mRNA and protein level and hence might impact immunotherapeutic approaches when used as adjuvant therapy.

In **chapter 5** we wonder whether HDAC expression is influenced by the presence of infiltrating lymphocytes and macrophages. We find that HDAC expression is positively correlated with the presence of infiltrating immune cells; we show that HDACs are induced by IFNɣ and therefore suggest a role for infiltrating cells in the regulation of HDAC enzymes.

In **chapter 6** we focus on miRNA's as another set of epigenetic regulators of inflammation. We investigate the potential relation of a set of 125 miRNA's with HLA Class I expression and the presence of an infiltrate in UM and report two patterns of miRNA expression.

In **chapter 7** we study the LAG3 immune checkpoint in UM tumours. As immune checkpoints might be responsible for the T cell exhaustion which is observed in UM, we investigate the involvement of LAG in prognostication and study how LAG3 and its ligands are distributed among different UM tumours in order to increase knowledge towards the design of new adjuvant therapy for the treatment of UM metastasis.

Finally in **chapter 8,** we summarize the different chapters and report our general conclusion based on what we have understood from the findings of this thesis.

References

1. Metzelaar-Blok, J.A.W.; Ter Huurne, J.A.C.; Hurks, H.M.H.; et al. Characterization of Melanocortin-1 Receptor gene variants in uveal melanoma patients. Invest. Ophthalmol. Vis. Sci. 2001, 42, 1951-1954.
2. Virgili, G.; Gatta, G.; Ciccolallo, L.; et al. EUROCARE Working Group. Incidence of uveal melanoma in Europe. Ophthalmology. 2007, 114 (12), 2309-2315.
3. Singh, A.D.; Topham, A. Incidence of uveal melanoma in the United States: 1973–1997. Ophthalmology. 2003, 110(5), 956-961.
4. Singh, A.D.; Turell, M.E.; Topham, A.K. Uveal melanoma: trends in incidence, treatment, and survival. Ophthalmology 2011, 118(9), 1881-1885.
5. Houtzagers, L.E.; Ruys, A.; Wierenga, A.P.A.; et al. The relation between iris colour and the development of uveal melanoma. Int J Molecular Sciences 2020, 21(19), 7172.
6. Weis, E.; Shah, C.P.; Lajous, M.; Shields, J.A.; Shields, C.L. The association between host susceptibility factorsand uveal melanoma: A meta-analysis.Arch. Ophthalmol. 2006,124, 54–60.
7. Damato, E.M.; Damato, B.E. Detection and time to treatment of uveal melanoma in the United Kingdom: an evaluation of 2384 patients. Ophthalmology. 2012, 119(8), 1582-1589.
8. Dogrusöz, M.; Jager, M.J.; Damato, B. Uveal melanoma treatment and prognostication. Asia Pac. J. Ophthalmol.2017,6, 186–196.
9. Jager, M.J.; Shields, C.L.; Cebulla, C.M.; et al. Uveal melanoma. Nat. Rev. Dis. Prim. 2020, 6(1), 1-25.
10. Augsburger, J.J.; Corrêa, Z.M.; Shaikh, A.H. Quality of evidence about effectiveness of treatments for metastatic uveal melanoma. Trans Am Ophthalmol Soc. 2008, 106, 128-137.
11. Roelofsen, C.D.M.; Wierenga, A.P.A.; van Duinen, S.; et al. Five Decades of Enucleations for Uveal Melanoma in One Center: More Tumors with High Risk Factors, No Improvement in Survival over Time. Ocul Oncol Pathol. 2021, 7(2), 133-141.
12. McLaughlin, C.C.; Wu, X.C.; Jemal, A.; et al. Incidence of noncutaneous melanomas in the US. Cancer: Interdisciplinary International Journal of the American Cancer Society. 2005, 103(5), 1000-1007.
13. Shields, C.L.; Kaliki, S.; Furuta, M.; et al. Clinical spectrum and prognosis of uveal melanoma based on age at presentation in 8,033 cases. Retina 2012, 32, 1363–1372.
14. Miyamoto, C.; Balazsi, M.; Bakalian, S.; et al. Uveal melanoma: Ocular and systemic disease. Saud. J. Ophthalm. 2012, 26(2), 145-149.

15. Callender, G. R. Malignant melanotic tumors of the eye: a study of histologic types in 111 cases. Trans. Am. Acad. 1931, Ophthalmol. Otolaryngol. 36, 131–140.
16. Kujala, E.; Mäkitie, T.; Kivelä, T. Very long-term prognosis of patients with malignant uveal melanoma. Invest Ophthalmol Vis Sci 2003, 44, 4651–4659.
17. Shields, C.L.; Furuta, M.; Thangappan, A.; et al. Metastasis of uveal melanoma millimeter-by-millimeter in 8033 consecutive eyes. Arch Ophthalmol 2009, 127,989–998.
18. Edge, S.B.; Compton, C.C. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Annals of surgical oncology. 2010, 17(6), 1471-1474.
19. Kivelä, T.; Kujala, E. Prognostication in eye cancer: the latest tumor, node, metastasis classification and beyond. Eye. 2013, 27(2), 243-252.
20. Zloto, O.; Pe'er, J.; Frenkel, S. Gender differences in clinical presentation and prognosis of uveal melanoma. Invest Ophthalmol Vis Sci 2013, 54, 652–656.
21. Prescher, G.; Bornfeld, N.; Horsthemke, B.; et al. Chromosomal aberrations defining uveal melanoma of poor prognosis. Lancet. 1992, 339, 691–692.
22. White, V. A.; Chambers, J. D.; Courtright, P. D.; et al. Correlation of cytogenetic abnormalities with the outcome of patients with uveal melanoma. Cancer. 1998, 83, 354-359.
23. Kilic, E.; Naus, N.C.; van Gils, W.; et al. Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients. Invest Ophthalmol Vis Sci. 2005, 46(7), 2253-2257.
24. Damato, B.; Coupland, S.E. Translating uveal melanoma cytogenetics into clinical care.Arch. Ophthalmol 2009,127, 423–429.
25. Dogrusöz, M.; Jager, M.J. Genetic prognostication in uveal melanoma. Acta Ophthalmol 2018, 96, 331–347.
26. Shields, C.L.; Say, E.A.; Hasanreisoglu, M.; et al. Cytogenetic abnormalities in uveal melanoma basedon tumor features and size in 1059 patients: The 2016 W. Richard Green Lecture.Ophthalmology. 2017,124, 609–618.
27. Kilic, E.; van Gils, W.; Lodder, E.; et al. Clinical and cytogenetic analyses in uveal melanoma. Investig. Ophthalmol. Vis. Sci 2006, 47, 3703–3707.
28. Scholes, A.G.; Damato, B.E.; Nunn, J.; et al. Monosomy 3 in uveal melanoma: correlation with clinical and histologic predictors of survival. Invest Ophthalmol Vis Sci. 2003, 44, 1008-1011.

29. De Lange, M.J.; van Pelt, S.I.; Versluis, M.; et al. Heterogeneity revealed by integrated genomic analysis uncovers a molecular switch in malignant uveal melanoma. Oncotarget. 2015, 6(35), 37824-37835.
30. Versluis, M.; de Lange, M.J.; van Pelt, S.I., et al. Digital PCR validates 8q dosage as prognostic tool in uveal melanoma. PLoS One. 2015, 10(3):e0116371.
31. Sisley, K.; Rennie, I.G.; Parsons, M.A.; et al. Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis. Genes Chromosomes Cancer. 1997, 19(1), 22-8.
32. Cassoux, N.; Rodrigues, M.J.; Plancher, C.; et al. Genome-wide profiling is a clinically relevant and affordable prognostic test in posterior uveal melanoma. Br. J. Ophthal. 2014, 98(6), 769-774.
33. Gezgin, G.; Dogrusöz. M.; van Essen, T.H.; et al. Genetic evolution of uveal melanoma guides the development of an inflammatory microenvironment. Can. Immunol. Immunother. 2017, 66(7), 903-912.
34. Vader, M.J.C.; Madigan, M.C.; Versluis, M.; et al. GNAQ and GNA11 mutations and downstream YAP activation in choroidal nevi. Br. J. Cancer. 2017, 117(6), 884-887.
35. Van Raamsdonk, C.D.; Bezrookove, V.; Green Get Bauer, J.; et al. Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. Nature 2009, 457, 599–602.
36. Van Raamsdonk, C.D.; Griewank, K.G.; Crosby, M.B.; et al. Mutations in GNA11 in uveal melanoma. NEJM. 2010, 363(23), 2191-2199.
37. De Lange, M.J.; Razzaq, L.; Versluis, M.; et al. Distribution of GNAQ and GNA11 mutation signatures in uveal melanoma points to a light dependent mutation mechanism. PloS one. 2015, 10(9), e0138002.
38. Piaggio, F.; Tozzo, V.; Bernardi, C.; et al. Secondary Somatic Mutations in G-Protein-Related Pathways and Mutation Signatures in Uveal Melanoma. Cancers (Basel). 2019, 11(11), 1688.
39. O'Hayre, M.; Degese, M.S.; Gutkind, J.S. Novel insights into G protein and G protein-coupled receptor signaling in cancer. Curr Opin Cell Biol. 2014, 27, 126-35. doi: 10.1016/j.ceb.2014.01.005.
40. Jensen, D.E.; Proctor, M.; Marquis, S.T.; et al. BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression.Oncogene. 1998 Mar 5; 16(9):1097-112.
41. Harbour, J.W.; Onken, M.D.; Roberson, E.D.; et al. Frequent mutation of BAP1 in metastasizing uveal melanomas. Science. 2010, 330(6009), 1410-1413.

42. Van Essen, T.H.; van Pelt, S.I.; Versluis, M.; et al. Prognostic parameters in Uveal Melanoma and their association with BAP1 expression. Br J Ophthalmol, 2014, 98 (12), 1738-1743.
43. Koopmans, A.E.; Verdijk, R.M.; Brouwer, R.W.; et al. Clinical significance of immunohistochemistry for detection of BAP1 mutations in uveal melanoma. Modern pathology. 2014, 27(10), 1321-1330.
44. Robertson, A.G.; Shih, J.; Yau, C.; et al. Integrative analysis identifies four molecular and clinical subsets in uveal melanoma. Cancer Cell 2017, 32, 204-220
45. Stein-Streilein, J; Taylor, A.W. An eye's view of T regulatory cells. J. Leukoc. Biol 2007 81, 593-598.
46. Apte, R.S.; Sinha, D.; Mayhew, E.; et al. Cutting edge: role of macrophage migration inhibitory factor in inhibiting NK cell activity and preserving immune privilege. J. Immunol 1998 160(12).5693-5696.
47. Yamada, Y.; Sugita, S.; Horie S.; et al. Mechanisms of immune suppression for CD8+ T cells by human corneal endothelial cells via membrane-bound TGFβ. IOVS 2010, 51(5). 2548–2557.
48. Sugita, S. Role of ocular pigment epithelial cells in immune privilege. Archivum immunologiae et therapiae experimentalis. 2009, 57(4), 263-268.
49. Sugita S., Streilein J. W. Iris pigment epithelium expressing CD86 (B7-2) directly suppresses T cell activation in vitro via binding to cytotoxic T lymphocyte-associated antigen 4. Journal of Experimental Medicine. 2003, 198(1):161–171.
50. Sugita S., Futagami Y., Smith S. B., Naggar H., Mochizuki M. Retinal and ciliary body pigment epithelium suppress activation of T lymphocytes via transforming growth factor beta. Exp. Eye Res 2006, 83(6), 1459–1471.
51. Streilein, J.W. Ocular immune privilege and the Faustian dilemma. The Proctor lecture. Investigative ophthalmology & visual science. 1996, 37(10), 1940-1950.
52. Colotta, F.; Allavena, P.; Sica, A.; et al. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis. 2009, 30(7), 1073-1081.
53. Mlecnik, B.; Bindea, G.; Pagès, F.; et al. Tumor immunosurveillance in human cancers. Cancer Metastasis Rev. 2011, 30(1), 5-12.
54. De Cruz, J.r. P.O.; Specht, C.S.; McLean, I.W. Lymphocytic infiltration in uveal malignant melanoma. Cancer. 1990, 65(1), 112-115.
55. Mäkitie, T.; Summanen, P.; Tarkkanen, A.; et al. Tumor-infiltrating macrophages (CD68+ cells) and prognosis in malignant uveal melanoma. Investigative ophthalmology & visual science. 2001, 42(7), 1414-1421.

56. Meecham, W.J.; Char, D.H.; Kaleta-Michaels, S. Infiltrating lymphocytes and antigen expression in uveal melanoma. Ophthalmol. Res. 1992, 24, 20–26.
57. Tobal, K.; Deuble, K.; McCartney, A; et al .Characterization of cellular infiltration in choroidal melanoma. Melanoma Res. 3, 63–65 (1993).
58. De Waard-Siebinga, I.; Hilders, C.G.; Hansen, B.E.; et al. HLA expression and tumor-infiltrating immune cells in uveal melanoma. Graefe's Arch. Clin. Exp. Ophthalmol. 1996, 234, 34–42.
59. Maat, W.; Ly, L.V.; Jordanova, E.S.; et al. Monosomy of chromosome 3 and an inflammatory phenotype occur together in uveal melanoma. Invest Ophthalmol Vis Sci. 2008, 49(2), 505-10.
60. De Waard-Siebinga, I.; Houbiers, J.G.; Hilders, C.G.; et al. Differential expression of HLA-A and B-alleles on uveal melanoma as determined by immuno-histology. Ocul Immunol Inflamm. 1996, 4(1), 1-14.
61. Jager, M.J.; Hurks, H.M.; Levitskaya, J.; et al. HLA expression in uveal melanoma: there is no rule without some exception. Human immunology. 2002, 63(6), 444-451.
62. Bronkhorst, I.H.; Ly, L.V.; Jordanova, E.S.;et al.. Detection of M2-macrophages in uveal melanoma and relation with survival. Investigative ophthalmology & visual science. 2011, 52(2), 643-650.
63. Ksander, B.R.; Rubsamen, P.E.; Olsen, K.R.; et al. Studies of tumor-infiltrating lymphocytes from a human choroidal melanoma. Invest. Ophthalmo. Vis. Sci. 1991 32(13), 3198-3208.
64. Apte, R. S.; Niederkorn, J. Y. Isolation and characterization of a unique natural killer cell inhibitory factor present in the anterior chamber of the eye. J. Immunol 1996, 156, 2667–2673.
65. Ma, D.; Niederkorn, J.Y. Transforming growth factor-β down-regulates major histocompatibility complex class I antigen expression and increases the susceptibility of uveal melanoma cells to natural killer cell-mediated cytolysis. Immunology. 1995, 86, 263–269.
66. Niederkorn, J.Y. Ocular immune privilege and ocular melanoma: parallel universes or immunological plagiarism?. Frontiers in immunology. 2012, 13; 3, 148.
67. Mills, C.D.; Kincaid, K.; Alt, J.M.; et al. M-1/M-2 macrophages and the Th1/Th2 paradigm. The Journal of immunology. 2000, 164(12), 6166-6173.
68. Mantovani, A.; Sozzani, S.; Locati, M.; et al. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends in immunology. 2002, 23(11), 549-555.
69. Nesbit, M.; Schaider, H.; Miller, T.H.; et al. Low-level monocyte chemoattractant protein-1 stimulation of monocytes leads to tumor formation in nontumorigenic melanoma cells. J. Immunol. 2001, 166, 6483–6490.

70. Azenshtein, E.; Luboshits, G.; Shina, S.; et al. The CC chemokine RANTES in breast carcinoma progression: regulation of expression and potential mechanisms of promalignant activity. Cancer Res.2002, 62, 1093–1102
71. Bronkhorst, I.H.; Vu, T.K.; Jordanova, E.S.; et al. Different subsets of tumor-infiltrating lymphocytes correlate with macrophage influx and monosomy 3 in uveal melanoma. Invest. Ophthal. Vis. Sci. 2012, 53(9), 5370-5378.
72. De Lange, M.J.; Nell, R.J.; Lalai, R.N.; et al. Digital PCR-Based T-cell Quantification–Assisted Deconvolution of the Microenvironment Reveals that Activated Macrophages Drive Tumor Inflammation in Uveal Melanoma. Mol. Canc. Res. 2018, 16(12), 1902-1911.
73. Biswas, S.K.; Mantovani, A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol. 2010, 11(10), 889-896.
74. Yu, P.; Fu, Y.X. Tumor-infiltrating T lymphocytes: friends or foes? Lab Invest. 2006, 86, 231–245.
75. Mihm, M.C.; Mulé, J.J. Reflections on the histopathology of tumor-infiltrating lymphocytes in melanoma and the host immune response. Cancer Immunol Res. 2015, 3, 827–835.
76. De la Cruz, P.O.J.r.; Specht, C.S.; et al. Lymphocytic infiltration in uveal malignant melanoma. Cancer. 1990, 65(1), 112-115.
77. Whelchel, J.C.; Farah, S.E.; McLean, I.W.; et al. Immunohistochemistry of infiltrating lymphocytes in uveal malignant melanoma. Invest Ophthalmol Vis Sci. 1993, 34(8), 2603-2606.
78. Lagouros, E.; Salomao, D.; Thorland, E.; et al. Infiltrative T regulatory cells in enucleated UMs. Trans Am Ophthalmol Soc. 2009, 107, 223–228.
79. Goldsby, R.A.; Kindt, T.J.; Osborne, B.A.; et al. Immunology. 5th ed. Mc Graw Hill; 2003, 171–194.
80. Szeto, C.; Lobos, C.A.; Nguyen, A.T.; et al. TCR Recognition of Peptide–MHC-I: Rule Makers and Breakers. Int. J. Mol. Scie. 2021, 22(1), 68-94.
81. Roche, P.A.; Furuta, K. The ins and outs of MHC class II-mediated antigen processing and presentation. Nature Reviews Immunology. 2015, 15(4), 203-216.
82. Garrido, F.; Cabrera, T.; Concha, A.; et al. Natural history of HLA expression during tumour development. Immunol Today. 1993, 14(10), 491-499.
83. Blom, D.J.; Luyten, G.P.; Mooy, C.; et al. Human leukocyte antigen class I expression. Marker of poor prognosis in uveal melanoma. Invest. Ophthalmo. Visual sci. 1997, 38(9), 1865-1872.
84. Ericsson, C.; Seregard, S.; Bartolazzi, A.; et al. Association of HLA class I and class II antigen expression and mortality in uveal melanoma. Invest Ophthalmol Vis Sci. 2001, 42(10), 2153-2156.

85. Hurks, H.M.H.; Metzelaar-Blok, J.A.W.; Mulder, A. et al. High frequency of allele-specific down-regulation of HLA Class I expression in uveal melanoma cell lines. Int. J. Cancer 2000, 85, 697-702.
86. Anastassiou, G.; Rebmann, V.; Wagner, S.; et al. Expression of classic and nonclassic HLA class I antigens in uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2003, 44, 2016–2019.
87. De Waard-Siebinga, I.; Creyghton, W.M.; Kool, J.; et al. Effects of interferon alfa and gamma on human uveal melanoma cells in vitro. Br. J. Ophthalmol. 1995, 79, 847–855.
88. Lawrence, T. The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol. 2009, 1(6), a001651.
89. Oh, H.; Ghosh, S. NF-κB: roles and regulation in different CD4 (+) T-cell subsets. Immunol Rev. 2013, 252(1), 41-51.
90. Pires, B.R.B.; Silva, R.C.M.C.; Ferreira, G.M.;. NF-kappaB: Two Sides of the Same Coin. Genes (Basel). 2018, 9(1), 24.
91. Meir, T.; Dror, R.; Yu, X.; et al. Molecular characteristics of liver metastases from uveal melanoma. Invest Ophthalmol Vis Sci. 2007, 48(11), 4890-4896.
92. Dror, R.; Lederman, M.; Umezawa, K.; et al. Characterizing the involvement of the nuclear factor-kappa B (NF kappa B) transcription factor in uveal melanoma. Invest Ophthalmol Vis Sci. 2010, 51(4), 1811-1816.
93. Halkidou, K.; Gaughan, L.; Cook, S. et al. Upregulation and nuclear recruitment of HDAC1 in hormone refractory prostate cancer. Prostate. 2004, 59, 177–189.
94. Song, J.; Noh, J.H.; Lee, J.H.; et al. Increased expression of histone deacetylase 2 is found in human gastric cancer. APMIS. 2005;113:264–268
95. Wahid F, Shehzad A, Khan T, Kim YY. MicroRNAs: synthesis, mechanism, function, and recent clinical trials. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 2010; 1803(11):1231-43.
96. Kim, J.; Ya, F.; Xiao, Z.; et al. MicroRNAs and metastasis: small RNAs play big roles. Cancer. Met. Rev. 2018, 37(1), 5-15.
97. Volinia, S.; Calin, G.A.; Liu, C.G.; et al. A microRNA expression signature of human solid tumors defines cancer gene targets. PNAS. 2006, 103(7), 2257-2261.
98. Wu, F.; Zikusoka, M.; Trindade, A.; et al. MicroRNAs are differentially expressed in ulcerative colitis and alter expression of macrophage inflammatory peptide-2α. Gastroenterology. 2008, 135(5), 1624-1635.

99. Worley, L.A.; Long, M.D.; Onken, M.D.; Harbour, J.W. Micro-RNAs associated with metastasis in uvealmelanoma identified by multiplexed microarray profiling.Melanoma Res.2008,18, 184–190.

100. Smit, K.N.; Chang, J.; Derks, K.; Vaarwater, J.; Brands, T.; Verdijk, R.M.; Wiemer, E.A.C.; Mensink, H.W.;Pothof, J.; de Klein, A.; et al. Aberrant MicroRNA Expression and Its Implications for Uveal Melanoma Metastasis. Cancers 2019,11, 815.

101. Aughton, K.; Kalirai, H.; Coupland, S.E. MicroRNAs and Uveal Melanoma: Understanding the Diverse Role of These Small Molecular Regulators. Int. J. Mol. Sci. 2020, 21(16), 5648.

102. Alegre, M. L.; Frauwirth, K. A.;Thompson, C. B., T-cell regulation by CD28 and CTLA-4. Nat. Rev. Immunol. 2001. 1, 220–228.

103. Topalian, S. L.; Drake, C. G.; Pardoll, D. M. Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity. Curr. Opin. Immunol. 2012. 24, 207–212

104. Rodrigues, M.; Koning, L.; Coupland, S.E.; et al. UM Cure 2020 Consortium. So Close, yet so Far: Discrepancies between Uveal and Other Melanomas. A Position Paper from UM Cure 2020. Cancers (Basel). 2019, 11(7), 1032.

105. Durante, M.A.; Rodriguez, D.A.; Kurtenbach, S.; et al. Single-cell analysis reveals new evolutionary complexity in uveal melanoma. Nat. Commun. 2020, 11(1), 1-10.

106. Triebel, F.; Jitsukawa, S.; Baixeras, E.; et al. "LAG-3, a novel lymphocyte activation gene closely related to CD4." TJ Exp Med. 1990, 171(5), 1393-1405

107. Krishnakumar, S.; Abhyankar, D.; Lakshmi, S.A.; et al. HLA class II antigen expression in uveal melanoma: Correlation with clinicopathological features. Exp. Eye Res. 2003, 77:175–180.

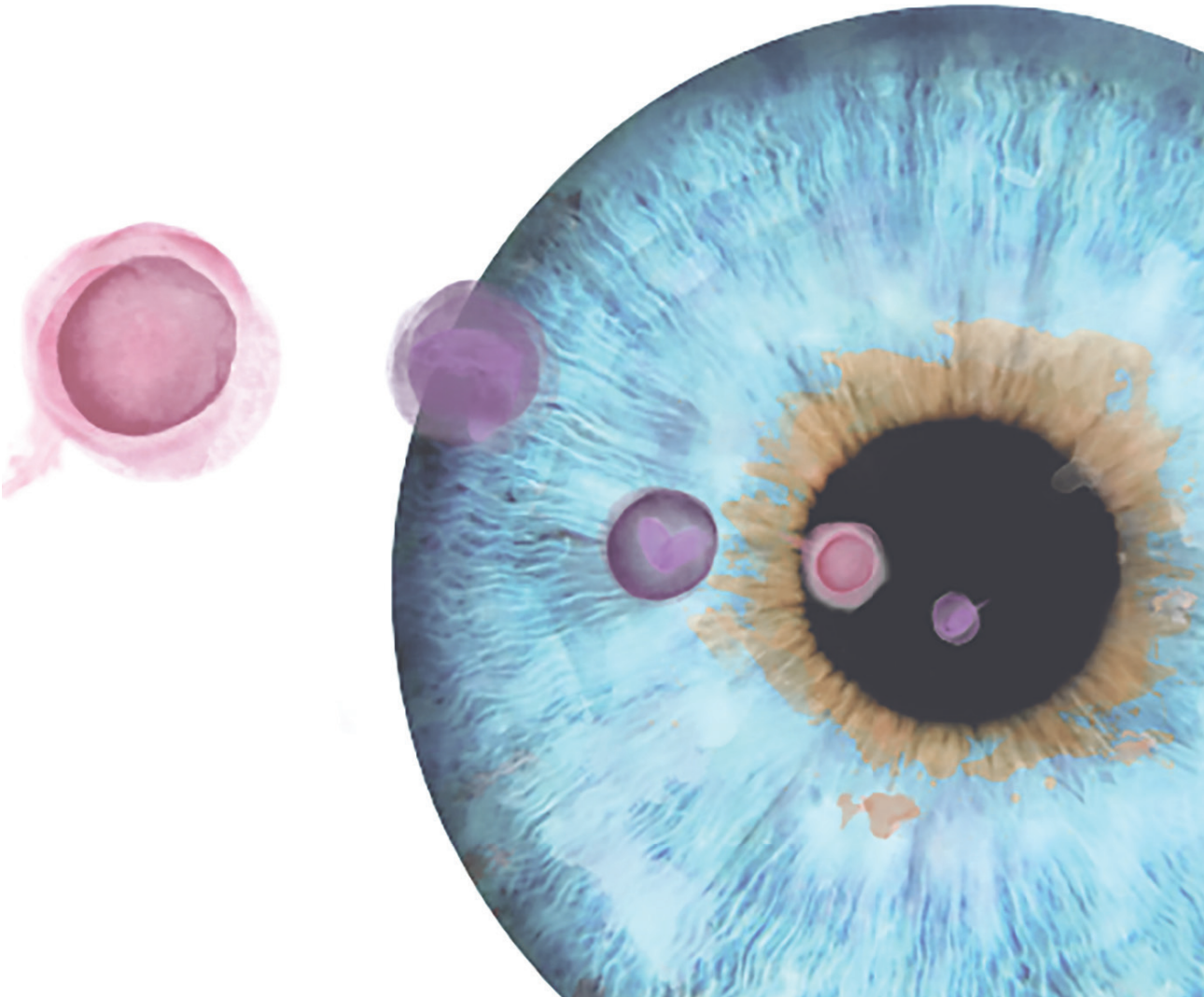
108. Jager, M.J.; van der Pol, J.P.; de Wolff-Rouendaal, D.; et al. Decreased expression of HLA Class II antigens on human uveal melanoma cells after in vivo X-ray irradiation. Am. J. Ophthalmol. 1988, 105:78–8

Chapter 2

HLA expression in Uveal Melanoma: an indicator of malignancy and a modifiable immunological target

Zahra Souri, Annemijn P.A. Wierenga, Arend Mulder, Aart G. Jochemsen, Martine J. Jager.

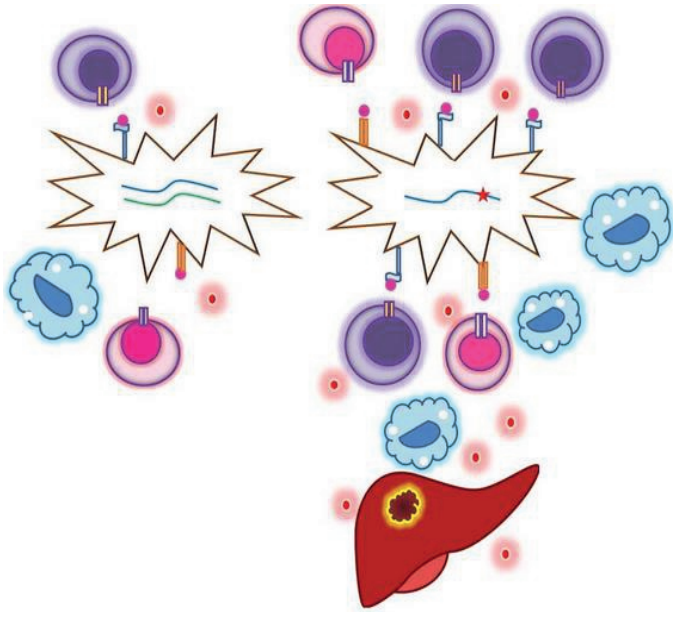
Cancers 2019, 11, 1132-1150



Abstract

Uveal melanoma (UM) is the most common primary intraocular malignancy in adults, and gives rise to metastases in 50% of cases. The presence of an inflammatory phenotype is a well-known risk factor for the development of metastases. This inflammatory phenotype is characterized by the presence of high numbers of lymphocytes and macrophages, and a high expression of the HLA Class I and II antigens. An abnormal expression of HLA Class I may influence cytotoxic T lymphocyte (CTL) as well as Natural Killer (NK) cell responses.We provide a comprehensive review regarding the inflammatory phenotype in UM and the expression of locus- and allele-specific HLA Class I and of Class II antigens in primary UM and its metastases. Furthermore, we describe the known regulators and the role of genetics (especially chromosome 3 and BRCA-Associated Protein 1 (BAP1 status)), and, last but not least, the effect of putative therapeutic treatments on HLA expression.

Keywords: Uveal Melanoma, Inflammation, HLA Class I, HLA Class II, Chromosome 3, BAP1



1. Introduction

Uveal melanoma (UM) is an intraocular tumor which arises from the uveal tract, with 3–5% of tumors involving the iris, 5–8% the ciliary body and 90% the choroid [1,2]. The disease occurs mainly in Caucasians, where it is associated with blond hair, light eyes and a fair skin [3,4]. Depending on the size and location of the tumor, the clinical presentation differs. Symptoms may include loss of vision and pain, or the tumor may be found by chance during a routine eye examination [5,6]. The diagnosis is made upon clinical examination by an ophthalmologist using indirect ophthalmoscopy, ultrasonography and sometimes fluorescein angiography. Treatment consists of irradiation (local brachytherapy, proton beam therapy, stereotactic irradiation), or enucleation of the eye. Histopathological examination of tumor material is performed for confirmation of the diagnosis as well as for determining prognostic parameters [6–8]. The hematogenous spread of UM specifically targets the liver and up to 50% of patients ultimately die due to metastatic disease [9]. One of the hallmarks of cancer is inflammation [10–12]: inflammation stimulates tumor cell proliferation, survival, angiogenesis, metastasis formation, and may cause a decreased response to treatments. Although the eye is an immune-privileged site, where inflammatory responses are limited [13], immune cell infiltration frequently occurs in the intraocular UM [14]. During the course of malignant progression of the tumor, an increase in infiltrating immune cells (high numbers of infiltrating tumor-associated macrophages (TAM's) and tumor-infiltrating leukocytes (TIL's) and HLA Class I expression) is observed [15], which combination is known as the inflammatory phenotype [16–19]. This inflammatory phenotype has been identified as being associated with an infaust prognosis. It is associated with loss of one chromosome 3 and loss of expression of BRCA-Associated Protein 1 (BAP1), the product of a gene located on chromosome 3, which encodes an ubiquitin protease [20]. Loss of expression of BAP1 and loss of one chromosome 3 often occur together and both are well-known risk factors for the development of metastases in UM patients [21, 22]. HLA molecules are glycoproteins expressed on the cell surface. The HLA genes are located on chromosome 6. HLA Class I proteins are present on almost all nucleated cells and serve to present foreign peptides to cytotoxic T cells (CTLs, CD8+) or bind to killer inhibitory receptors (KIR's) of Natural Killer (NK) cells, which may lead to suppression of these NK cells (Figure 1).

HLA Class II proteins are mainly expressed on B cells, a subset of T cells, and on antigen-presenting cells and interact with regulatory Treg cells (CD4+) [23]. As HLA expression is known as one of the prognostic factors in UM while it is also important for the effectiveness of immunotherapeutic approaches, we here provide an overview of the expression of these molecules in UM, their regulation, and function.

2. HLA Expression in Uveal Melanoma

2.1. Variability of HLA Expression in UM

Studies in the 1980s on cutaneous melanoma showed that specific expression patterns of HLA Class I and HLA Class II might be associated with progression to metastasis. HLA Class I, beta-2 microglobulin (B2M) and HLA Class II were not detected on the majority of nevus cells but were found on primary cutaneous melanomas and metastatic lesions [24]. The difference in the expression of these molecules suggested a role in the progression of malignancy. This led to a study on HLA expression in UM, which examined the expression of monomorphic and locus-specific HLA Class I and II antigens on paraffin sections of 27 human UM, using a panel of monoclonal antibodies directed against B2M, HLA Class I and HLA Class II [25]. HLA Class I antigens were expressed on most tumors and more cells expressed Class I than Class II. HLA Class I expression was higher on (high risk) epithelioid tumors than on tumors with a spindle or mixed cell type. There was a great variability in HLA expression among the tumors which theoretically could be caused by different developmental states, different oncogenic mutations and also different lymphokines present in the tumor microenvironment. In 1996, De Waard-Siebinga et al. [26] compared the expression of HLA-A versus HLA-B on paraffin sections of 23 HLA-typed UM and found that HLA-A expression was higher than HLA-B expression.

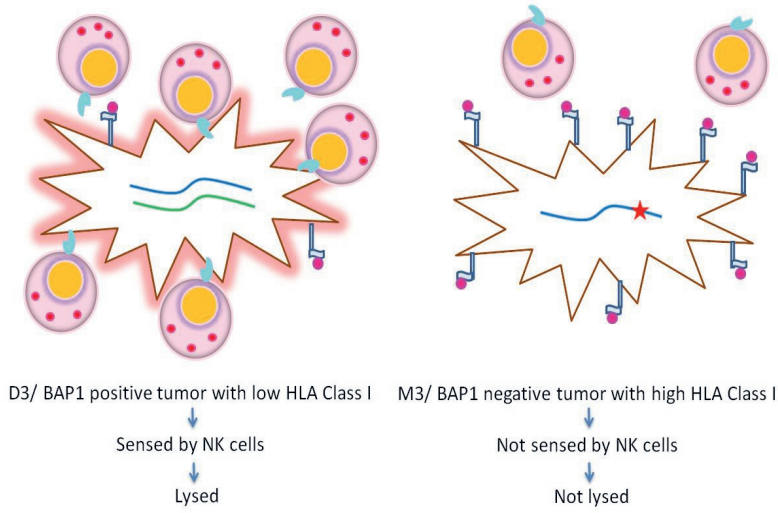


Figure 1. Schematic illustration of the relationship between the tumor's chromosome3/BRCA-Associated Protein 1 (BAP1) status, its HLA expression and Natural Killer (NK) cell recognition. (a) Disomy 3/BAP1-wild type tumor: low HLA Class I expression, killed by NK cells. (b) Monosomy3/BAP1-mutated tumor: high HLA Class I expression, escapes from NK cell-mediated lysis in the blood stream.

2.2. HLA Expression and Metastatic Potential

The studies by Jager et al. in 1986 [25] and De Waard-Siebinga et al. [26] in 1996 lacked proper information on the relation with survival. To answer the question whether HLA expression is related to survival, Blom et al. [27] set out to determine the expression of locus-specific HLA-A and HLA-B expression using immunohistochemistry on paraffin sections of 30 UM with good clinical information and excellent follow-up. Confirming earlier findings, they found variability among different tumors for HLA-A and HLA-B expression. However, within individual tumors, HLA-A and HLA-B expression were highly correlated. While the paradigm and expectation was that a low HLA expression would allow tumor cells to escape from CTL-mediated lysis, and thus lead to metastases, this study showed for the first time that the opposite was true: in UM, a high HLA-A/B expression was associated with a low patient survival. The assumption arose that a decreased expression of HLA-A and HLA-B could prevent the development of metastasis because of NK cell-mediated lysis of migrating tumor cells in the blood, preventing the tumor cells from reaching the liver.

An earlier study by Ma and Niederkorn in 1995 [28] had already indicated the possible role of NK cell-mediated lysis against UM cells. The group of Niederkorn tested Transforming Growth Factor beta (TGF-β), a down-regulator of HLA Class I, on two melanoma cell lines (OCM1, OCM8) which were known to have high levels of HLA Class I, in order to investigate the effect of NK-mediated lysis. Incubation with TGF-β resulted in a significant downregulation of HLA Class I (52–62%) and an increase of NK cell cytolysis. As an analogous experiment they applied Interferon gamma (IFNγ), an inducer of HLA Class I, on cell lineOCM3, which had a low HLA Class I level. This led to an 80% increase in HLA Class I expression and a 10% reduction in NK cell-mediated lysis. Taken together these results confirmed the strong role of different external factors in the tumor microenvironment which could influence the survival and metastasis of melanoma cells, and the role of HLA expression in the effector function of immune cells. The hypothesis became that NK cells in the blood would kill UM cells with a low HLA Class I expression prior to their settlement in the liver, while the HLA Class I expressing cells would not be lysed. These studies stimulated an interest in the function of HLA in UM. A few years later, papers from three different countries confirmed the findings of Blom et al. [27]. A study by Ericsson et al. [29] not only confirmed the findings with regard to HLA Class I, but also provided information about the association of HLA Class II expression and the progression of the disease in 70 patients. A low expression of HLA Class I and HLA Class II was significantly more frequent in more benign spindle cell UM ($p = 0.006$ and $p = 0.01$, respectively). A high expression of HLA Class I, B2M and HLA Class II correlated significantly with the development of metastases ($p = 0.013$, $p = 0.001$, and $p = 0.02$, respectively). These findings support the role of NK cell-mediated protection against systemic spreading of UM. In 2002, Dithmar et al. [30] looked at the association between HLA Class I locus-specific expression and melanoma cell type: 22 tumors were divided into those with a spindle or those with an epithelioid cell type. HLA Class I expression was analyzed by immune histochemistry, using the same antibodies as used previously by De Waard-Siebinga et al. [26] and Blom et al. [27]. 18% of the spindle type UM versus 82% of the epithelioid type UM stained positively with (HCA)-10, an antibody that recognizes specifically HLA-A antigens [31]. They confirmed that the more malignant epithelioid cell type tumors had a higher HLA expression than non-epithelioid tumors.

2.3. Coordination between HLA Expression and Tumor Infiltrates.

An early study in 1992 used monoclonal antibodies and flow cytometry to study the lymphocyte and monocyte population and HLA expression in 41 UM samples [32]. Meecham et al. determined the presence of melanoma cells using antibody 13A3E (an anti-melanocyte antibody) which stained on average 82% of the cells (31–98%). Staining for HLA-A, HLA-B and HLA-C was positive in on average 85% of the tumor cells (25–98%) and for the Class II antigen HLA-DR in 7% (0–58%). The amount of lymphocytic infiltrate was variable among the samples with predominantly CD3+T cells, accounting for 4.5% of the total cell population (range 0.1–29%). Other immune cells such as NK cells, B cells and macrophages comprised less than 2.5%. They found that the ratio of the CD4+/CD8+ population was not constant, as in some tumors the CD4+ population was dominant while in others CD8+T cells were the most common. This study showed that (in irradiated tumors), an increase in age was associated with an increase in the CD4+ population, while the number of CD8+ cells decreased (p = 0.02). In 1996 [14], De Waard-Siebinga et al. found significant positive correlations between the presence of CD3+ cells and HLA Class I, HLA-A2, HLA-Bw4 and HLA Class II expression. In addition, CD4+ T cells and CD11B+ cells (granulocytes, monocytes/macrophages, NK cells) were significantly correlated to HLA class I expression. All UM samples contained some infiltrating cells although often in small amounts, with a predominance of T cells. Van Essen et al. [33] compared the presence of molecules of the Antigen Processing Machinery (APM) with the quantity of infiltrating cells and similarly observed that a high number of macrophages (CD68+) and lymphocytes (CD3+) correlated positively to the level of HLA Class I expression as they did with the status of chromosome 3. HLA-A, HLA-B, and B2M were all higher in tumors with a high CD3+ infiltrate. When fresh tumor material was implanted as a xenograft into Severe Combined Immuno-deficient (SCID) mice, leukocyte infiltration was lost and subsequently, the expression of HLA Class I and its regulators became downregulated. These findings show that it is the presence of leukocytes that causes the upregulation of HLA Class I and II antigens, and that is not the high level of HLA antigens attracting the infiltrating immune cells.

2.4. Locus and Allele-Specific Loss as an Escape Strategy

During the last decades, several studies have confirmed that UM cells may lack expression of locus or allele-specific HLA antigens, which may help cells to escape from T cell-mediated responses while the normal expression of the remaining antigens would give the cells the ability to be protected against NK cells inside the bloodstream. In 2002, Anastassiou et al. [34] studied HLA-A, HLA-B, and HLA-C expression using immunoprecipitation and Western blot on tissues of 18 HLA-typed patients. Half of the samples showed full HLA-A and HLA-B expression, while HLA allotype loss was found in 33%, with three cases affecting the HLA-A locus (HLA-A2, A28, and A29) and three the HLA-B locus (HLA-B18, B35 and B55). Two tumors showed a haplotype loss (HLA-A2, B44 and HLA-A2, B13) and one tumor showed a complete HLA-A (HLA-A26, -A32) loss together with one HLA-B allotype loss (HLA-B41). HLA-A2 expression was variable, with homogenous expression in three samples (>75% of the tumor cells), heterogeneous expression in two samples (25–75% of the tumor cells) and negative expression in only one sample. The cause for lack of expression was not investigated. In order to find out whether immunotherapy would be feasible on UM, our lab studied the reason why specific T cells would not recognize tumor cells in spite of high levels of HLA Class I expression in UM [35]. It was suggested that there might be an association between loss of specific HLA haplotypes and T cell recognition. A series of molecularly HLA-typed cell lines (92.1, Mel202, OCM-1, EOM3, OCM-3 and OMM1) was tested using Fluorescence-Activated Cell Sorting (FACS) in order to find defects in polymorphic HLA-A and HLA-B expression. HLA-A expression was high in all the tested cell lines and could be further (and similarly) induced by both IFNγ and IFNα treatment [36,37]. Both allele-specific and locus-specific loss of HLA expression were observed in UM cell lines, and some could be restored by IFN treatment, others could not. Cell line 92.1 had lost HLA-B44, and Mel202 had lost its HLA-B5-allele specific expression while OMM1 had lost all B locus-specific expression (HLA-B27, -B40). This loss of expression may have helped the tumor to prevent recognition by CTLs, helping metastasis to develop [16]. Recently, we tested four UM cell lines and used IFNγ to induce HLA Class I expression (Figure 2).

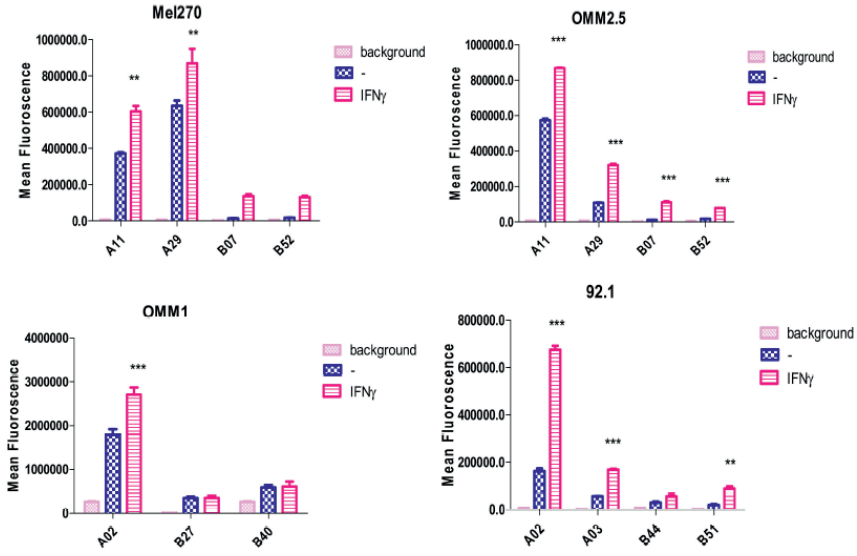


Figure 2. HLA-typed Uveal melanoma (UM) cell lines can be analysed by Fluorescence-Activated Cell Sorting (FACS) using HLA antigen specific monoclonal antibodies to find defects in the expression of HLA alleles, and to determine the sensitivity of induction by Interferon gamma (IFNγ). Loss of HLA Class I allele expression in UM cell lines: B7 and B52 loss in Mel270, B27 and B40 loss in OMM1, B44 loss in 92.1 (**$p = 0.01$; *** $p = 0.001$).

3. HLA Class I Regulation in UM

3.1. HLA Expression is related to Genetic Factors

In other tumors such as cutaenous melanoma, several different mechanisms have been found responsible for a loss in HLA antigen expression. We asked the question whether genetic determinants influenced the level of expression of HLA. Van Essen et al. [38] compared HLA expression with the distribution of the HLA allele frequencies in 50 patients who had undergone enucleation for UM in Leiden between 1999 –2004. Before correction for multiple testing, a lower macrophage infiltration was seen for tumors that were HLA-A2, and a higher HLA-DR expression in tumors of patients with HLA-DR6, although after correction for the number of analyses, no significance was found. This study showed that the HLA genotype does not affect overall HLA expression or macrophage infiltration in UM.

An inverse correlation between HLA Class I expression and expression of a well-known oncogene, c-myc, has been observed in cutaneous melanoma [39]. In 1997, Blom et al. asked whether they could find a similar inverse association in UM, and studied paraffin sections from 30 UM, where they measured HLA-A, HLA-B and c-myc expression by immunohistochemistry [40]: a low level of HLA-B was significantly correlated to a high level of c-myc expression in the cytoplasm (p = 0.03), which was similar to the findings that had been reported regarding cutaneous melanoma. In the UM sections, a high level of HLA-B was associated with an epithelioid cell type (p = 0.004). In other malignancies, loss of HLA Class I antigens is often caused by loss of essential molecules of the HLA antigen-processing and presentation system, such as the Transporter Associated with Antigen Processing (TAP1) protein in colorectal cancer [41,42] and primary cutaneous melanoma [43,44]. Studies were undertaken to determine whether the same held true for UM. A study in 2003 assessed the relation between HLA Class I and the presence of Antigen-Processing Molecules (APM) in 41 primary UM specimens from Asian-Indians [45]. HLA Class I, Low Molecular Mass Polypeptide 2 (LMP2), LMP10, TAP1, tapasin and calnexin were all low in tumors without extra-scleral extension and high in tumors which gave rise to liver metastasis. Later, van Essen [33] not only analyzed peptide-loading components but also some other regulators of expression, and (similarly) found significant associations between the presence of TAP1 and TAP2 and expression of HLA Class I, HLA Class II and B2M. In addition, the HLA expression regulators Interferon Regulatory Factor 1 (IRF1) and IRF8 were found to correlate positively with HLA-A, HLA-B and B2M. Regulator NOD-like Receptor family CARD domain containing 5 (NLRC5) correlated with HLA-B and B2M expression, and Class II major histocompatibility complex Transactivator (CIITA) with HLA-B as well as with B2M. This study compared expression with the most important genetic prognostic factor, monosomy for chromosome 3. The relation between HLA-A and HLA-B expression with chromosome 3 status was investigated by Illumina microarray, immunohistochemistry and qPCR. Illumina data showed a strong difference between disomy 3 (D3) and monosomy 3 (M3) for HLA-A and HLA-B, with a higher expression in M3 tumors. Immunohistochemistry revealed only a difference for HLA-A, while qPCR revealed differences for HLA-A, HLA-B and B2M between D3 and M3 tumors. While an increased level of IRF1 was associated with M3, expression levels of IRF2, IRF8, NLRC5, and CIITA were not significantly related to M3.

However, the group of analyzed tumors was quite small (n=13). A high expression of TAP1 and IRF1 was associated with death due to metastases, while NLRC5, CIITA, IRF2, and IRF8 expression levels were not. This study confirmed that UM cells contain a proper functioning HLA antigen-processing system which therefore do not cause loss of HLA expression, and that expression of HLA proteins on the cell surface, and several HLA transcription regulators and the peptide loading machinery are co-regulated.

3.2. Locus-Specific Differences between HLA-A and HLA-B

Low constitutive HLA Class I expression is observed among different types of cells with some being restored by the addition of IFN's and tumor necrosis factor (TNF). In 1993, Girdlestone et al. investigated the reason for a different regulation of HLA-A and HLA-B [46]. They found a role for Nuclear Factor kappa-light-chain-enhancer of activated B cells (NFkB) in the regulation of HLA-A and HLA-B expression in MOLT4 and YHHH cell lines, which had been derived from lymphoblastic leukemia. Two main upstream control elements are known to be responsible for basal HLA Class I expression. The HLA-A enhancer contains two Rel (kBF/NFkB) binding motifs while the HLA-B enhancer has only one and transcription of the HLA-B gene is therefore less transactivated by the NFkB p65 subunit. In contrast, HLA-B transcription was stronger induced by IFNγ than HLA-A transcription and the HLA-B promoter was found to have a higher affinity for the IRF1 and IRF2 transcription regulators. They also found that in cutaneous melanoma, IRF1 binds to HLA-A Interferon Response Element (IRE) with a lower affinity than to the HLA-B IRE, explaining why HLA-A and B genes are distinctly transcriptionally regulated by Rel family members and IFN's. Also in another study, Johnson et al. reported that HLA-A, B and C differ in their regulation by cytokines and expression indifferent tissues [47].

4. Other Types of HLA Class I in UM

In addition to HLA-A and -B, several other Class I molecules can be expressed, with different functions. HLA-C is a member of the HLA Class I family with a relatively low cell surface expression. The main function of HLA-C is its ability to bind as a ligand to KIRs of the NK cells and suppress their cytotoxicity [48]. HLA-G together with HLA-E are members of the non-classic HLA Class I family expressed mainly on fetal cells of human placenta.

These molecules are known to have the ability to suppress immune cell functions such as NK and CTL-mediated cytolysis [49–51]. HLA-G has so far not been detected in UM [52]: a study in 2002 used a variety of methods to analyse HLA-G expression in 11 human UM cell lines with different HLA-A and -B expression levels and metastatic potential. In addition, HLA-G expression was investigated on 17 frozen primary UM sections. The trophoblast cell line JEG-3 was used as a control. No HLA-G protein or RNA was found in any UM cell line or tissue even after treatment with IFNγ. Different levels of HLA-E expression were observed, which could be further induced after IFNγ treatment, suggesting that HLA-G has no role in a tumor's escape from the immune system, while HLA-E should be further investigated. As the previous studies demonstrated a low expression of HLA-G in UM it is conceivable that NK cell-based lysis may be effective in destroying UM cells and that these molecules are not involved in the immune-biology of UM.

5. Genetics Play a Role in the Development of UM

5.1. HLA Allele Frequencies in UM

Several investigators in Leiden studied the genetic distribution of HLA Class I, II and MICA genes in UM patients. In 2005, Metzelaar-Blok et al. analyzed 159 cases of UM which were typed for HLA Class I and II and 168 cases which were evaluated for the MICA gene by microsatellite typing, and compared their HLA genotypes to 2440 healthy controls [53]. No significant differences were observed between the two populations. In the UM population, the highest allele frequency was seen for A2 (55% in UM and 53% in control population), a gene that is often used for inclusion in immunotherapy trials. No significant associations were noticed between HLA and MICA genetic polymorphisms and the development of a UM. An extension of this study to 235 cases did not change the conclusions [54], but did find some associations between tumor characteristics (HLA-DR13 with large tumor size, HLA-B35 with spindle cell type and HLA-B60 with ciliary body involvement). While the first study on the relation between HLA type and prognosis showed an association between the presence of HLA-B40 and the development of metastases [55], the later study by Maat in 2006 did not reproduce this, but rather found an association between B44 and a worse survival [54].

5.2. Somatic Genetic Abnormalities and HLA Expression in Uveal Melanoma

Somatic genetic factors are known to influence the development and behavior of UM, and have been found to play a major role in creating the inflammatory microenvironment. Loss of chromosome 1p, 3, or 6q and gain in 6p or 8q are among the most frequently occurring chromosomal changes identified in UM. These genetic abnormalities affect different aspects of UM [7, 8, 56–58]. Loss of one chromosome 3 (Monosomy 3, M3) is found in 50% of UM patients and is associated with a bad prognosis. Almost all M3 tumors also show gain in chromosome 8q, while this aberration can also be present in Disomy 3 (D3) tumors [59–61]. Gain of chromosome 8q is considered an early event, developing prior to the loss of chromosome 3, is a bad prognostic factor and is associated with an increased macrophage infiltration [15]. As the HLA genes are located on chromosome 6 and both losses and gains of parts of this chromosome are common in UM, the relation between these genetic aberrations and HLA expression was investigated by several authors. Blok et al. used three microsatellite markers on chromosome 6 to see whether Loss of Heterozygozity (LOH) of chromosome 6p might affect HLA Class I expression [62]. She analyzed DNA extracted from 20 formalin-fixed paraffin-embedded primary UM, and found that 65% of the studied tumors showed LOH of at least one locus on chromosome 6p. However, no correlation between LOH on 6p and HLA-A and HLA-B monomorphic expression was observed. Van Essen et al. [33] investigated putative associations between copy numbers of multiple chromosomes and HLA expression. Half of the studied UM showed M3 according to Single Nucleotide Polymorphism (SNP) analysis, which was associated with death due to metastasis (Kaplan-Meier, $p < 0.001$). Gain in 6p was reported in 29% and was associated with good survival (Kaplan-Meier: $p = 0.049$). M3 was associated with elevated levels of HLA Class I and B2M. When looking at all tumors together, an association was observed between 6p gain and a low HLA-B expression ($p = 0.049$), which was probably due to the negative association between M3 and 6p gain: among the D3 tumors, no dosage effect of 6p was found on HLA Class I, II or B2M gene expression while M3 tumors had higher mean level of HLA Class I compared to D3 tumors. As shown in Figure 3, HLA-A, HLA-B and HLA-DR are significantly higher in M3 tumors compared to D3 tumors according to data analysed from The Cancer Genome Atlas (TCGA) database [18].

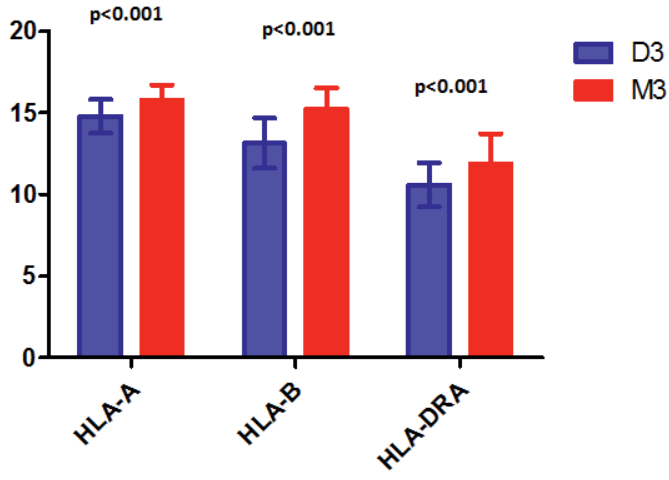


Figure 3. Comparison between HLA-A, HLA-B and HLA-DR expression in disomy 3 (D3) vs monosomy3 (M3) tumors of The Cancer Genome Atlas (TCGA) database.

6. Metastases and HLA Expression

When comparing expression of monomorphic HLA Class I on a primary UM sample, and several metastases, Blom et al. [63] noticed that in the metastatic lesions, expression of polymorphic HLA-A2 and HLA-A3 was decreased. Changes in HLA-B expression could not be assessed as HLA-Bw4 was low in all lesions. All metastases contained high amounts of CD3+ and CD4+ and a lower amount of CD8+ cells. The tissue of the metastasis was epithelioid, which was the same as the primary tissue but with less coherence and atypical mitoses. Verbik et al. [64] compared the ability of T cell stimulation in primary melanoma cell lines derived from either eye or skin melanoma. The expression of HLA Class I and II was assessed either by absence or presence of IFNγ. Also, various concentrations of tumor cells were added to lymphocyte cultures and their stimulatory capacity on T cells was determined. Primary cutaneous melanoma cells induced T cell proliferation while UM cells did not, although the UM cells expressed high levels of HLA Class I and II after stimulation with IFNγ.

The inhibitory effect was lost when ocular cells formed hepatic metastasis. This suggested that primary ocular tumor cells were poorly immunogenic and this immunogenicity alters when they move to a new microenvironment. In 2017, Gezgin et al. reported on a series of metastases [65]. Previously, Melanoma Antigen Preferentially Expressed in Tumors (PRAME) was found to be expressed on primary UM [66]. Gezgin observed that PRAME is indeed expressed in almost half of UM cases and is associated with largest basal diameter (15 mm vs 12 mm $p = 0.005$), ciliary body involvement (59% vs 26%, $p = 0.008$) and gain of chromosome 8 (66% vs 23%, $p = 0.002$). PRAME-specific T cells reacted with four out of seven UM cell lines. Of UM metastases, 69% were positive for PRAME mRNA, and 63% positive for HLA Class I, with a total of 50% samples co-expressing HLA and PRAME. This study confirmed that PRAME is expressed in UM and that some metastatic samples co-express HLA and PRAME, which makes the PRAME antigen a putative target for PRAME-directed immunotherapy. In order to study changes during tumor progression and discover potential pathways in the development of metastasis in UM, Meir et al. [67] analyzed seven metastases and seven primary UM by microarray analysis, validating their study by qPCR and immunohistochemistry. Microarray analysis showed that 193 genes were differently expressed between metastasis and primary UM, with 184 increased in the metastasis. NFkB2 was increased in the metastasis, with an increased expression of downstream genes which are involved in the progression of the disease such as Growth Arrest and DNA-Damage-inducible, Beta (GADD45B) and Hedgehog Interacting Protein (HHIP).

7. The Effect of Different Treatments on HLA Expression in UM

Interferon Induces HLA Expression In order to achieve an effective immune response against tumor cells it is crucial that tumor antigens get displayed on the cell surface by sufficient numbers of HLA Class I molecules. The cytokine IFNγ has the ability to up-regulate HLA expression on UM cell lines in vitro [36], which could suggest that environmental factors may influence HLA expression as well. De Waard-Siebinga et al. [68] compared HLA expression in short term UM cultures with the expression in the original tumors from which the cultures had been derived. Immunohistochemistry was used to measure HLA expression on tissue sections (HCA2, HC10 and allele-specific B8.11.2, BB7.2, GAP.A3, 116/5/28, SFR-8-B6) and monoclonal antibodies W6/32 and BBM1 were used to evaluate HLA Class I expression on cultured cells.

HLA-A expression in the cultured UM cells correlated to the tissue expression (R=0.77) while HLA-B was less correlated (R=0.68). HLA-DR expression was decreased during culture, probably because the new microenvironment of the cells did not contain interferon-producing leukocytes. In a later study, De Waard-Siebinga et al. [69] established UM cell line 92.1 at the Leiden University Medical Center. Immunohistochemistry, flow cytometry and Northern blot analysis for HLA Class I revealed that this cell line had a low amount of HLA-B compared to HLA-A which, however, could be further induced by IFNγ. The effect of IFNγ and IFNα on the growth and expression of HLA Class I and II was studied on cell lines 92.1 and Mel202 by cytospins and flow cytometry [36]. Treatment with either IFNγ or IFNα decreased the growth of 92.1 but only IFNγ was able to inhibit the growth of Mel202. IFNα increased only HLA Class I on the two cell lines. At that time the exact mechanism of action of IFN on the cell lines was not well known but as differences were observed with regard to the induction of HLA among cell lines, this study reflected a potential individual difference in response to treatment with interferon's in vivo. We investigated the expression of HLA Class I (using monoclonal antibody W6/32) on UM cell line 92.1 (Figure 4) and show by FACS that IFNγ induces HLA Class I expression.

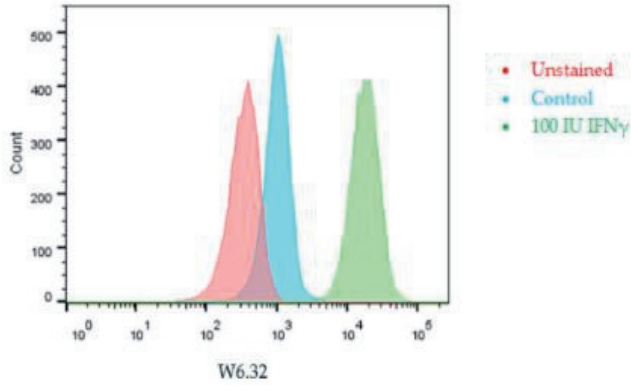


Figure 4. Interferon gamma (IFNγ) treatment induces HLA Class I expression, determined with monoclonal antibody W6/32 and Fluorescence-Activated Cell Sorting (FACS) analysis on Uveal melanoma (UM) cell line 92.1, after 48h treatment.

8. HLA Class II in UM

Under normal physiological conditions, constitutive HLA Class II expression mainly occurs on the surface of antigen presenting cells and thymic epithelial cells [70]. Krishnakumar et al. analysed HLA Class II expression on 45 primary UM from Asian-Indian patients [71], using the anti-HLA Class II mAb LGII 612.14. In their study, 17 of the tumors were spindle cell type, 16 mixed cell type and 12 epithelioid cell type. Thirty-five UM had no extrascleral extension and showed a low HLA Class II expression. Among the remaining 10 melanomas with extrascleral extension, 60% had developed metastases and revealed a high HLA Class II expression, while 40% did not develop metastases and had a low HLA Class II expression ($p < 0.001$).

8.1. Irradiation and HLA Class II Expression

In 1988, it was shown that expression of HLA-DQ was correlated with the presence of a lymphocyte infiltrate, suggesting a potential regulatory relationship between HLA Class II and infiltrates [72]. Comparing non-irradiated tumors with irradiated ones showed differences in all three HLA Class II antigens: DR, DP and DQ, although the level of HLA Class I, B2M and various melanoma-associated antigens remained the same. This suggested that irradiation might directly influence the tumor cells or indirectly influence HLA Class II expression via alteration in the extent of the infiltrate, suggesting that radiotherapy decreases the amount of lymphocytic infiltration: less IFN is then produced that could upregulate HLA Class II expression. Ericsson et al. [29] found that HLA Class II expression was expressed on 30 out of 65 UM samples and expression was associated with a lower survival. This study found higher levels of HLA Class II expression than the study of Jager in 1988 and it was suggested that in the previous study, irradiation might have decreased the expression.

8.2. Regulation of HLA Class II

Radosevich et al. found that UM cells have resistance to IFN induction of HLA Class II [73], but that this was not due to blocking of the biosynthetic pathway or the IFNγ signal transduction pathway. They used the Mel202, Mel270 and 92.1 cell lines, with Jurkat cells as control and found that, similar to other reports, CIITA expression was decreased in the UM cell lines. After addition of the DNA methylation inhibitor 5-aza-2'-deoxycytidine, the cells again expressed CIITA mRNA

and also HLA-DRA mRNA. They therefore concluded that DNA methylation is a strategy for the tumor cells to maintain themselves in the immune-privileged eye, by downregulating their HLA Class II expression. When examining leukemic T cells, Holling et al. found that CIITA gene expression is silenced by an epigenetic mechanism rather than lack of transcription factors [74].

They subsequently investigated melanoma cell lines (Mel285, OMM1.3, OCM-1, OCM-3) [75] and observed a lack of response to IFNγ induction when there were high levels of DNA methylation of the MHC2TA promotor IV (CIITA-pIV), and with high levels of tri-methylated histone H3-lysine 27. This resulted in low CIITA and HLA Class II expression. Histone methyltransferase EZH2 (Enhancer of Zeste Homolog 2) contributed to this silencing of IFNγ-inducible transcription of CIITA. Boyd et al. described the role of two other DNA-binding proteins that interact with CIITA pIV, namely Yin Yang 1 (YY1) and Jumonji domain containing protein 2 (JARID2) [76]. They show that these proteins are involved in recruitment of the silencing complex Polycomb Repressive Complex 2 (PRC2) (which contains EZH2) to the pIV promoter. Jumonji domain containing protein 2 (JARID2) knockdown resulted in elevated levels of CIITA mRNA upon IFNγ stimulation, suggesting that JARID2 is an inhibitor of HLA Class II activation. Interestingly, other studies have also described a role for Yin Yang 1 (YY1) in regulating HLA [77, 78].

9. Environmental Influences

Several studies have analyzed environmental influences, such as photodynamic therapy and hyperthermia, on expression of HLA in cultured UM cells. An early study [32] analyzed tumors that had previously been treated with either helium ions or I125 plaque. These tumors had a reduced staining with anti-melanoma antibody 13A3E because of tumor cell destruction or showed an altered antigen expression. No clear relationship was observed between HLA Class I expression and cell type. Blom et al. evaluated the effect of photodynamic therapy (PDT), using the hematoporphyrin ester bacteriochlorin on UM cell line 92.1 [79]. Flow cytometry analyses showed that HLA-A,-B,-C and B2M microglubulin expression was reduced after PDT treatment, increased after 2h and normalized after 6h of treatment. Blom et al. similarly tested the effect of hyperthermia on HLA Class I, B2M, Heat Shock Protein (HSP)-60 and HSP-70 [80].

They found a time and temperature-dependent effect of this treatment on HLA Class I and HSP-70: exposure to 45°C increased HSP-70, but not HSP-60 and reduced HLA Class I expression. However, no effect was observed on NK cell susceptibility. Recently, we determined whether certain therapeutic drugs, which are being tested to treat metastasis of UM, influence HLA Class I expression (Figure 5). Our results show that 48h treatment with silmitasertib (an inhibitor of Casein Kinase 2 and Clks) reduced HLA Class I expression in OMM2.5 while foretinib (an inhibitor of c-Met, Vascular Endothelial Growth Factor (VEGF) receptor-2 and Tumor Associated Macrophage (TAM) receptors) increased HLA Class I expression. More small molecule compounds are being tested, but these data show it should be realized that (chemo) therapy can influence the expression of immunologically relevant molecules such as the HLA antigens.

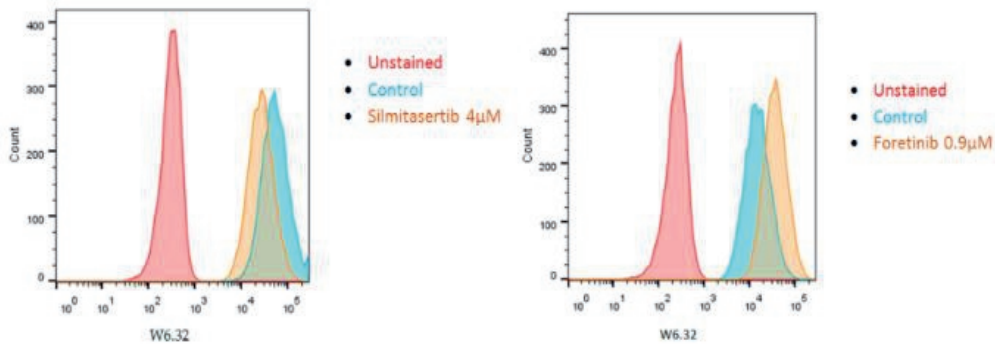


Figure 5. Treatment of cell line OMM2.5 with two different kinase inhibitors for 48h either reduced (silmitasertib) or increased (foretinib) HLA Class I expression (monoclonal antibody W6/32, measured by Fluorescence-Activated Cell Sorting (FACS) analysis). In the control, no drug was added.

10. Potential for Immunotherapy: Function of HLA Antigens in Antigen Presentation

Murine experiments demonstrated that UM cells in the eye are potential candidates for Tcell mediated therapy [81]. Sutmuller et al. immunized HLA-A*0201/Kb (A2/Kb)-transgenic mice with recombinant canarypox virus (ALVAC-gp100) and isolated HLA-A*0201-restricted CTL against human gp100. After injection of human HLA-A2 positive UM cells into the anterior chamber of A2/Kb-transgenic mice, these CTL's were injected systemically, which resulted in a rapid elimination of the UM cells from the murine eye.

These data show that immunological treatment of intraocular tumors should be possible in spite of the immune privilege. Luyten et al. studied eight UM cell lines to assess their capacity to be used as stimulators in immunotherapy [82]. The expression of human Melanoma Associated Antigen (MAGE), -1, -2 and-3, gp100 and Tyrosinase was measured in UM cell lines. Cell lines OCM.1 and OMM1 expressed MAGE-1,-2 and -3, whereas EOM-3, Mel202, 92.1, and OMM3 did not. Gp100 was expressed in all cell lines, while Tyrosinase was not expressed in EOM.29, OMM2 and OMM3. They tested the effect of various CTLs in a complement-dependent micro-lympho cytotoxicity assay and showed the presence of HLA Class I expression on the primary UM cell lines and HLA-A1 or HLA-A2 allelic expression on the metastatic cell lines. Because the cell lines expressed HLA Class I molecules and at least two melanoma-associated antigens, they could be used as targets to improve experimental immunotherapy. Bosch et al. designed Major Histocompatibility Complex II (MHCII)-matched vaccines from UM individuals which could cross react with HLA-DR-restricted Tregs of other UM patients and induce IFNγ secretion in them, allowing for a CD8+ cell immune response in the eye [83]. In order to develop MHC II vaccines they used cell lines Mel202, Mel270 and OMM2.3 which express HLA Class I but not Class II or the invariant chain. Expression of HLA-DR and the co-stimulatory molecule CD80 was induced by transduction with retroviruses encoding HLA-DRB1*0101 (DR1) and/or the co-stimulatory molecule CD80. The MHC II generating vaccine cells were able to stimulate T cell responses, and were stable for 6 months in culture. Indoleamine-2,3-dioxygenase 1 (IDO1) is a potential immune modulator. Mondanelli et al. focused on two arginase 1 (Arg1) enzymes, both of which are involved in the regulation of the immune system [84]. When studying their activity in dendritic cells, they observed that the cytokine TGFβ was able to upregulate Arg1 and IDO1 together, with Arg1 getting upregulated earlier than IDO1. Their study led to their understanding that Arg1 is essential for the activity of IDO1 and both are expressed at the same time in dendritic cells. Li et al. used a special vector to induce IDO expression and observed that IDO suppressed HLA Class I expression in keratinocytes [85]. IDO1 is expressed in primary UM and at low levels also in metastases [86, 87]. It would be interesting to see whether induction of IDO could serve as a potential mechanism to downregulate HLA Class I and lower the tumor cell's metastatic potential in UM.

11. New Technologies Might Serve to Improve Knowledge about HLA Expression in UM

Many of the studies on HLA expression in UM used monoclonal antibodies on fresh-frozen tissue sections and mRNA expression. Nowadays, new techniques have become available that may help to further our knowledge of the immune system and improve cancer therapy. De Lange et al. showed that droplet PCR that identifies DNA differences is a very useful technique to determine tumor heterogeneity; this technique is being used to determine the percentage tumor cells in a sample, as well as the percentage of T and B cells [88, 89]. Mass cytometery (CyTOF) is another method which can assess different subpopulations of infiltrating immune cells in small tissue samples [90]. Using this technology, antigen-presenting cells can be categorized into different subsets according to their phenotype and function. Lingblom et al. used this technology to characterize immune cell populations in the blood before and after administration of a vaccine against the respiratory syncytial virus (RSV). They found differences between HLA-DR expression on both CD4+ as well as CD8+ cells between responders and non-responders to the vaccine [91]. Other new molecular techniques use genetic and RNA information to precisely obtain information of the genome and transcriptome and classify the characteristics of different diseases. RNAseq analysis of primary UM and its metastases may help to assess the differential expression of different HLA alleles, and its association with different infiltrating leukocyte types, and molecular pathways. Robertson et al. not only used RNA but also microRNA and long non-coding RNAs (lncRNA) to subdivide UM tumors according to their molecular characteristics in order to better understand how each population's molecular signature is related to clinical outcome [18]. However, this study still needed fresh-frozen tumor tissue. A recent study used paraffin-embedded tissues when applying nano-string technology for the analysis of RNA in addition to microarray data in cutaneous melanoma patients who had been treated with anti-PD1. Using this new technology, they found that a special pathway related to hypoxia was activated in hypoxic regions. This pathway induces autophagy and eventually leads to resistance of the tumor cells against T cell therapy [92]. Recently, hypoxia as defined by expression of Hypoxia-Inducible Factor 1 (HIF1a) was found to be associated with loss of BAP1 expression in UM, and may become a target for therapy [93, 94].

Using these new techniques to develop an insight in the pathophysiology of the relation between the function of BAP1, the immune system, and the development of metastasis may help to develop a treatment for UM patients.12.

Conclusions

Several studies have been performed on the role of HLA in UM and all confirm that a high HLA Class I expression is associated with a bad prognosis, in contrast to the situation in many other malignancies. A high HLA expression is associated with loss of one chromosome 3/loss of BAP1 expression, and is associated with the presence of infiltrating lymphocytes and macrophages, in spite of the eye being an immune-privileged site. It is likely that a genetically-determined upregulation of HLA antigens attracts leukocytes, which produce IFN, which further stimulates HLA Class I expression [95].

Epigenetic modifications influence expression, and loss of expression of the ubiquitin protease BAP1 is associated with an increased expression of HLA Class I antigens. It is as yet unknown why BAP1 has such a great influence on HLA expression and infiltration of immune cells in UM. Further studies are necessary to determine whether this is also relevant inside metastases. The inflammatory microenvironment in the primary tumor creates an opportunity for the UM cells to evade NK cells when they migrate haematogenously from the eye to the liver. As another strategy to avoid recognition from the immune system, UM cells may lose expression of their genetically-determined HLA antigens, with allele-, locus- or haplotype loss, which helps tumor cells to escape from cytotoxic T cells. The cell lines which are currently used in research laboratories represent many of the characteristics of primary and metastatic UM and serve as good and comparable models of UM tumors for future observations, helping to understand the regulation of HLA antigen expression. Cell line studies may be useful to identify the effect of different drugs on the expression of HLA antigens and immune-modulating proteins such as IDO1 and PD-L1. As HLA antigens are necessary for T cell therapy, knowing how they are expressed and regulated helps to understand which patients are good candidates for immunotherapy.

Author Contributions: Conceptualization, Z.S., A.G.J. and M.J.J.; Methodology, Z.S., A.P.A.W., A.M., A.G.J. and M.J.J.; Investigation, Z.S., A.M., A.G.J. and M.J.J.; Writing, Z.S., M.J.J.; Review and editing, A.P.A.W, A.M., A.G.J. and M.J.J.; Supervision, A.G.J. and M.J.J.; Project administration, M.J.J.; Funding acquisition, M.J.J.

Funding: This research was funded by a grant to Z.S. From The Iranian Ministry of Science Research and Technology and a Horizon 2020 grant nr: 667787, UM CURE 2020 from the European Community.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Krantz, B.A.; Dave, N.; Komatsubara, K.M.; Marr, B.P.; Carvajal, R.D. Uveal melanoma: Epidemiology, etiology, and treatment of primary disease. Clin. Ophthalmol. 2017, 11, 279–289.
2. Singh, A.D.; Bergman, L.; Seregard, S. Uveal Melanoma: Epidemiologic Aspects. Ophthalmol. Clin. North Am. 2005, 18, 75–84.
3. Metzelaar-Blok, J.A.W.; Ter Huurne, J.A.C.; Hurks, H.M.H.; Keunen, J.E.E.; Jager, M.J.; Gruis, N.A. Characterization of Melanocortin-1 Receptor gene variants in uveal melanoma patients. Investig. Ophthalmol. Vis. Sci. 2001, 42, 1951–1954.
4. Weis, E.; Shah, C.P.; Lajous, M.; Shields, J.A.; Shields, C.L. The association between host susceptibility factors and uveal melanoma: A meta-analysis. Arch. Ophthalmol. 2006, 124, 54–60.
5. Dogrusöz, M.; Jager, M.J.; Damato, B. Uveal melanoma treatment and prognostication. Asia Pac. J. Ophthalmol. 2017, 6, 186–196. [
6. Kaliki, S.; Shields, C.S. Uveal melanoma: Relatively rare but deadly cancer. Eye 2017, 31, 241–257.
7. Dogrusöz, M.; Jager, M.J. Genetic prognostication in uveal melanoma. Acta Ophthalmol. 2018, 96, 331–347.
8. Damato, B.; Coupland, S.E. Translating uveal melanoma cytogenetics into clinical care. Arch. Ophthalmol. 2009, 127, 423–429.
9. Kujala, E.; Mäkitie, T.; Kivelä, T. Very long-term prognosis of patients with malignant uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2003, 44, 4651–4659.
10. Hanahan, D.; Weinberg, R.A. Hallmarks of cancer: The next generation. Cell 2011, 144, 646–674.
11. Fouad, Y.A.; Aanei, C. Revisiting the hallmarks of cancer. Am. J. Cancer Res. 2017, 7, 1016–1036.

12. Colotta, F.; Allavena, P.; Sica, A.; Garlanda, C.; Mantovani, A. Cancer-related inflammation, the seventh hallmark of cancer: Links to genetic instability. Carcinogenesis 2009, 30, 1073–1081.
13. Streilein, J.W. Unraveling immune privilege. Science 1995, 270, 1158–1159.
14. De Waard-Siebinga, I.; Hilders, C.G.; Hansen, B.E.; van Delft, J.L.; Jager, M.J. HLA expression and tumor-infiltrating immune cells in uveal melanoma. Graefe's Arch. Clin. Exp. Ophthalmol. 1996, 234, 34–42.
15. Gezgin, G.; Dogrusöz, M.; van Essen, T.H.; Kroes, W.G.; Luyten, G.P.; van der Velden, P.A.; Walter, V.; Verdijk, R.M.; van Hall, T.; van der Burg, S.H.; et al. Genetic evolution of uveal melanoma guides the development of an inflammatory microenvironment. Cancer Immunol. Immunother. 2017, 66, 903–912.
16. Jager, M.J.; Hurks, H.M.; Levitskaya, J.; Kiessling, R. HLA expression in uveal melanoma: There is no rule without some exception. Hum. Immunol. 2002, 63, 444–451.
17. Maat, W.; Ly, L.V.; Jordanova, E.S.; de Wolff-Rouendaal, D.; Schalij-Delfos, N.E.; Jager, M.J. Monosomy of chromosome 3 and an inflammatory phenotype occur together in uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2008, 49, 505–510.
18. Robertson, A.G.; Shih, J.; Yau, C.; Gibb, E.A.; Oba, J.; Mungall, K.L.; Hess, J.M.; Uzunangelov, V.; Walter, V.; Danilova, L.; et al. Integrative analysis identifies four molecular and clinical subsets in uveal melanoma. Cancer Cell 2017, 32, 204–220.
19. Bronkhorst, I.H.; Jager, M.J. Uveal melanoma: The inflammatory microenvironment. J. Innate Immunol. 2012, 4, 454–462.
20. Harbour, J.W.; Onken, M.D.; Roberson, E.D.; Duan, S.; Cao, L.; Worley, L.A.; Council, M.L.; Matatall, K.A.; Helms, C.; Bowcock, A.M. Frequent mutation of BAP1 in metastasizing uveal melanomas. Science 2010, 330, 1410–1413.
21. Van Essen, T.H.; van Pelt, S.I.; Versluis, M.; Bronkhorst, I.H.G.; van Duinen, S.G.; Marinkovic, M.; Kroes, W.G.M.; Ruivenkamp, C.A.L.; de Klein, A.; Kilic, E.; et al. Prognostic parameters in Uveal Melanoma and their association with BAP1 expression. Br. J. Ophthalmol. 2014, 98, 1738–1743.
22. Szalai, E.; Wells, J.R.; Ward, L.; Grossniklaus, H.E. Uveal melanoma nuclear BRCA-1-Associated Protein-1 Immunoreactivity is an indicator of metastasis. Ophthalmology 2018, 125, 203–209.
23. Mosaad, Y.M. Clinical role of human leukocyte antigen in health and disease. Scand J. Immunol. 2015, 82, 283–306.

24. Ruiter, D.J.; Bergman, W.; Welvaart, K.; Scheffer, E.; van Vloten, W.A.; Russo, C.; Ferrone, S. Immunohistochemical analysis of malignant melanomas and nevocellular nevi with monoclonal antibodies to distinct monomorphic determinants of HLA antigens. Cancer Res. 1984, 44, 3930–3935.
25. Jager, M.J.; de Wolff-Rouendaal, D.; Breebaart, A.C.; Ruiter, D.J. Expression of HLA antigens in paraffin sections of uveal melanomas. Doc. Ophthalmol. 1986, 64, 69–76.
26. De Waard-Siebinga, I.; Houbiers, J.G.; Hilders, C.G.; de Wolff-Rouendaal, D.; Jager, M.J. Differential expression of HLA-A and B-alleles on uveal melanoma as determined by immuno-histology. Ocul. Immunol. Inflamm. 1996, 4, 1–14.
27. Blom, D.J.; Luyten, G.P.; Mooy, C.; Kerkvliet, S.; Zwinderman, A.H.; Jager, M.J. Human leukocyte antigen class I expression. Marker of poor prognosis in uveal melanoma. Investig. Ophthalmol. Vis. Sci. 1997, 38, 1865–1872.
28. Ma, D.; Niederkorn, J.Y. Transforming growth factor-beta down-regulates major histocompatibility complex class I antigen expression and increases the susceptibility of uveal melanoma cells to natural killer cell-mediated cytolysis. Immunology 1995, 86, 263–269.
29. Ericsson, C.; Seregard, S.; Bartolazzi, A.; Levitskaya, E.; Ferrone, S.; Kiessling, R.; Larsson, O. Association of HLA class I and class II antigen expression and mortality in uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2001, 42, 2153–2156.
30. Dithmar, S.; Crowder, J.; Jager, M.J.; Vigniswaran, N.; Grossniklaus, H.E. HLA class I antigen expression correlates with histological cell type in uveal melanoma. Der Ophthalmol. 2002, 99, 625–628.
31. Stam, N.J.; Vroom, T.M.; Peters, P.J.; Pastoors, E.B.; Ploegh, H.L. HLA-A-and HLA-B-specific monoclonal antibodies reactive with free heavy chains in western blots, in formalin-fixed, paraffin-embedded tissue sections and in cryo-immuno-electron microscopy. Int. Immunol. 1990, 2, 113–125.
32. Meecham, W.J.; Char, D.H.; Kaleta-Michaels, S. Infiltrating lymphocytes and antigen expression in uveal melanoma. Ophthalmol. Res. 1992, 24, 20–26.
33. Van Essen, T.H.; van Pelt, S.I.; Bronkhorst, I.H.; Versluis, M.; Némati, F.; Laurent, C.; Luyten, G.P.; van Hall, T.; van den Elsen, P.J.; van der Velden, P.A.; et al. Upregulation of HLA expression in primary uveal melanoma by infiltrating leukocytes. PLoS ONE 2016, 11.

34. Anastassiou, G.; Rebmann, V.; Wagner, S.; Bornfeld, N.; Grosse-Wilde, H. Expression of classic and nonclassic HLA class I antigens in uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2003, 44, 2016–2019.
35. Hurks, H.M.; Metzelaar-Blok, J.A.; Mulder, A.; Claas, F.H.; Jager, M.J. High frequency of allele-specific down-regulation of HLA class I expression in uveal melanoma cell lines. Int. J. Cancer 2000, 85, 697–702.
36. De Waard-Siebinga, I.; Creyghton, W.M.; Kool, J.; Jager, M.J. Effects of interferon alfa and gamma on human uveal melanoma cells in vitro. Br. J. Ophthalmol. 1995, 79, 847–855.
37. Jager, M.J.; Magner, J.A.; Ksander, B.R.; Dubovy, S.R. Uveal melanoma cell lines: Where do they come from? (An American Ophthalmological Society Thesis). Trans. Am. Ophthalmol. Soc. 2016, 114, T5.
38. Van Essen, T.H.; Bronkhorst, I.H.; Maat, W.; Verduyn, W.; Roelen, D.L.; Luyten, G.P.; Jager, M.J. A comparison of HLA genotype with inflammation in uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2012, 53, 2640–2646.
39. Versteeg, R.; Krüse-Wolters, K.M.; Plomp, A.C.; Van Leeuwen, A.; Stam, N.J.; Ploegh, H.L.; Ruiter, D.J.; Schrier, P.I. Suppression of class I human histocompatibility leukocyte antigen by c-myc is locus specific. J. Exp. Med. 1989, 170, 621–635.
40. Blom, D.J.; Mooy, C.M.; Luyten, G.P.; Kerkvliet, S.; Ouwerkerk, I.; Zwinderman, A.H.; Schrier, P.I.; Jager, M.J. Inverse correlation between expression of HLA-B and c-myc in uveal melanoma. J. Pathol. 1997, 181, 75–79.
41. Kaklamanis, L.; Townsend, A.; Doussis-Anagnostopoulou, I.A.; Mortensen, N.; Harris, A.L.; Gatter, K.C. Loss of major histocompatibility complex-encoded transporter associated with antigen presentation (TAP) in colorectal cancer. Am. J. Pathol. 1994, 145, 505–509.
42. Ling, A.; Löfgren-Burström, A.; Larsson, P.; Li, X.; Wikberg, M.L.; Öberg, Å.; Stenling, R.; Edin, S.; Palmqvist, R. TAP1 down-regulation elicits immune escape and poor prognosis in colorectal cancer. Oncoimmunology 2017, 6.
43. Dissemond, J.; Götte, P.; Mörs, J.; Lindeke, A.; Goos, M.; Ferrone, S.; Wagner, S.N. Association of TAP1 downregulation in human primary melanoma lesions with lack of spontaneous regression. Melanoma Res. 2003, 13, 253–258.

44. Kamarashev, J.; Ferrone, S.; Seifert, B.; Böni, R.; Nestle, F.; Burg, G.; Dummer, R. TAP1 down-regulation in primary melanoma lesions: An independent marker of poor prognosis. Int J. Cancer 2001, 95, 23–28.
45. Krishnakumar, S.; Abhyankar, D.; Sundaram, A.L.; Pushparaj, V.; Shanmugam, M.P.; Biswas, J. Major histocompatibility antigens and antigen-processing molecules in uveal melanoma. Clin. Cancer Res. 2003, 9, 4159–4164.
46. Girdlestone, J.; Isamat, M.; Gewert, D.; Milstein, C. Transcriptional regulation of HLA-A and-B: Differential binding of members of the Rel and IRF families of transcription factors. Proc. Natl. Acad. Sci. USA 1993, 90, 11568–11572.
47. Johnson, D.R.; Pober, J.S. HLA class I heavy-chain gene promoter elements mediating synergy between tumor necrosis factor and interferons. Mol. Cell. Biol. 1994, 14, 1322–1332.
48. Blais, M.E.; Dong, T.; Rowland-Jones, S. HLA-C as a mediator of natural killer and T-cell activation: Spectator or key player? Immunology 2011, 133, 1–7.
49. Ugurel, S.; Rebmann, V.; Ferrone, S.; Tilgen, W.; Grosse-Wilde, H.; Reinhold, U. Soluble human leukocyte antigen-G serum level is elevated in melanoma patients and is further increased by interferon-α immunotherapy. Cancer 2001, 92, 369–376.
50. Derré, L.; Corvaisier, M.; Charreau, B.; Moreau, A.; Godefroy, E.; Moreau-Aubry, A.; Jotereau, F.; Gervois, N. Expression and release of HLA-E by melanoma cells and melanocytes: Potential impact on the response of cytotoxic effector cells. J. Immunol. 2006, 177, 3100–3107.
51. Rouas-Freiss, N.; Moreau, P.; Ferrone, S.; Carosella, E.D. HLA-G proteins in cancer: Do they provide tumor cells with an escape mechanism? Cancer Res. 2005, 65, 10139–10144.
52. Hurks, H.M.; Valter, M.M.; Wilson, L.; Hilgert, I.; van den Elsen, P.J.; Jager, M.J. Uveal melanoma: No expression of HLA-G. Investig. Ophthalmol. Vis. Sci. 2001, 42, 3081–3084.
53. Metzelaar-Blok, J.A.; Hurks, H.M.; Naipal, A.; de Lange, P.; Keunen, J.E.; Claas, F.H.; Doxiadis, I.; Jager, M.J. Normal HLA class I, II, and MICA gene distribution in uveal melanoma patients. Mol. Vis. 2005, 11, 1166–1172.
54. Maat, W.; Haasnoot, G.W.; Claas, F.H.; Schalij-Delfos, N.E.; Schreuder, G.M.; Jager, M.J. HLA Class I and II genotype in uveal melanoma: Relation to occurrence and prognosis. Investig. Ophthalmol. Vis. Sci. 2006, 47, 3–6.
55. Jager, M.J.; Völker-Dieben, H.J.; de Wolff-Rouendaal, D.; Kakebeeke-Kemme, H.; D'Amaro, J. Possible relation between HLA and ABO type and prognosis of uveal melanoma. Doc. Ophthalmol. 1992, 82, 43–47.

56. Helgadottir, H.; Höiom, V. The genetics of uveal melanoma: Current insights. Appl. Clin. Genet. 2016, 9, 147–155.
57. Kilic, E.; van Gils, W.; Lodder, E.; Beverloo, H.B.; van Til, M.E.; Mooy, C.M.; Paridaens, D.; de Klein, A.; Luyten, G.P.M. Clinical and cytogenetic analyses in uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2006, 47, 3703–3707.
58. Shields, C.L.; Say, E.A.; Hasanreisoglu, M.; Saktanasate, J.; Lawson, B.M.; Landy, J.E.; Badami, A.U.; Sivalingam, M.D.; Mashayekhi, A.; Shields, J.A.; et al. Cytogenetic abnormalities in uveal melanoma based on tumor features and size in 1059 patients: The 2016 W. Richard Green Lecture. Ophthalmology 2017, 124, 609–618.
59. Cassoux, N.; Rodrigues, M.J.; Plancher, C.; Asselain, B.; Levy-Gabriel, C.; Lumbroso-Le Rouic, L.; Piperno-Neumann, S.; Dendale, R.; Sastre, X.; Desjardins, L.; et al. Genome-wide profiling is a clinically relevant and affordable prognostic test in posterior uveal melanoma. Br. J. Ophthalmol. 2014, 98, 769–774.
60. Versluis, M.; de Lange, M.J.; van Pelt, S.I.; Ruivenkamp, C.A.; Kroes, W.G.; Cao, J.; Jager, M.J.; Luyten, G.P.M.; van der Velden, P.A. Digital PCR validates 8q dosage as prognostic tool in uveal melanoma. PLoS ONE 2015, 10.
61. Singh, N.; Singh, A.D.; Hide, W. Inferring an evolutionary tree of uveal melanoma from genomic copy number aberrations. Investig. Ophthalmol. Vis. Sci. 2015, 56, 6801–6809.
62. Metzelaar-Blok, J.A.W.; Jager, M.J.; Hanifi Moghaddam, P.; van der Slik, A.; Giphart, M.J. Frequent loss of heterozygosity on chromosome 6p in uveal melanoma. Hum. Immunol. 1999, 60, 962–969.
63. Blom, D.J.; Schurmans, L.R.; de Waard-Siebinga, I.; de Wolff-Rouendaal, D.; Keunen, J.E.; Jager, M.J. HLA expression in a primary uveal melanoma, its cell line, and four of its metastases. Br. J. Ophthalmol. 1997, 81, 989–993.
64. Verbik, D.J.; Murray, T.G.; Tran, J.M.; Ksander, B.R. Melanomas that develop within the eye inhibit lymphocyte proliferation. Int J. Cancer 1997, 73, 470–478.
65. Gezgin, G.; Luk, S.J.; Cao, J.; Dogrusöz, M.; van der Steen, D.M.; Hagedoorn, R.S.; Krijgsman, D.; van der Velden, P.A.; Field, M.G.; Luyten, G.P.; et al. PRAME as a potential target for immunotherapy in metastatic uveal melanoma. JAMA Ophthalmol. 2017, 135, 541–549.

66. Field, M.G.; Decatur, C.L.; Kurtenbach, S.; Gezgin, G.; van der Velden, P.A.; Jager, M.J.; Kozak, K.N.; Harbour, J.W. PRAME as an independent biomarker for metastasis in uveal melanoma. Clin. Cancer Res. 2016, 22, 1234–1242.
67. Meir, T.; Dror, R.; Yu, X.; Qian, J.; Simon, I.; Pe'er, J.; Chowers, I. Molecular characteristics of liver metastases from uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2007, 48, 4890–4896.
68. De Waard-Siebinga, I.; Kool, J.; Jager, M.J. HLA antigen expression on uveal melanoma cells in vivo and in vitro. Hum. Immunol. 1995, 44, 111–117.
69. De Waard-Siebinga, I.; Blom, D.J.; Griffioen, M.; Schrier, P.I.; Hoogendoorn, E.; Beverstock, G.; Danen, E.H.; Jager, M.J. Establishment and characterization of an uveal-melanoma cell line. Int. J. Cancer 1995, 62, 155–161.
70. Seliger, B.; Kloor, M.; Ferrone, S. HLA class II antigen-processing pathway in tumors: Molecular defects and clinical relevance. Oncoimmunology 2017, 6.
71. Krishnakumar, S.; Abhyankar, D.; Lakshmi, S.A.; Shanmugam, M.P.; Pushparaj, V.; Biswas, J. HLA class II antigen expression in uveal melanoma: Correlation with clinicopathological features. Exp. Eye Res. 2003, 77, 175–180.
72. Jager, M.J.; van der Pol, J.P.; de Wolff-Rouendaal, D.; de Jong, P.T.V.M.; Ruiter, D.J. Decreased expression of HLA Class II antigens on human uveal melanoma cells after in vivo X-ray irradiation. Am. J. Ophthalmol. 1988, 105, 78–86.
73. Radosevich, M.; Jager, M.; Ono, S.J. Inhibition of MHC class II gene expression in uveal melanoma cells is due to methylation of the CIITA gene or an upstream activator. Exp. Mol. Pathol. 2007, 82, 68–76.
74. Holling, T.M.; Schooten, E.; Langerak, A.W.; van den Elsen, P.J. Regulation of MHC class II expression in human T-cell malignancies. Blood 2004, 103, 1438–1444.
75. Holling, T.M.; Bergevoet, M.W.; Wilson, L.; van Eggermond, M.C.; Schooten, E.; Steenbergen, R.D.; Snijders, P.J.; Jager, M.J.; van den Elsen, P.J. A role for EZH2 in silencing of IFN-γ inducible MHC2TA transcription in uveal melanoma. J. Immunol. 2007, 179, 5317–5325.
76. Boyd, N.H.; Morgan, J.E.; Greer, S.F. Polycomb recruitment at the Class II transactivator gene. Mol. Immunol. 2015, 67, 482–491.
77. Hehlgans, T.; Strominger, J.L. Activation of transcription by binding of NF-E1 (YY1) to a newly identified element in the first exon of the human DR alpha gene. J. Immunol. 1995, 154, 5181–5187.

78. Griffioen, M.; Ouwerkerk, I.J.; Harten, V.; Schrier, P.I. HLA-B locus-specific downregulation in human melanoma requires enhancer A as well as a sequence element located downstream of the transcription initiation site. Immunogenetics 2000, 52, 121–128.
79. Blom, D.J.; Schuitmaker, H.J.; de Waard-Siebinga, I.; Dubbelman, T.M.; Jager, M.J. Decreased expression of HLA class I on ocular melanoma cells following in vitro photodynamic therapy. Cancer Lett. 1997, 112, 239–243.
80. Blom, D.J.; de Waard-Siebinga, I.; Apte, R.S.; Luyten, G.P.; Niederkorn, J.Y.; Jager, M.J. Effect of hyperthermia on expression of histocompatibility antigens and heat-shock protein molecules on three human ocular melanoma cell lines. Melanoma Res. 1997, 7, 103–109.
81. Sutmuller, R.P.; Schurmans, L.R.; van Duivenvoorde, L.M.; Tine, J.A.; van der Voort, E.I.; Toes, R.E.; Melief, C.J.; Jager, M.J.; Offringa, R. Adoptive T cell immunotherapy of human uveal melanoma targeting gp100. J. Immunol. 2000, 165, 7308–7315.
82. Luyten, G.P.M.; van der Spek, C.W.; Brand, I.; Sintnicolaas, K.; de Waard-Siebinga, I.; Jager, M.J.; de Jong, P.T.; Schrier, P.I.; Luider, T.M. Expression of MAGE, gp100 and tyrosinase genes in uveal melanoma cell lines. Melanoma Res. 1998, 8, 11–16.
83. Bosch, J.J.; Thompson, J.A.; Srivastava, M.K.; Iheagwara, U.K.; Murray, T.G.; Lotem, M.; Ksander, B.R.; Ostrand-Rosenberg, S. MHC Class II-transduced tumor cells originating in the immune-privileged eye prime and boost CD4+ T lymphocytes that cross-react with primary and metastatic uveal melanoma cells. Cancer Res. 2007, 67, 4499–4506.
84. Mondanelli, G.; Bianchi, R.; Pallotta, M.T.; Orabona, C.; Albini, E.; Iacono, A.; Belladonna, M.L.; Vacca, C.; Fallarino, F.; Macchiarulo, A.; et al. A relay pathway between Arginine and Tryptophan metabolism confers immunosuppressive properties on dendritic cells. Immunity 2017, 46, 233–244.
85. Li, Y.; Tredget, E.E.; Ghahary, A. Cell surface expression of MHC Class I antigen is suppressed in Indoleamine 2,3-Dioxygenase genetically modified keratinocytes: Implications in allogeneic skin substitute engraftment. Hum. Immunol. 2004, 65, 114–123.
86. Chen, P.W.; Mellon, J.K.; Mayhew, E.; Wang, S.; He, Y.G.; Hogan, N.; Niederkorn, J.Y. Uveal melanoma expression of indoleamine 2,3-deoxygenase: Establishment of an immune privileged environment by tryptophan depletion. Exp. Eye Res. 2007, 85, 617–625.
87. Stalhammer, G.; Seregard, S.; Grossniklaus, H.E. Expression of immune checkpoint receptors Indolamine 2,3-dioxygenase and T cell ig and ITIM domain in metastatic versus nonmetastatic choroidal melanoma. Cancer Med. 2019, 8, 2784–2792.

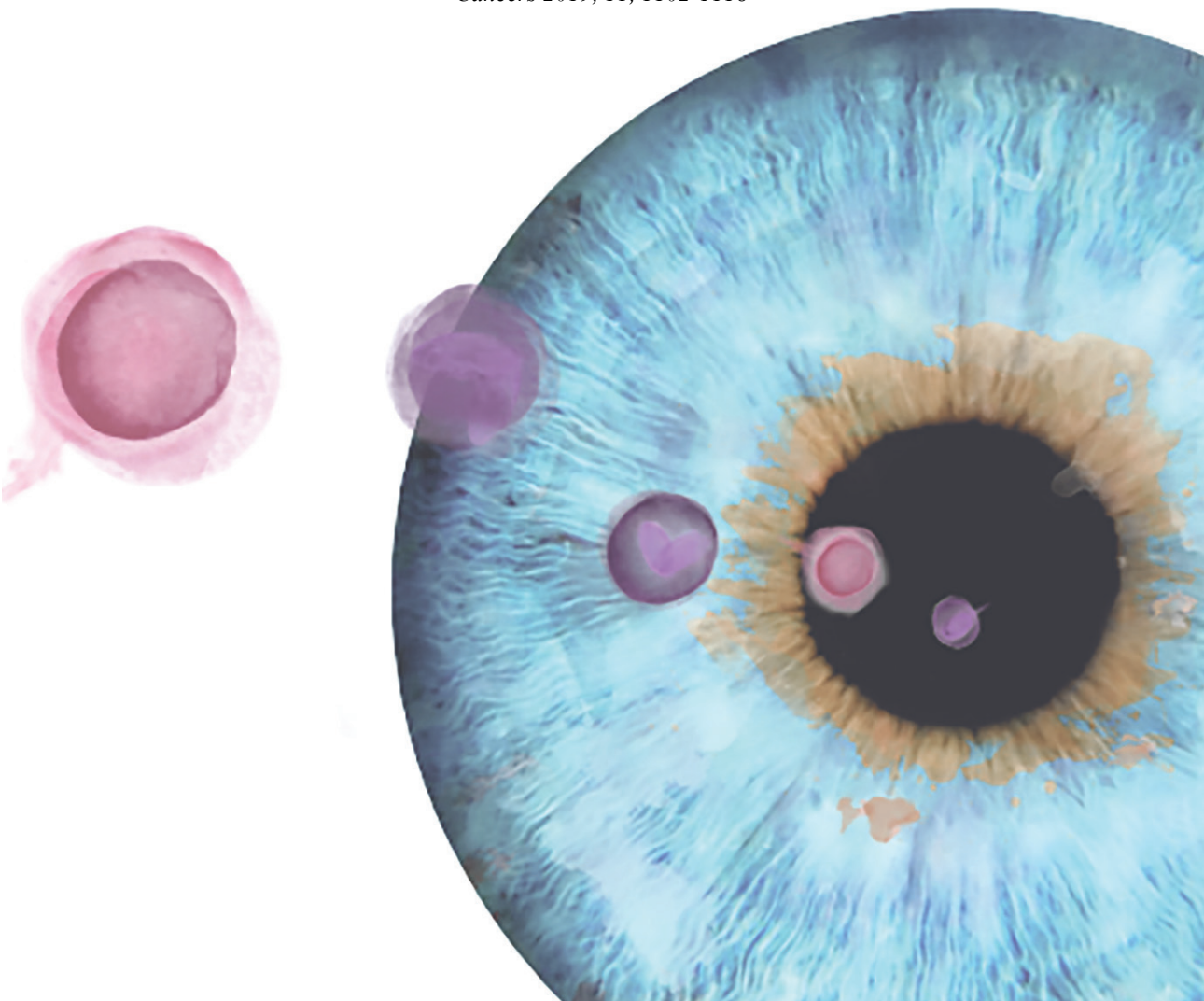
88. Zoutman, W.H.; Nell, R.J.; van der Velden, P.A. Usage of Droplet Digital PCR (ddPCR) assays for T cell quantification in cancer. Methods Mol. Biol. 2019, 1884, 1–14.
89. De Lange, M.J.; Nell, R.J.; Lalai, R.N.; Versluis, M.; Jordanova, E.S.; Luyten, G.P.M.; Jager, M.J.; van der Burg, S.H.; Zoutman, W.H.; van Hall, T.; et al. Digital PCR-based T cell quantification-assisted deconvolution of the microenvironment reveals that activated macrophages drive tumor inflammation in uveal melanoma. Mol. Cancer Res. 2018, 16, 1902–1911.
90. Subrahmanyam, P.B.; Maecker, H.T. CyTOF measurement of immunocompetence across Major Immune Cell Types. Curr. Protoc. Cytom. 2018, 82, 9–54.
91. Lingblom, C.M.D.; Kowli, S.; Swaminathan, N.; Maecker, H.T. Baseline immune profile by CyTOF can predict response to an investigational adjuvanted vaccine in elderly adults. J. Transl. Med. 2018, 16, 153–164.
92. Buart, S.; Terry, S.; Noman, M.Z.; Lanoy, E.; Boutros, C.; Fogel, P.; Dessen, P.; Meurice, G.; Gaston-Mathé, Y.; Vielh, P.; et al. Transcriptional response to hypoxic stress in melanoma and prognostic potential of GBE1 and BNIP3. Oncotarget 2017, 8, 108786–108801.
93. Brouwer, N.J.; Wierenga, A.P.A.; Gezgin, G.; Marinkovic, M.; Luyten, G.P.M.; Kroes, W.G.M.; Versluis, M.; van der Velden, P.A.; Verdijk, R.M.; Jager, M.J. Ischemia Is Related to Tumour Genetics in Uveal Melanoma. Cancers 2019, 11, 1004.
94. Dong, L.; You, S.; Zhang, Q.; Osuka, S.; Devi, N.S.; Kaluz, S.; Ferguson, J.H.; Yang, H.; Chen, G.; Wang, B.; et al. Arylsulfonamide 64B inhibits hypoxia/HIF-induced expression of c-Met and CXCR4 and reduces primary tumor growth and metastasis of uveal melanoma. Clin Cancer Res. 2019, 25, 2206–2218.
95. Souri, Z.; Wierenga, A.P.A.; van Weeghel, C.; van der Velden, P.A.; Kroes, W.G.M.; Luyten, G.P.M.; van der Burg, S.H.; Jochemsen, A.G.; Jager, M.J. Loss of BAP1 Is Associated with Upregulation of the NFkB Pathway and Increased HLA Class I Expression in Uveal Melanoma. Cancers 2019, 11, 1102.

Chapter 3

Loss of BAP1 is associated with upregulation of the NFkB pathway and increased HLA Class I expression in Uveal Melanoma

Zahra Souri, Annemijn P. A. Wierenga, Christiaan van Weeghel, Pieter A. van der Velden,Wilma G. M. Kroes, Gregorius P. M. Luyten, Sjoerd H. van der Burg, Aart G. Jochemsen, Martine J. Jager

Cancers 2019, 11, 1102-1118



Abstract

One of the characteristics of prognostically infaust uveal melanoma (UM) is an inflammatory phenotype, which is characterized by high numbers of infiltrating T cells and macrophages, and a high HLA Class I expression. We wondered how this inflammation is regulated, and considered that one of the most important regulators of inflammation, the NFkB pathway, might play a role. We analyzed 64 UM samples for expression of HLA Class I, its regulators, and of members of the NFkB transcription family, using an Illumina HT12V4 array. HLA Class I expression and infiltrating immune cells were also determined by immune histochemical staining. Information was obtained regarding chromosome status by Affymetrix Nsp array. Our analysis shows that expression of NFkB1, NFkB2 and RELB positively correlates with the level of HLA Class I expression and the number of infiltrating T cells and macrophages, while SPP1 and PPARγ are negatively correlated. Increased levels of NFkB1 and NFkB2 and decreased levels of SPP1 and PPARγ are seen in Monosomy 3/BAP1-negative tumors. This is also the case in non-inflammatory UM, indicating that our observation not only involves infiltrating leukocytes but the tumor cells themselves. We report that the NFkB pathway is associated with inflammation and HLA Class I expression in UM, and is upregulated when BAP1 expression is lost.

Keywords: Uveal Melanoma, Inflammation, HLA Class I, NFkB pathway, BAP1, Oncology

1. Introduction

Uveal melanoma (UM) is a malignancy that originates from melanocytes in the eye, and 50% of the patients will ultimately develop metastases [1,2]. Immune cell infiltration, secretion of inflammatory cytokines and an increase in HLA expression are frequently observed in UM, leading to an inflamed tumor environment, inside the immune-privileged eye [3]. This inflammatory phenotype is linked to an increased risk for metastasis [4,5,6,7], and has been associated with loss of chromosome 3 [8,9]. It was recently noticed that an extra copy of chromosome 8q in the tumor is associated with an influx of macrophages, while loss of chromosome 3 (Monosomy 3, M3) correlates with an increased influx of T cells [10]. M3 occurs in almost 50% of patients and is associated with a bad prognosis [11-13]. Almost all cases with

M3 also show gain of chromosome 8q, but this may even occur in tumors with two chromosomes 3 (Disomy 3, D3); it is considered a bad prognostic sign [14,15,16]. Another alteration is a gain of 6p, which occurs in 25–40% of cases, and is a sign of good prognosis [17,18].

Besides chromosomal changes, important genetic driver mutations have been identified: these include activating mutations in GNAQ and GNA11 [19,20], which are thought to lead to the transformation of melanocytic cells by upregulation of YAP1 [21]. These mutations are already seen in choroidal nevi, where YAP upregulation is also present [22]. Loss of expression of a key tumor suppressor gene, BAP1, encoding a deubiquitinating protein and located on chromosome 3, together with the loss of the other chromosome 3, is strongly correlated with the development of metastases [23]. Major signalling pathways such as the Rb, PI3K/Akt, MAPK and NFkB pathways are frequently dysregulated in prognostically bad UM [24,25,26]. This dysregulation will lead to inhibition of apoptosis and stimulation of cell proliferation, while the cells will become more invasive and tend to migrate to other organs.

Inflammation is considered as one of the hallmarks of cancer [27], but its regulation is not always clear. Although in UM a relation is seen between M3/loss of BAP1 expression and the presence of an inflammatory phenotype, little is known about the pathways which regulate this inflammation. One of the major regulators of inflammation in cancer is the NFkB-signalling pathway [28]. Members of the NFkB transcription family include RELA (p65), RELB, c-REL, NFkB1 (p105/p50), and NFkB2 (p100/p52), which are regulated by proteins such as IKK and NEMO (IkBkG). The canonical pathway, in which NFkB1 is the main player, is involved in many different aspects of the immune system while the non-canonical pathway, in which NFkB2 is involved, regulates specific functions of the adaptive immune system.

During tumorigenesis, the NFkB pathway is often upregulated and leads to uncontrolled proliferation, apoptosis resistance, angiogenesis, inhibition of senescence, invasion, metastasis and resistance to different treatments [28]. P53 and PTEN proteins can function as negative regulators of NFkB signalling and mutations in these genes can affect the pathway's activity. Other oncogenic mutations, such as amplifications and point mutations in RELA and other NFkB signalling genes, have been identified in several lymphoid malignancies, and give rise to inflammation [29]. Stromal M2 macrophages produce various cytokines and play a major role in NFkB upregulation.

In cutaneous melanoma, the NFkB pathway is known as a molecular switch which is turned on during tumor progression [30,31].

Both the canonical and non-canonical NFkB pathway have been shown to be active in primary and metastatic UM and are related to progression of the disease [32,33,34], while inhibition of NFkB has been shown to reduce cell proliferation. As far as we know, the relation between NFkB signalling, HLA Class I expression and genetic tumor progression in UM has not been reported. As we observed in our previous studies that genetic aberrations in UM are related to progression of the inflammatory environment, we set out to investigate whether NFkB plays a role in the expression of HLA Class I and whether the expression of NFkB itself is related to the tumor's chromosome 3/BAP1 status.

2. Results

2.1. HLA Expression is related to NFkB Pathway Molecules

As an increased HLA expression is one of the hallmarks of the inflammatory phenotype in UM, we wondered whether the NFkB pathway plays a role in the expression of HLA Class I (A, B) molecules. We investigated this by analysis of Illumina mRNA expression data. As we had several probes for the HLA Class I markers, we compared these probes with previously acquired immunohistochemistry data (Supplementary Table S1) [8, 35, and 36]. Three of four mRNA probes for gene expression of HLA-A correlated with the immune histochemical data on HLA-A. The probe for HLA-B correlated with HC10 staining ($p = 0.001$). Using the three best HLA-A probes and the HLA-B probe, we determined the relation between the NFKB pathway and HLA expression in UM (Table 1).

Significant positive correlations were seen between most of the NFkB markers and HLA Class I: NFkB1, NFkB2-pr1 (probe 1) and RELB had a significant positive correlation with HLA-A and HLA-B. SPP1-pr2 (probe 2) showed a negative correlation with HLA-B, while PPARγ-pr1 showed a negative correlation with HLA-A.

Table 1. Correlation between mRNA expression levels of different NFkB pathway molecules with mRNA expression markers of HLA-A and HLA-B. r = two-tailed Spearman correlation coefficient. $p \leq 0.05$ is considered significant. Bonferroni correction was applied.

Markers	HLA-A Probe 1		HLA-A Probe 2		HLA-A Probe 3		HLA-B	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
NFkB1	0.438	0.001	0.454	0.001	0.358	0.04	0.433	0.001
NFkB2, pr1	0.417	0.01	0.403	0.01	0.258	0.40	0.395	0.01
NFkB2, pr2	0.239	0.57	0.285	0.22	0.171	1	0.254	0.43
RELA	−0.055	1	−0.270	0.31	−0.280	0.25	−0.147	1
RELB	0.343	0.05	0.327	0.08	0.429	0.001	0.419	0.01
SPP1, pr1	−0.285	0.23	−0.303	0.15	−0.241	0.55	−0.325	0.09
SPP1, pr2	−0.311	0.12	−0.335	0.07	−0.263	0.36	−0.346	0.05
PPARγ, pr1	−0.325	0.09	−0.355	0.04	−0.363	0.03	−0.327	0.08
PPARγ, pr2	−0.294	0.18	−0.338	0.06	−0.327	0.08	−0.265	0.35
IkBkG	0.188	1	0.039	1	0.250	0.47	0.235	0.62

2.2. NFkB Pathway is Associated with HLA Class I Regulatory Factors and the Antigen-Loading Machinery

In order to further investigate the relationship between HLA Class I expression and the NFkB pathway, we investigated a putative relation between the NFkB-signalling pathway molecules and HLA Class I regulatory factors (Table 2). CIITA and NLRC5 are both known as regulators of HLA Class I expression. IRF1, IRF2 and IRF8 are all transcription factors which specifically bind to the interferon response element and are known to play a role in the induction of HLA Class I expression in many tissues [35]. TAP1, TAP2, tapasin and calreticulin all belong to the antigen-loading machinery and play a role in the assembly of HLA Class I molecules on the cell surface [37].NFkB1 showed a correlation with IRF1 and TAP1, while NFkB2 had positive correlations with TAP1 and calreticulin. RELA did not show any significant correlations while RELB showed positive correlations with IRF1, IRF8, TAP1 and TAP2. Both probes of SPP1 showed negative correlations with IRF1 and TAP1. Both probes of PPARγ showed negative correlations with IRF1, IRF8 and TAP1.

Table 2. Correlation between mRNA expression of NFkB pathway molecules and the antigen-presenting machinery and known regulators of HLA Class I expression, in a cohort of 64 primary UM. r = two-tailed Spearman correlation coefficient; $p \leq 0.05$ is considered significant. Bonferroni correction was applied.

Markers	CIITA		NLRC5		IRF1		IRF2		IRF2, pr2		IRF8		TAP1		TAP2		Tapasin		Calreticulin	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
NFkB1	0.212	0.93	0.091	1	0.346	0.05	0.123	1	0.248	0.48	0.236	0.61	0.396	0.01	0.086	1	0.240	0.56	0.342	0.06
NFkB2, pr1	0.261	0.37	0.205	1	0.327	0.08	0.143	1	0.081	1	0.242	0.54	0.409	0.01	0.052	1	0.208	0.99	0.393	0.01
NFkB2, pr2	0.290	0.20	0.191	1	0.238	0.59	0.215	0.89	0.194	1	0.219	0.82	0.320	0.10	0.055	1	0.326	0.09	0.344	0.05
RELA	−0.249	0.48	−0.122	1	−0.180	1	0.145	1	−0.010	1	−0.175	1	−0.206	1	0.118	1	−0.011	1	0.281	0.24
RELB	0.106	1	0.262	0.37	0.449	0.001	0.122	1	0.082	1	0.439	0.001	0.440	0.001	0.444	0.001	0.267	0.33	0.134	1
SPP1, pr1	−0.255	0.42	−0.175	1	−0.394	0.01	−0.119	1	−0.081	1	−0.305	0.14	−0.439	0.001	−0.258	0.40	−0.102	1	−0.081	1
SPP1, pr2	−0.252	0.45	−0.174	1	−0.406	0.01	−0.134	1	−0.067	1	−0.294	0.18	−0.462	0.001	−0.260	0.38	−0.107	1	−0.089	1
PPARγ, pr1	−0.172	1	−0.229	0.68	−0.372	0.02	−0.062	1	0.058	1	−0.394	0.01	−0.443	0.001	−0.299	0.17	−0.259	0.39	0.147	1
PPARγ, pr2	−0.152	1	−0.218	0.83	−0.346	0.05	−0.082	1	−0.020	1	−0.345	0.05	−0.384	0.02	−0.263	0.36	−0.215	0.88	0.138	1
IkBkG	0.022	1	0.215	0.87	0.167	1	0.035	1	0.266	0.34	0.279	0.25	0.084	1	0.298	0.17	0.209	0.98	0.067	1

2.3. Expression of NFkB Pathway Molecules is related to the Presence of Infiltrating Leukocytes

In order to investigate whether the NFkB pathway is involved in the regulation of leukocyte influx, we determined whether any correlation could be found between expression of members of the NFkB pathway and markers of infiltrate (determined by immunohistochemistry and mRNA analysis). Since several probes for T cell and macrophage markers were available, we looked at data from a comparison between previous immunofluorescence results and gene expression data and selected the probes with the highest correlation [9,10,38]. We decided to use the following markers to determine the presence of infiltrating leukocytes: CD3E and CD3D1 (part of the T-cell receptor-CD3 complex as a marker for T cells), CD4 as a marker for T-helper cells and macrophages, CD8A as a marker for cytotoxic T cells, and CD68 and CD163 as markers for monocytes and macrophages (Table 3).

Table 3. Correlation between mRNA expression levels of various NFkB pathway molecules with mRNA expression markers of different types of infiltrating immune cells. r = two-tailed Spearman correlation coefficient. $p \leq 0.05$ is considered significant. Bonferroni correction was applied.

Markers	CD3E		CD3D1		CD4		CD8A		CD68		CD163	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
NFkB1	0.398	0.01	0.298	0.17	0.227	0.71	0.365	0.03	0.172	1	0.009	1
NFkB2, pr1	0.378	0.02	0.213	0.91	0.195	1	0.258	0.39	0.375	0.02	−0.086	1
NFkB2, pr2	0.361	0.03	0.242	0.54	0.278	0.26	0.267	0.33	0.411	0.01	0.040	1
RELA	0.110	1	−0.095	1	−0.119	1	−0.125	1	−0.062	1	−0.065	1
RELB	0.363	0.03	0.365	0.03	0.308	0.13	0.327	0.08	0.149	1	0.317	0.11
SPP1, pr1	−0.295	0.18	−0.247	0.49	−0.337	0.06	−0.280	0.25	−0.442	0.001	−0.265	0.35
SPP1, pr2	−0.297	0.17	−0.251	0.46	−0.330	0.08	−0.281	0.25	−0.447	0.001	−0.243	0.53
PPARγ, pr1	−0.174	1	−0.327	0.08	−0.475	0.001	−0.256	0.41	−0.221	0.79	−0.399	0.01
PPARγ, pr2	−0.195	1	−0.311	0.12	−0.460	0.001	−0.276	0.27	−0.209	0.97	−0.353	0.04
IkBkG	0.184	1	0.196	1	0.233	0.64	0.188	1	−0.009	1	0.354	0.04

Expression of NFkB1 was positively correlated with the presence of CD3 and CD8 T cells (CD3E $p = 0.01$, CD8A $p = 0.03$). Similarly, both probes for NFkB2 had a positive correlation with CD3E and CD68. RELA did not show any significant correlation, while expression of RELB was significantly correlated to CD3E and CD3D1. Both SPP1 probes showed a negative correlation with CD68, while expression of PPARγ (both probes) showed a negative correlation with CD4 and CD163. Expression of IkBkG showed a positive correlation with CD163.

2.4. Confirmation of Associations Found by Heatmap and Survival

2.4.1. Distribution of NFkB Pathway and HLA Class I Gene Expression in Primary UM

We put all different analyses together in a heat map, which displays the data of 64 UM, highlighting mRNA expression levels of relevant immune markers and tumor characteristics. According to the heatmap (Figure 1), most D3/BAP1-positive tumors (indicated as blue in the legend) had low expression of HLA-A, HLA-B, NFkB1, NFkB2 (indicated as blue in the expression array) and high levels of SPP1 and PPARγ (indicated as yellow-orange) while most M3/BAP1-negative tumors (indicated as red in the legend) had a high expression of HLA-A, HLA-B, NFkB1, NFkB2 (indicated as yellow-orange) and low levels of SPP1 and PPARγ (indicated as blue).

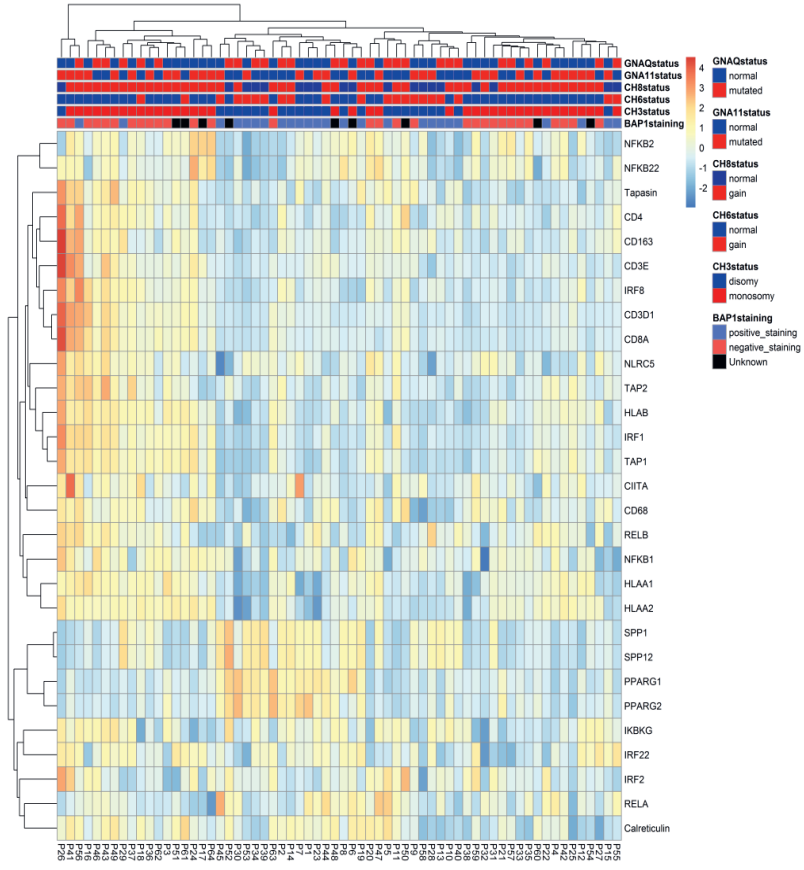


Figure 1. Heat map of 64 UM, highlighting mRNA expression levels of inflammatory markers. The presence of BAP1 staining, the status of chromosome 3, 6p and 8q, and the presence of GNAQ and GNA11 mutations are also shown. Clusters were created by unsupervised clustering of the tumors.

2.4.2. Associations between Survival in UM and Chromosome 3, BAP1 and Inflammatory Factors

Next, we investigated whether any differences could be found between survival with regard to the level of expression of HLA Class I and NFkB pathway molecules. Different levels of expression in HLA-A, HLAB, RELB and PPARγ significantly differed in survival (Figure 2). Tumors with a low expression of HLA-A, HLA-B and RELB led to a better overall survival ($p < 0.001$, $p < 0.001$, $p < 0.001$), while tumors with a high level of PPARγ correlated with better survival ($p = 0.006$). We also checked survival in relation to chromosome 3 status and BAP1 staining. Patients with M3 tumors showed a much shorter survival compared to patients with D3 tumors ($p < 0.001$), and those with tumors that lacked BAP1 staining had a shorter survival compared to those with BAP1-positive tumors ($p < 0.001$).

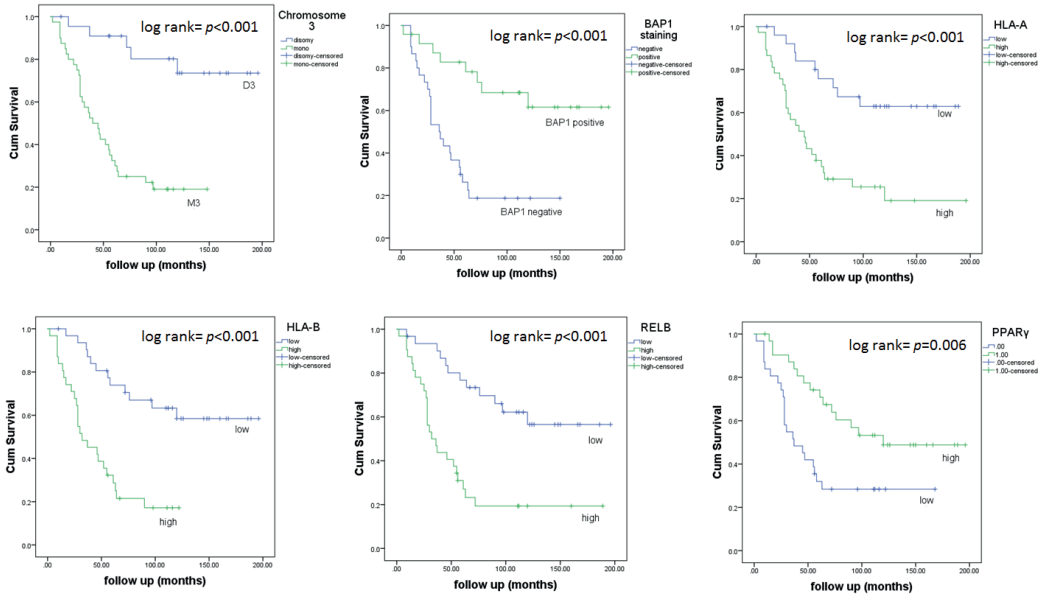


Figure 2. Kaplan–Meier survival curves based on chromosome 3 status, BAP1 staining and mRNA expression of HLA-A, HLA-B, RELB and PPARγ. A log-rank test was used for the significance analysis.

2.5. NFkB Pathway and Chromosome 3/BAP1 Status

As M3 and loss of BAP1 expression are well known risk factors for the development of metastasis in UM, we determined whether these genetic aberrations might be associated with expression of the NFkB-related molecules directly and, therefore, with the generation of the inflammatory environment. First, we analysed the gene expression levels of the NFkB-related markers versus chromosome 3 status in our cohort of 64 tumors (Figure 3a). M3 was associated with an increase in NFkB1, NFkB2, and RELB and a decrease in SPP1 (both probes) and PPARγ (both probes).

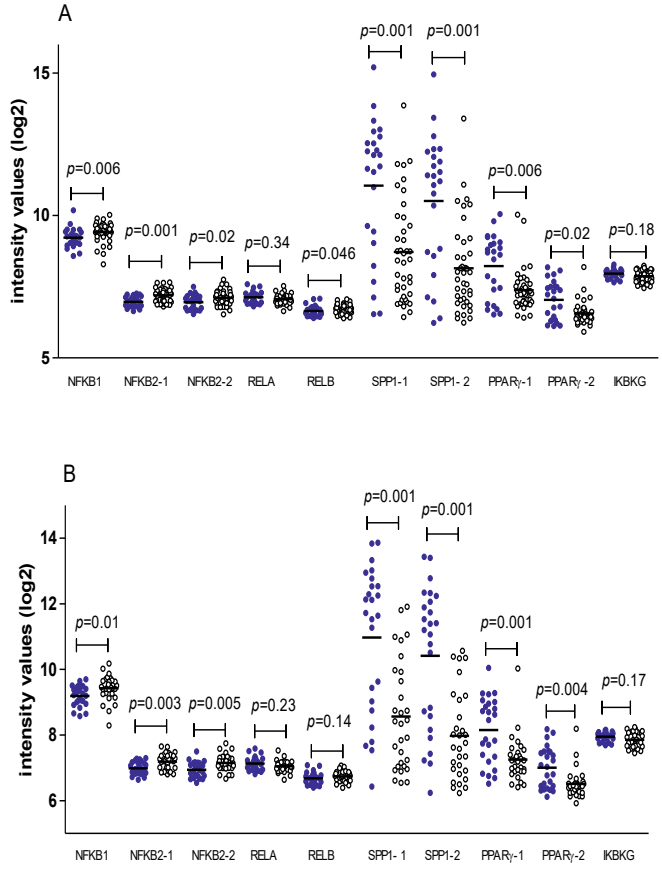


Figure 3. Comparison of expression of factors involved in NFkB signalling pathways in (a) D3 (● blue) (n = 24) versus M3 (o white) (n = 40), and (b) BAP1-positive (● blue) (n = 25) versus BAP1-negative (o white) (n = 30) tumors. Mann–Whitney U test, $p \leq 0.05$ is considered significant. Thick bars indicate the means.

Next, we analysed a group of 55 tumors with information on BAP1 staining (Figure 3b). Levels of NFkB1 and NFkB2 (both probes) were increased in tumors with BAP1 loss, while SPP1 (both probes) and PPARγ (both probes) were decreased in these tumors.

2.6. NFkB Upregulation is Present in Tumor Cells

However, the positive correlations may be (partially) due to the expression of NFkB in infiltrating lymphocytes and macrophages. In order to determine whether the association between loss of BAP1 and changes in the NFkB pathway occurs in the tumor cells themselves, we excluded the 33% of tumors with the highest CD8 score, leaving a total of 43 tumors. Even with the decreased number of cases, one probe for NFkB2 was still positively correlated with two HLA-A probes and with HLA-B, while SPP1 and PPARγ probes remained significantly negatively correlated to some or all of the HLA probes (Table 4).

Table 4. Correlation between the NFkB pathway molecules and HLA Class I expression, after exclusion of one-third of the tumors with the highest infiltrate, based on CD8 expression (n = 43). r = two-tailed Spearman correlation coefficient. $p \leq 0.05$ is considered significant.

Markers	HLA-A Probe 1		HLA-A Probe 2		HLA-A Probe 3		HLA-B	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
NFkB1	0.292	0.06	0.255	0.10	0.139	0.37	0.269	0.08
NFkB2, pr1	0.408	0.007	0.431	0.004	0.251	0.11	0.463	0.002
NFkB2, pr2	0.212	0.17	0.257	0.10	0.113	0.47	0.281	0.07
RELA	0.095	0.55	−0.067	0.67	−0.153	0.33	−0.012	0.94
RELB	0.097	0.54	0.188	0.23	0.199	0.20	0.248	0.11
SPP1, pr1	−0.238	0.12	−0.372	0.01	−0.196	0.21	−0.286	0.06
SPP1, pr2	−0.289	0.06	−0.425	0.01	−0.245	0.11	−0.331	0.03
PPARγ, pr1	−0.354	0.02	−0.431	0.004	−0.445	0.003	−0.320	0.04
PPARγ, pr2	−0.341	0.03	−0.403	0.007	−0.421	0.005	−0.228	0.14
IkBkG	0.001	1,0	−0.023	0.89	0.188	0.23	0.061	0.70

Most of the associations also remained significant when we performed the same comparison after exclusion of one-third of the tumors with the highest number of macrophages (CD68) (Supplementary Table S2).

We subsequently compared the levels of expression of NFkB pathway molecules in BAP1-positive and -negative tumors, using the whole panel, as well as after exclusion of one-third of the tumors with the highest CD8 scores. BAP1-negative tumors still had higher levels of NFkB2 and IkBkG, while the negative correlations with SPP1 and PPARγ remained (Table 5).

Table 5. Correlation between BAP1 staining as defined by immunohistochemical staining and NFkB pathway molecules expression in all tumors for which BAP1 staining was available (n = 55) and again after exclusion of one-third of the tumors with the highest infiltrate, based on CD8 expression (n = 37). One-Way ANOVA was used to calculate the means. $p \leq 0.05$ is considered significant.

Markers	All Tumors (n = 55)			Exclusion of High CD8 Tumors (n = 37)		
	BAP1 + (Mean n = 25)	BAP1 - (Mean n = 30)	p	BAP1 + (Mean n = 22)	BAP1 - (Mean n = 15)	p
NFKB1	9.19	9.42	0.018	9.16	9.26	0.37
NFKB2-pr1	6.98	7.19	0.001	6.98	7.16	0.014
NFKB2-pr2	6.94	7.14	0.003	6.94	7.15	0.01
RELA	7.13	7.05	0.12	7.14	7.09	0.47
RELB	6.68	6.73	0.23	6.66	6.70	0.48
SPP1-pr1	10.97	8.56	<0.001	10.91	8.38	0.001
SPP1-pr2	10.41	7.97	<0.001	10.34	7.84	<0.001
PPARγ-pr1	8.15	7.25	<0.001	8.22	7.15	0.001
PPARγ-pr2	7.00	6.51	0.001	7.03	6.46	0.003
IkBkG	7.94	7.85	0.085	7.93	7.78	0.006

3. Discussion

We hypothesized that the inflammatory phenotype with an increased HLA Class I expression in UM is linked to the activation of the NFkB pathway. Therefore, we explored the expression of some of the most important components of the NFkB signalling pathway and investigated their association with HLA-A and HLA-B expression and with different types of infiltrating leukocytes. In accordance with previous studies, we noticed an association between upregulation of most of the factors involved in the NFkB pathway with inflammatory factors [33,34]. We found that NFkB1, NFkB2 and RELB expression were positively correlated with the expression levels of HLA-A and HLA-B, their regulators and their antigen-loading machinery, suggesting that these NFkB pathway molecules may be positive regulators of HLA-A and HLA-B transcription. It has been suggested that NFkB is responsible for the secretion of several proinflammatory cytokines and chemokines and thereby guarantees the attraction, activation, survival and differentiation of

immune cells [39] which might lead to the increase of HLA Class I expression in the tumor cells. Singh et al. [34], in a recent study, similarly observed that infiltration increases during progression of UM and that the canonical NFkB pathway is more active in high-risk inflamed UM. They did not report on the chromosome or BAP1 status of the tumors they studied.

We observed that M3 tumors showed higher expression levels of NFkB1, NFkB2, and RELB. Similarly, BAP1-negative tumors showed higher levels of NFkB1 and NFkB2. We previously demonstrated that M3/loss of BAP1 is related to increased leukocytic infiltration [8, 9], and now show an association between BAP1 loss and upregulation of the NFkB pathway. As the link between the expression of the NFkB molecules and T cell infiltrate may also be caused by the expression of these molecules in the immune infiltrate itself, we repeated the analyses on the same set but now without one-third of the tumors, which based on the expression of CD8, were highly infiltrated with T cells. We again looked for associations between HLA and NFkB, and the genetic constitution. In this group, we similarly observed that the tumors with M3/loss of BAP1 had an increased NFkB and HLA Class I expression, indicating that the infiltrating leukocytes may enhance expression, but that the genetic basis determines expression in the first place. We also explored the presence of negative regulators of inflammation in UM: SPP1 and PPAR-γ. The SPP1/osteopontin protein is involved in different aspects of tumor biology such as cell survival and proliferation, invasion and metastasis, and is involved in neural crest development. Kadkol et al. [40] reported that the level of SPP1 expression was increased in the serum of patients with UM metastasis, and that tumors with looping high-risk vasculogenic mimicry patterns had increased expression of osteopontin. Another study [41] also looked at protein expression using a monoclonal antibody and did not observe a relation between expression and survival. In contrast to Kadkol and Simoes, we looked at mRNA and observed that decreased levels of SPP1 mRNA were associated with an unfavorable prognosis in UM. Onken et al. have similarly reported a decreased expression of SPP1 mRNA in high-risk UM [42]. It is of interest to further investigate the mechanism of SPP1 downregulation during UM progression to determine its function.

The upregulation of the NFkB pathway upon loss of one copy of chromosome 3 could possibly be explained by the fact that the PPAR-γ gene is located on chromosome 3. The PPAR-γ protein is a negative regulator of the NFkB pathway, so it has been hypothesized that loss of chromosome 3 leads to insufficient inactivation of NFkB by PPAR-γ [43]. In our study, SPP1 and PPAR-γ expression were negatively correlated to T lymphocyte and macrophage markers. As expected, we found a positive correlation between the expression of these two factors with BAP1 expression. We previously observed [35] that a high expression of HLA-A, HLA-B, B2M, TAP1, IRF1 and IRF8 was related to an increase in the presence of T cells. Van Essen and colleagues compared the expression levels of these genes in primary tumors with their corresponding xenograft in immune-deficient mice, in which, of course, no human leukocytes are present. They showed that the expression of HLA-A, HLA-B, B2M, TAP1, TAP2, IRF1, IRF8, CIITA and NLRC5 were all down-regulated in xenografts compared to the primary tumors, suggesting that the increased expression depended on the presence of infiltrate. Cytokines potentially involved in the upregulation of these genes were interferon-gamma and CCL2. In the current study, we observed a correlation between the expression of the HLA and HLA-associated genes, the presence of infiltrating immune cells, and the NFkB pathway, but we also see this correlation in tumors without inflammation. From this, we conclude that the genetic basis of the tumor, i.e., Monosomy 3 with BAP1 loss, determines the primary upregulation of the NFkB pathway, which leads to an increase in HLA Class I expression in the tumor cells and production of cytokines and chemokines, which then attracts immune cells, further upregulating expression of HLA Class I. These data indicate that absence of the deubiquitinating function of BAP1 allows the NFkB pathway to be active, potentially by affecting the regulators of NFkB directly (Figure 4).

These findings imply that BAP1 (UCHL2) might have a similar function in UM as observed for UCHL1 in Human papilloma virus (HPV)-infected keratinocytes. UCHL1 and UCHL2 are both ubiquitin hydrolase enzymes which belong to the ubiquitin hydrolase family [44]. HPV-infected keratinocytes produce increased levels of the ubiquitin protease UCHL1, which reduces tumor necrosis factor receptor-associated factor 3 (TRAF3) K63 poly-ubiquitination, resulting in the suppression of the production of interferon and other pro-inflammatory cytokines [45].

Higher UCHL1 expression also resulted in increased degradation of an essential NFkB modulator, IkBkG. In this manner, canonical NFkB signalling was reduced, thereby suppressing a potential anti-viral immune response. HPV-infected keratinocytes with a high UCHL1 expression showed greatly decreased HLA-A and -B levels [46]. The virus uses UCHL1 to escape from the immune system, while in UM, the presence of an inflammatory infiltrate helps to produce blood vessels [9].

Taken together, our data suggest that the main regulator of the NFkB pathway in uveal melanoma is loss of chromosome 3 and BAP1: loss of chromosome 3/loss of BAP1 expression correlates with upregulation of the NFkB pathway and affects the activity of the NFkB pathway in UM tumors, which leads to upregulation of HLA Class I expression and attraction of infiltrating cells to the tumor environment which is a well-known factor in the development of metastasis in this disease.

a

b

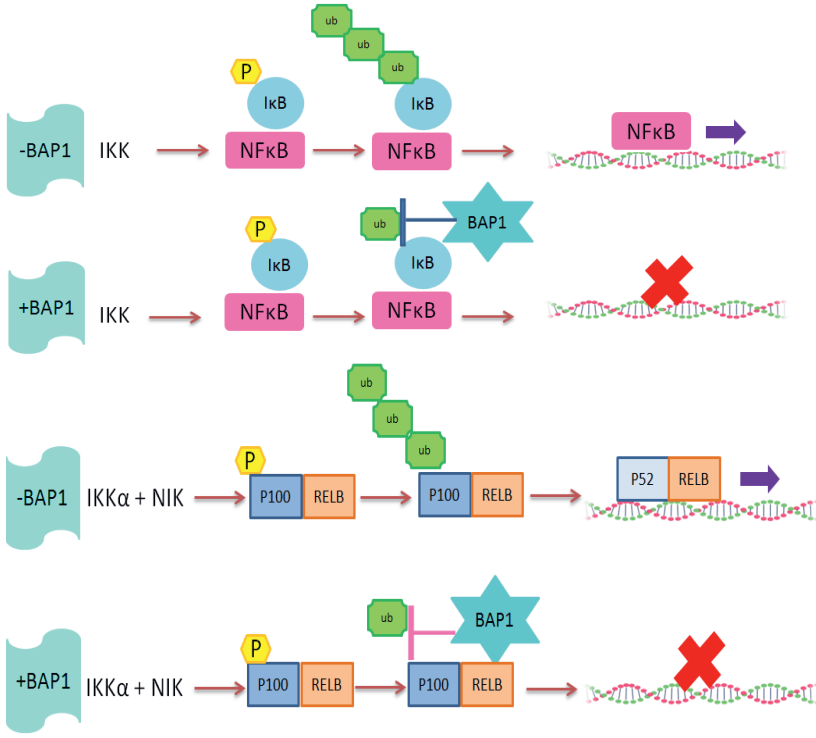


Figure 4. Schematic illustration of the potential role of BAP1 protein in (a) Canonical and (b) non-Canonical NFkB pathway. We propose that BAP1, as a deubiquitinating enzyme, inhibits NFkB activity.

4. Materials and Methods

4.1. Study Population

Tumor tissues were obtained from 64 enucleated eyes that underwent an enucleation for UM between 1999 and 2008 at the Leiden University Medical Center (LUMC) in Leiden, The Netherlands. In our cohort of 64 patients, 51% were male and 49% were female (see Table 6 for patient and tumor characteristics of this cohort). The mean age at the time of enucleation was 61 years. The mean follow-up time (defined as the time period between enucleation and death) was 77 months (range 2 to 196 months). At the end of follow-up in 2018, 58% of patients had died because of UM metastasis, 10% because of other causes, 5% due to an unknown cause and 28% were still alive.

The collection of materials and the research protocol is compliant with the tenets of the Declaration of Helsinki (World Medical Association of Declaration 2013; ethical principles for medical research involving human subjects). Tumor material was handled in accordance with the Dutch National Ethical Guidelines ('Code for Proper Secondary Use of Human Tissue').

The Medisch Ethische Toetsingscommissie (METC) declared that it did not object to performing this study (19/10/2016, code G16.076/NV/gk).

Table 6. Characteristics of the cohort of uveal melanoma (UM) patients enucleated in the LUMC between 1999 and 2008 (n = 64).

		Number of Cases or Mean	% of Cases or SD
Gender	Male	33	51%
	Female	31	49%
Age at enucleation (in years)		61	±15.7
Cell type	Spindle	22	33%
	Mixed/epithelioid	41	62%
Ciliary body involvement	No	40	61%
	Yes	24	36%
Chromosome 3 status	Disomy	24	46%
	Monosomy	40	61%
BAP1 Staining	Positive	25	46%
	Negative	30	55%
	Unknown	9	16%
GNA11 mutation	No	31	48%
	Yes	33	52%
GNAQ mutation	No	36	56%
	Yes	28	44%
Chromosome 6p gain	No	43	65%
	Yes	21	32%
Chromosome 8q gain	No	19	29%
	Yes	45	68%
Metastasis	No	27	42%
	Yes	37	58%
Follow-up time (in months)		77	±55
Vital status	Dead due to UM metastasis	37	58%
	Dead due to other causes	6	10%
	Cause of death unknown	3	5%
	Alive at last follow-up date	18	28%

4.2. Immunohistochemistry and Immunofluorescence

Immunohistochemical staining was previously performed for HLA-A, HLA-B and BAP1. Mouse monoclonal antibodies HCA2 (staining HLA-A heavy chains) and HC10 (staining HLA-B/C) (produced by the Netherlands Cancer institute, Amsterdam, The Netherlands), were used [36]. Tumors were divided into BAP1-positive or -negative based on nuclear staining [47]. An example of positive and negative staining is shown in van Essen et al. [48]. Briefly, tissues were incubated with mouse monoclonal antibody against human BAP1 (clone sc-28383, 1:50 dilution, Santa Cruz Biotechnology, Dallas, TX, USA). Tumor cells positive for each marker were counted at 100X magnification and were shown as a percentage of the total number of the tumor cells.

Immunofluorescence staining was performed for T cell and macrophage markers as described [9,38] with anti-CD3 (ab828; Abcam, Cambridge, MA, USA), anti-CD8 (4B11, IgG2b; Novocastra Valkenswaard, The Netherlands), anti-CD68 (514H12; Abcam, Cambridge, UK) and anti-CD163 (Clone 10D6; Novocastra, Newcastle-upon-Tyne, UK). Counts were expressed as pixels per millimeter2. Monoclonal antibodies were selected as described previously, based on correlations between mRNA expression and immunofluorescence staining [10].

4.3. Chromosome Analysis

DNA from samples collected at the Leiden University Medical Center was purified using QIAmp DNA Mini kit (Qiagen, Venlo, The Netherlands). Affymetrix 250K Nsp array (Affymetrix, Santa Clara, CA, USA) was performed in order to obtain a genome-wide micro-array of single nucleotide polymorphisms (SNPs) as described previously for chromosome 3 abnormalities [35].

4.4. Gene Expression

Gene expression profiling was performed with the Illumina HT12v4 array (Illumina, Inc., San Diego, CA, USA) for NFkB pathway markers and regulators of the pathway (NFkB1, NFkB2, RELA, RELB, SPP1, PPARγ, IKBKG), HLA genes and its regulators (HLA-A, HLA-B, CIITA, NLRC5, IRF1, IRF2, IRF8), and peptide-loading machinery molecules (TAP1, TAP2, Tapasin and Calreticulin), as described previously [35]. Illumina probe numbers are indicated in Supplementary Table S3.

4.5. Statistical Analysis

Data were analysed with SPSS software version 22.0 (SPSS, nc., Chicago, Il, USA). Graphs were obtained using GraphPad Prism version 5.0 for windows (GraphPad Software, La Jolla, CA, USA). One-Way ANOVA was used to calculate the means. Spearman correlation was performed in order to make correlations between data. Bonferroni correction was applied for multiple testing and after correction, all p values above one were rounded to one. The Mann–Whitney U test was used to compare non-normal groups. Kaplan–Meier survival curves were made and the log rank test was used for the significance analysis.

5. Conclusions

This study evaluated the association between HLA Class I expression, essential components of the NFkB signalling pathway and genetic evolution in UM. Loss of chromosome 3/loss of nuclear BAP1 protein in UM is associated with upregulation of the main components of the NFkB pathway (NFkB1-NFkB2 and RELB) and downregulation of two negative regulators of this pathway (SPP1 and PPARγ). It seems that under normal conditions, BAP1 helps to keep the uveal pigment cells immunologically quiet, but that during evolution of a UM, loss of BAP1 expression results in lack of suppression of the NFkB pathway and subsequent inflammation. Functional studies will show how BAP1 molecularly targets the NFkB pathway.

Author Contributions: Conceptualization, Z.S., M.J.J. and S.H.v.d.B.; methodology Z.S. and A.P.A.W.; software C.v.W.; formal analysis Z.S.; investigation Z.S.; resources A.P.A.W., P.A.v.d.V., A.G.J.; data curation Z.S.; writing—Original draft preparation Z.S.; writing—review and editing A.P.A.W., C.v.W., P.A.v.d.V., W.G.M.K., G.P.M.L., S.H.v.d.B., A.G.J., M.J.J.; visualization, Z.S., C.v.W.; supervision, A.G.J. and M.J.J.; project administration A.P.A.W., M.J.J.; funding acquisition G.P.M.L. and M.J.J.

Funding: This research was funded by a grant to Z.S. from The Iranian Ministry of Science Research and Technology and a Horizon 2020 grant from the European Community, CURE UM, nr. 667787.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Kujala, E.; Mäkitie, T.; Kivelä, T. Very long-term prognosis of patients with malignant uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2003, 44, 4651–4659, doi:10.1167/iovs.03-0538.
2. Dogrusöz, M.; Jager, M.J.; Damato, B. Uveal melanoma treatment and prognostication. Asia Pac. J. Ophthalmol. 2017, 6, 186–196, doi:10.22608/APO.201734.
3. De Waard-Siebinga, I.; Hilders, C.G.; Hansen, B.E.; van Delft, J.L.; Jager, M.J. HLA expression and tumor-infiltrating immune cells in uveal melanoma. Graefe's Arch. Clin. Exp. Ophthalmol. 1996, 234, 34–42.
4. Blom, DJ.; Luyten, GP.; Mooy, C.; Kerkvliet, S.; Zwinderman, AH.; Jager, M.J. Human leukocyte antigen class I expression. Marker of poor prognosis in uveal melanoma. Investig. Ophthalmol. Vis. Sci. 1997, 38, 1865–1872.
5. Ericsson, C.; Seregard, S.; Bartolazzi, A.; Levitskaya, E.; Ferrone, S.; Kiessling, R.; Larsson, O. Association of HLA class I and class II antigen expression and mortality in uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2001, 42, 2153–2156.
6. Jager, M.J.; Hurks, H.M.; Levitskaya, J.; Kiessling, R. HLA expression in uveal melanoma: There is no rule without some exception. Hum. Immunol. 2002, 63, 444–451.
7. Robertson, A.G.; Shih, J.; Yau, C.; Gibb, EA.; Oba, J.; Mungall, K.L.; Hess, J.M.; Uzunangelov, V.; Walter, V.; Danilova, L.; Lichtenberg, T.M. Integrative analysis identifies four molecular and clinical subsets in uveal melanoma. Cancer Cell 2017, 32, 204–220, doi:10.1016/j.ccell.2017.12.013.
8. Maat, W.; Ly, L.V.; Jordanova, E.S.; de Wolff-Rouendaal, D.; Schalij-Delfos, N.E.; Jager, M.J. Monosomy of chromosome 3 and an inflammatory phenotype occur together in uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2008, 49, 505–510, doi:10.1167/iovs.07-0786.
9. Bronkhorst, I.H.; Vu, T.H.; Jordanova, E.S.; Luyten, G.P.; Burg, S.H.; Jager, M.J. Different subsets of tumor-infiltrating lymphocytes correlate with macrophage influx and monosomy 3 in uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2012, 53, 5370–5378, doi:10.1167/iovs.11-9280.
10. Gezgin, G.; Dogrusöz, M.; van Essen, T.H.; Kroes, W.G.; Luyten, G.P.; van der Velden, P.A.; Walter,V.; Verdijk, R.M.; van Hall, T.; van der Burg, S.H.; Jager, M.J. Genetic evolution of uveal melanoma guides the development of an inflammatory microenvironment. Cancer Immunol. Immunother. 2017, 66, 903–912, doi:10.1007/s00262-017-1991-1.
11. Prescher, G.; Bornfeld, N.; Becher, R. Nonrandom chromosomal abnormalities in primary uveal melanoma. J. Natl. Cancer Inst. 1990, 82, 1765–1769, doi:10.1093/jnci/82.22.1765.

12. Horsthemke, B.; Prescher, G.; Becher, R.; Bornfeld, N. Loss of chromosome 3 alleles and multiplication of chromosome 8 alleles in uveal melanoma. Genes Chromosomes Cancer 1992, 4, 217–221.
13. Scholes, A.G.; Damato, B.E.; Nunn, J.; Hiscott, P.; Grierson, I.; Field, J.K. Monosomy 3 in uveal melanoma: Correlation with clinical and histologic predictors of survival. Investig. Ophthalmol. Vis. Sci. 2003, 44, 1008–1011, doi:10.1167/iovs.02-0159.
14. Aalto, Y.; Eriksson, L.; Seregard, S.; Larsson, O.; Knuutila, S. Concomitant loss of chromosome 3 and whole arm losses and gains of chromosome 1, 6, or 8 in metastasizing primary uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2001, 42, 313–317.
15. Cassoux, N.; Rodrigues, M.J.; Plancher, C.; Asselain, B.; Levy-Gabriel, C.; Lumbroso-Le, Rouic, L.; Piperno-Neumann, S.; Dendale, R.; Sastre, X.; Desjardins, L.; et al. Genome-wide profiling is a clinically relevant and affordable prognostic test in posterior uveal melanoma. Br. J. Ophthalmol. 2014, 98, 769–774, doi:10.1136/bjophthalmol-2013-303867.
16. Versluis, M.; de Lange, M.J.; van Pelt, S.I.; Ruivenkamp, C.A.; Kroes, W.G.; Cao, J.; Jager, M.J. ; Luyten, G.P.M.; van der Velden, P.A. Digital PCR validates 8q dosage as prognostic tool in uveal melanoma. PLoS ONE 2015, 10, e0116371, doi:10.1371/journal.pone.0116371.
17. Van Gils, W.; Kilic, E.; Brüggenwirth, H.T.; Vaarwater, J.; Verbiest, M.M.; Beverloo, B.; van Til-Berg, M.E.; Paridaens, D.; Luyten, G.P.M.; de Klein, A. Regional deletion and amplification on chromosome 6 in a uveal melanoma case without abnormalities on chromosomes 1p, 3 and 8. Melanoma Res. 2008, 18, 10–15, doi:10.1097/CMR.0b013e3282f1d4d9.
18. Damato, B.; Dopierala, J.; Klaasen, A.; van Dijk, M.; Sibbring, J.; Coupland, S.E. Multiplex ligation-dependent probe amplification of uveal melanoma: Correlation with metastatic death. Investig. Ophthalmol. Vis. Sci. 2009, 50, 3048–3055, doi:10.1167/iovs.08-3165.
19. Van Raamsdonk, C.D.; Bezrookove, V.; Green Get Bauer, J.; Gaugler, L.; O'brien, J.M.; Simpson, E.M.; Barsh, G.S.; Bastian, B.C. Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. Nature 2009, 457, 599–602, doi:10.1038/nature07586.
20. Van Raamsdonk, C.D.; Griewank, K.G.; Crosby, M.B.; Garrido, M.C.; Vemula, S.; Wiesner, T.; Obenauf, A.C.; Wackernagel, W.; Green, G.; Bouvier, N.; et al. Mutations in GNA11 in uveal melanoma. N. Engl. J. Med. 2010, 363, 2191–2199, doi:10.1056/NEJMoa1000584.
21. Yu, F.X.; Luo, J.; Mo, J.S.; Liu, G.; Kim, Y.C.; Meng, Z.; Zhao, L.; Peyman, G.; Ouyang, H.; Jiang, W.; et al. Mutant Gq/11 promote uveal melanoma tumorigenesis by activating YAP. Cancer Cell 2014, 25, 822–830, doi:10.1016/j.ccr.2014.04.017.

22. Vader, M.J.; Madigan, M.C.; Versluis, M.; Suleiman, H.M.; Gezgin, G.; Gruis, N.A.; Out-Luiting, J.J.; Bergman, W.; Verdijk, R.M.; Jager, M.J.; et al. GNAQ and GNA11 mutations and downstream YAP activation in choroidal nevi. Brit. J. Cancer 2017, 117, 884–887, doi:10.1038/bjc.2017.259.
23. Harbour, J.W.; Onken, M.D.; Roberson, E.D.; Duan, S.; Cao, L.; Worley, L.A.; Council, M.L.; Matatall, K.A.; Helms, C.; Bowcock, AM. Frequent mutation of BAP1 in metastasizing uveal melanomas. Science 2010, 330, 1410–1413, doi:10.1126/science.1194472.
24. Brantley, M.A.; Harbour, J.W. Inactivation of retinoblastoma protein in uveal melanoma by phosphorylation of sites in the COOH-terminal region. Cancer Res. 2000, 60, 4320–4323.
25. Babchia, N.; Calipel, A.; Mouriaux, F.; Faussat, A.M.; Mascarelli, F. The PI3K/Akt and mTOR/P70S6K signaling pathways in human uveal melanoma cells: Interaction with B-Raf/ERK. Investig. Ophthalmol. Vis. Sci. 2010, 51, 421–429, doi:10.1167/iovs.09-3974.
26. Coupland, S.E.; Lake, S.L.; Zeschnigk, M.; Damato, B.E. Molecular pathology of uveal melanoma. Eye 2013, 2, 230–242, doi:10.1038/eye.2012.255.
27. Hanahan, D.; Weinberg, RA. Hallmarks of cancer: The next generation. Cell 2011, 144, 646–674, doi:10.1016/j.cell.2011.02.013.
28. Pires, B.R.B.; Silva, R.C.M.C.; Ferreira, G.M.; Abdelhay, E. Nf-kappab: Two sides of the same coin. Genes 2018, 9, 1–24, doi:10.3390/genes9010024.
29. Karin, M. NF-kB as a critical link between inflammation and cancer. Cold Spring Harb. Perspect. Biol. 2009, 1, a000141, doi:10.1101/cshperspect.a000141.
30. McNulty, S.; Tohidian, N.B.; Meyskens Jr, F.L. RelA, p50 and inhibitor of kappa B alpha are elevated in human metastatic melanoma cells and respond aberrantly to ultraviolet light B. Pigment Cell Res. 2001, 14, 456–465.
31. Ueda, Y.; Richmond, A. NF-κB activation in melanoma. Pigment Cell Res. 2006, 19, 112–124, doi:10.1111/j.1600-0749.2006.00304.x.
32. Meir, T.; Dror, R.; Yu, X.; Qian, J.; Simon, I.; Pe'er, J.; Chowers, I. Molecular characteristics of liver metastases from uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2007, 48, 4890–4896, doi:10.1167/iovs.07-0215.
33. Dror, R.; Lederman, M.; Umezawa, K.; Barak, V.; Pe'er, J.; Chowers, I. Characterizing the Involvement of the Nuclear Factor-kappa B (NFκB) Transcription Factor in Uveal Melanoma. Investig. Ophthalmol. Vis. Sci. 2010, 51, 1811–1816, doi:10.1167/iovs.09-3392.
34. Singh, M.K.; Singh, L.; Pushker, N.; Saini, N.; Meel, R.; Chosdol, K.; Bakhshi, S.; Sen, S.; Venkatesh, P.; Chawla, B.; et al. Identification of canonical NFκB (C-NFκB) pathway in uveal

melanoma and their relation with patient outcome. Clin. Exp. Metastasis 2019, 36, 271–790, doi:10.1007/s10585-019-09969-y.

35. Van Essen, T.H.; van Pelt, S.I.; Bronkhorst, I.H.; Versluis, M.; Némati, F.; Laurent, C.; Luyten, G.P.M.; van Hall, T.; van den Elsen, P.J.; van der Velden, P.A.; et al. Upregulation of HLA expression in primary uveal melanoma by infiltrating leukocytes. PLoS ONE 2016, 11, e0164292, doi:10.1371/journal.pone.0164292.
36. Stam, N.J.; Vroom, T.M.; Peters, P.J.; Pastoors, E.B.; Ploegh, H.L. HLA-A-and HLA-B-specific monoclonal antibodies reactive with free heavy chains in western blots, in formalin-fixed, paraffin-embedded tissue sections and in cryo-immuno-electron microscopy. Int. Immunol. 1990, 2, 113–125, doi:10.1093/intimm/2.2.113.
37. Krishnakumar, S.; Abhyankar, D.; Sundaram, A.L.; Pushparaj, V.; Shanmugam, M.P.; Biswas, J. Major histocompatibility antigens and antigen-processing molecules in uveal melanoma. Clin. Cancer Res. 2003, 9, 4159–4164.
38. Bronkhorst, I.H.; Ly, L.V.; Jordanova, E.S.; Vrolijk, J.; Versluis, M.; Luyten, G.P.M.; Jager, M.J. Detection of M2-macrophages in uveal melanoma and relation with survival. Investig. Ophthalmol. Vis. Sci. 2011, 52, 643–650, doi:10.1167/iovs.10-5979.
39. Liu, T.; Zhang, L.; Joo, D.; Sun, S. NF-κB signaling in inflammation. Signal Transduct. Target Ther. 2017, 2, 17023–17029.
40. Kadkol, S.S.; Lin, A.Y.; Barak, V.; Kalickman, I.; Leach, L.; Valyi-Nagy, K.; Majumdar, D.; Setty, S.; Maniotis, A.J.; Folberg, R.; et al. Osteopontin expression and serum levels in metastatic uveal melanoma: A pilot study. Investig. Ophthalmol. Vis. Sci. 2006, 47, 802–806.
41. Simões, C.C.; Call, M.K.; Corrêa, Z.M.; Spaulding, A.G.; Augsburger, J.J. Clinical and histopathological features and immunoreactivity of human choroidal and ciliary melanomas as prognostic factors for metastasis and death. Graefe's Arch. Clin. Exp. Ophthalmol. 2011, 249, 1795–1803.
42. Onken, M.D.; Worley, L.A.; Ehlers, J.P.; Harbour, J.W. Gene expression profiling in uveal melanoma reveals two molecular classes and predicts metastatic death. Cancer Res. 2004, 64, 7205–7209.
43. Velho, T.R.; Kapiteijn, E.; Jager, M.J. New therapeutic agents in uveal melanoma. Anti-Cancer Res. 2012, 32, 2591–2598, doi:10.1158/0008-5472.CAN-04-1750.
44. Fang, Y.; Fu, D.; Shen, X.Z. The potential role of ubiquitin c-terminal hydrolases in oncogenesis. Biochem. Biophys. Acta Rev. Cancer 2010, 1806, 1–6, doi:10.1016/j.bbcan.2010.03.001.

45. Karim, R.; Tummers, B.; Meyers, C.; Biryukov, J.L.; Alam, S.; Backendorf, C.; Jha, V.; Offringa, R.; van Ommen, G.J.; Melief, C.J.; et al. Human papillomavirus (HPV) upregulates the cellular deubiquitinase UCHL1 to suppress the keratinocyte's innate immune response. PLoS Pathog. 2013, 9, e1003384.
46. Tummers, B.; Goedemans, R.; Pelascini, L.P.; Jordanova, E.S.; Van Esch, E.M.; Meyers, C.; Melief, C.J.; Boer, J.M.; van der Burg, S.H. The interferon-related developmental regulator 1 is used by human papillomavirus to suppress NFκB activation. Nat. Commun. 2015, 6, 6537–6549, doi:10.1038/ncomms7537.
47. Koopmans, A.E.; Verdijk, R.M.; Brouwer, R.W.; van Den Bosch, T.P.; van Den Berg, M.M.; Vaarwater, J.; Kockx, C.E.; Paridaens, D.; Naus, N.C.; Nellist, M.; et al. Clinical significance of immunohistochemistry for detection of BAP1 mutations in uveal melanoma. Modern Pathol. 2014, 27, 1321–1330, doi:10.1038/modpathol.2014.43.
48. Van Essen, T.H.; van Pelt, S.I.; Versluis, M.; Bronkhorst, I.H.; van Duinen, S.G., Marinkovic, M., Kroes, W.G.; Ruivenkamp, C.A.; Shukla, S.; De Klein, A.; et al. Prognostic parameters in uveal melanoma and their association with BAP1 expression. Br. J. Ophthalmol. 2014, 98, 1738–1743, doi:10.1136/bjophthalmol-2014-305047.

Supplementary Materials

The following are available online at https://www.mdpi.com/2072-6694/11/8/1102/s1, Table S1: Correlation between different probes obtained with an Illumina gene expression array with immunohistochemical data in the Leiden cohort (n = 28); Table S2: Correlation between NFkB signalling and HLA mRNA expression, after exclusion of the one-third tumors with the highest macrophage infiltrate, as indicated by CD68 mRNA levels (n = 43); Table S3. Illumina probe number of factors which have been used in this study.

Table S1. Correlation between different probes obtained with an Illumina gene expression array with immune histochemical data in the Leiden cohort (n =28) [35].

Gene-expression (Illumina)	mAb HCA2 (HLA-A)		mAb HC10 (HLA-B)	
	r	*p*	r	*p*
HLA-A probe 1 ◊	0.619	<0.001		
HLA-A probe 2 ◊	0.459	0.014		
HLA-A probe 3	0.055	0.781		
HLA-A probe 4 ◊	0.327	0.089		
HLA-B probe ◊			0.601	0.001

Table S2. Correlation between NFkB signalling and HLA mRNA expression, after exclusion of the one-third tumors with the highest macrophage infiltrate, as indicated by CD68 mRNA levels (n =43).

	HLA-A Probe 1		HLA-A Probe 2		HLA-A Probe 3		HLA-B	
	r	*p*	r	*p*	r	*p*	r	*p*
NFkB1	0.477	0.001	0.372	0.014	0.345	0.024	0.446	0.003
NFkB2, pr1	0.466	0.002	0.393	0.009	0.297	0.053	0.426	0.004
NFkB2, pr2	0.159	0.309	0.140	0.371	0.072	0.648	0.178	0.253
RELA	−0.014	0.931	−0.150	0.339	−0.245	0.113	−0.133	0.397
RELB	0.112	0.473	0.182	0.242	0.259	0.093	0.207	0.182
SPP1, pr1	−0.393	0.009	−0.472	0.001	−0.366	0.016	−0.419	0.005
SPP1, pr2	−0.415	0.006	−0.483	0.001	−0.381	0.012	−0.429	0.004
PPARγ, pr1	−0.305	0.046	−0.398	0.008	−0.344	0.024	−0.268	0.082
PPARγ, pr2	−0.311	0.042	−0.386	0.011	−0.324	0.034	−0.211	0.174
IkBkG	0.091	0.563	0.056	0.720	0.182	0.242	0.159	0.307

Table S3. Illumina probe number of factors which have been used in this study.

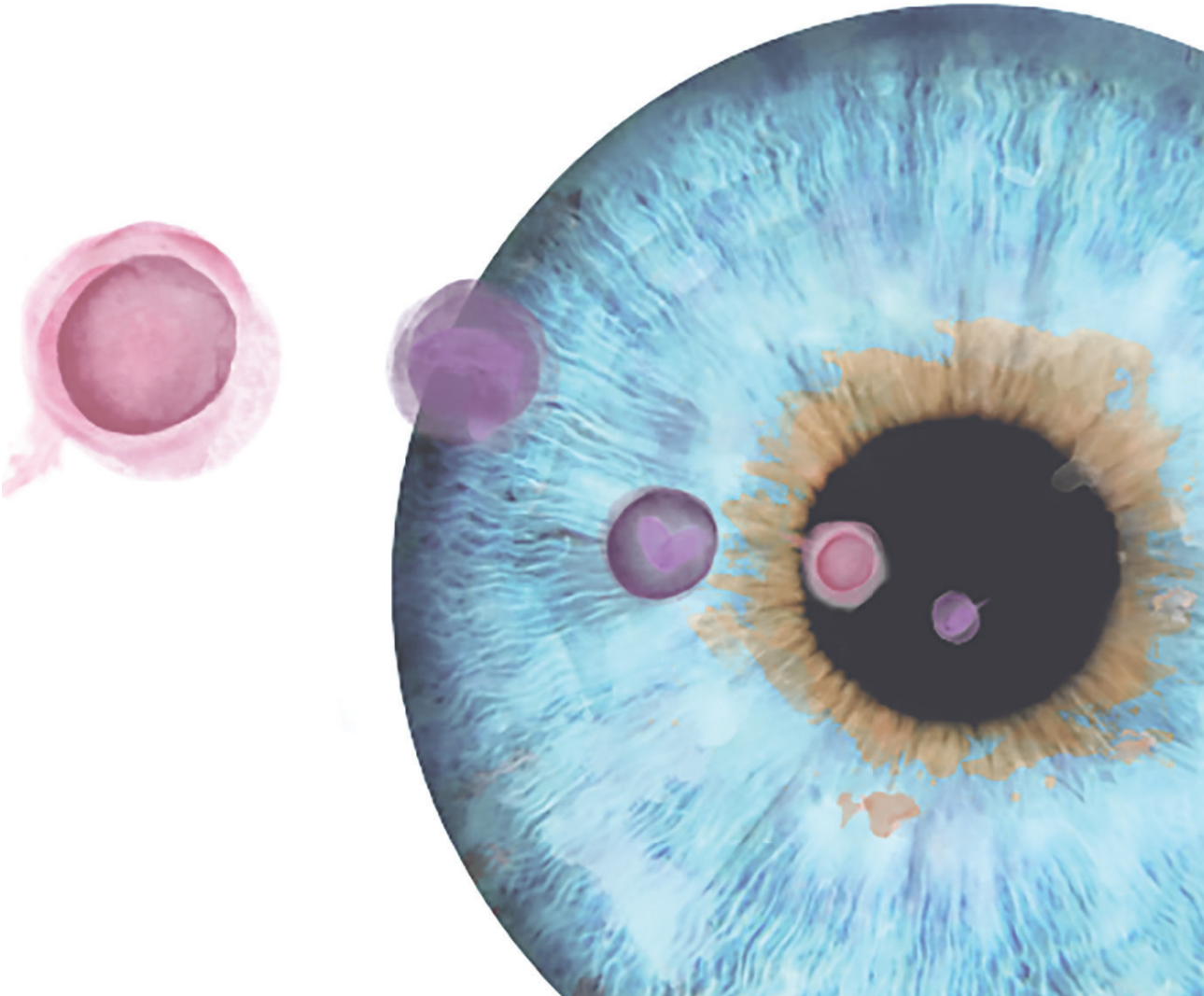
Gene-Expression (Illumina)		Probe Number
HLA-A	probe 1	ILMN_1671054
HLA-A	probe 2	ILMN_2203950
HLA-A	probe 3	ILMN_2186806
HLAB		ILMN_1778401
CD3E		ILMN_1739794
CD3D		ILMN_2261416
CD4		ILMN_1727284
CD8-A		ILMN_1768482
CD163		ILMN_1733270
NFkB1		ILMN_1714965
NFkB2	probe 1	ILMN_1799062
NFkB2	probe 2	ILMN_2390859
RELA		ILMN_1705266
RELB		ILMN_1811258
SPP1	probe 1	ILMN_1651354
SPP1	probe 2	ILMN_2374449
PPARγ	probe 1	ILMN_1800225
PPARγ	probe 2	ILMN_2364384
IkBkG		ILMN_1707308

Chapter 4

HDAC inhibition increases HLA Class I expression in Uveal Melanoma

Zahra Souri, Aart G. Jochemsen, Mieke Versluis, Annemijn P.A. Wierenga, Fariba Nemati, Pieter A. van der Velden, Wilma G.M. Kroes, Robert M. Verdijk, Gregorius P.M. Luyten, Martine J. Jager

Cancers 2020, 12, 3690-3704



Simple Summary

Chemotherapy and immunotherapy are both used to treat malignancies. The immunotherapy of cancer often involves T cells, which recognise the antigens presented in HLA molecules. Uveal melanoma (UM) is an intraocular malignancy, which often gives rise to metastases. We determined whether high-risk tumours expressed the target of two drugs, histone deacetylase (HDAC) inhibitor Quisinostat and Tazemetostat, an inhibitor of Enhancer of zeste homologue 2 (EZH2). We observed that especially high-risk UM tumours (monosomy 3, gain of 8q, loss of BAP1) expressed several HDACs, and showed a high HLA Class I expression. We further tested whether these drugs influenced HLA Class I expression on three UM cell lines. The drug Quisinostat led to an upregulation of HLA protein and mRNA levels in three UM cell lines, while Tazemetostat had little effect. We concluded that the use of drugs that influence epigenetic regulators may impact immunotherapy approaches.

Abstract

The treatment of uveal melanoma (UM) metastases or adjuvant treatment may imply immunological approaches or chemotherapy. It is to date unknown how epigenetic modifiers affect the expression of immunologically relevant targets, such as the HLA Class I antigens, in UM.

We investigated the expression of HDACs and the histone methyl transferase EZH2 in a set of 64 UMs, using an Illumina HT12V4 array, and determined whether a histone deacetylase (HDAC) inhibitor and EZH2 inhibitor modified the expression of HLA Class I on three UM cell lines. Several HDACs (HDAC1, HDAC3, HDAC4, and HDAC8) showed an increased expression in high-risk UM, and were correlated with an increased HLA expression. HDAC11 had the opposite expression pattern. While in vitro tests showed that Tazemetostat did not influence cell growth, Quisinostat decreased cell survival. In the three tested cell lines, Quisinostat increased HLA Class I expression at the protein and mRNA level, while Tazemetostat did not have an effect on the cell surface HLA Class I levels.

Combination therapy mostly followed the Quisinostat results. Our findings indicate that epigenetic drugs (in this case an HDAC inhibitor) may influence the expression of immunologically relevant cell surface molecules in UM, demonstrating that these drugs potentially influence immunotherapy.

Keywords: Eye diseases, Uveal Melanoma, Oncology, HLA, HDAC, Immunology, Inflammation

1. Introduction

Uveal melanoma (UM) is a rare malignancy of the eye, estimated to occur in 6–7 cases per million per year in northern Europe and the United States of America [1,2]. The risk of developing metastases is around 50% [3]. The tumour arises from the uveal tract, which involves the choroid, ciliary body, and the iris [4]. In spite of the immunologically privileged nature of the eye, high-risk UM may show inflammation, and the presence of an inflammatory phenotype is related to a bad prognosis [5,6,7]. This inflammatory phenotype is characterised by the presence of high numbers of lymphocytes and macrophages, and a high HLA Class I and II expression [7,8,9,10]. All of these are related to the loss of one chromosome 3, a well-known risk factor for the development of metastases in this malignancy.

A high HLA Class I surface expression may protect UM cells from killing by natural killer cells (NK), and as metastases in this disease occur hematogenously, where NK cells help to remove tumour cells, a high HLA expression may help tumour cells to escape destruction [11,12,13]. On the other hand, the loss of HLA Class I expression has been identified as a tumour-escape mechanism in for instance cutaneous melanoma [14]. Both genetic and epigenetic events have been identified as regulators of HLA Class I and II expression [15], which is also the case in UM [16,17]. A study from our lab showed that one epigenetic regulator, EZH2, part of the Polycomb Repressive Complex 2 (PRC2), was found to influence HLA Class II expression in UM through the histone methylation of promoter IV of CIITA [18]. In other malignancies, members of the histone deacetylase (HDAC) family were identified as HLA regulators [19- 22].

As HDACs are aberrantly expressed in UM [23], targeting these epigenetic regulators is considered for treatment [24]. In addition, the use of such inhibitors as adjuvant therapy in UM is being investigated [25, 26]. A trial for the treatment of UM metastases has been set up [27], in

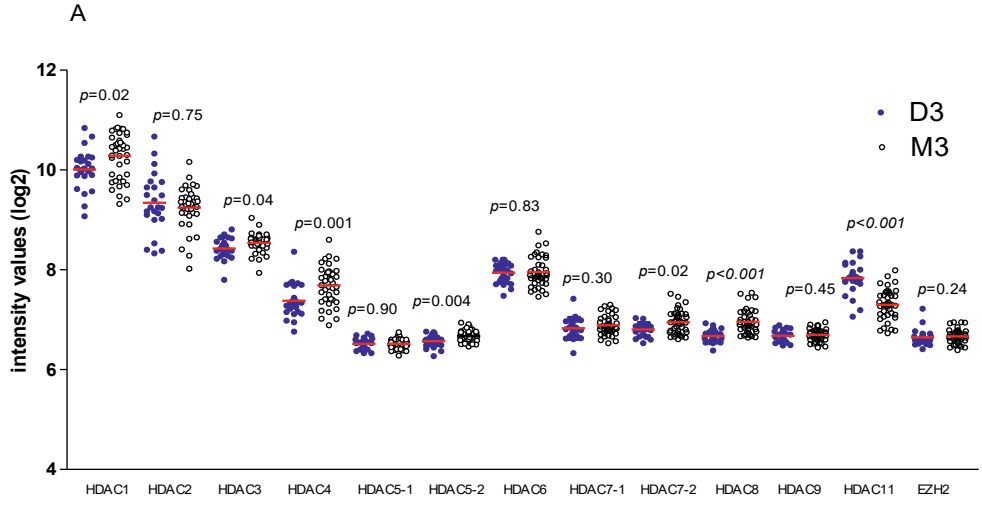
which an immune checkpoint inhibitor (Pembrolizumab) is combined with the HDAC inhibitor Entinostat, under the hypothesis that the inhibition of HDACs may lead to an enhanced expression of HLA and cancer antigens, and a decreased activity of myeloid-derived suppressor cells. While this may help T cell-mediated immunotherapy, it may negatively affect NK cell-mediated lysis.

We set out to investigate the effect of two clinically relevant inhibitors of epigenetic modifiers, Quisinostat, an HDAC inhibitor, and Tazemetostat, an EZH2 inhibitor, on the expression of HLA Class I molecules on UM cell lines. We first determined the expression of HDACs, EZH2, as well as HLA expression in a set of 64 UMs.

2. Results

2.1. HDAC and EZH2 Expression in UM, Association with High Risk

Inhibitors of HDACs may be used as adjuvant treatment in combination with immune checkpoint inhibitors; as especially high-risk UM tumours with an inflammatory phenotype that express HLA Class I give rise to metastases, we wondered whether these high-risk tumours would express HDACs. We studied the mRNA expression of HDACs in a panel of 64 primary UMs, and compared their expression with the tumour's chromosome 3, chromosome 8q and BAP1 status, which are indicators of a high risk of metastases formation (Figure 1A–C).



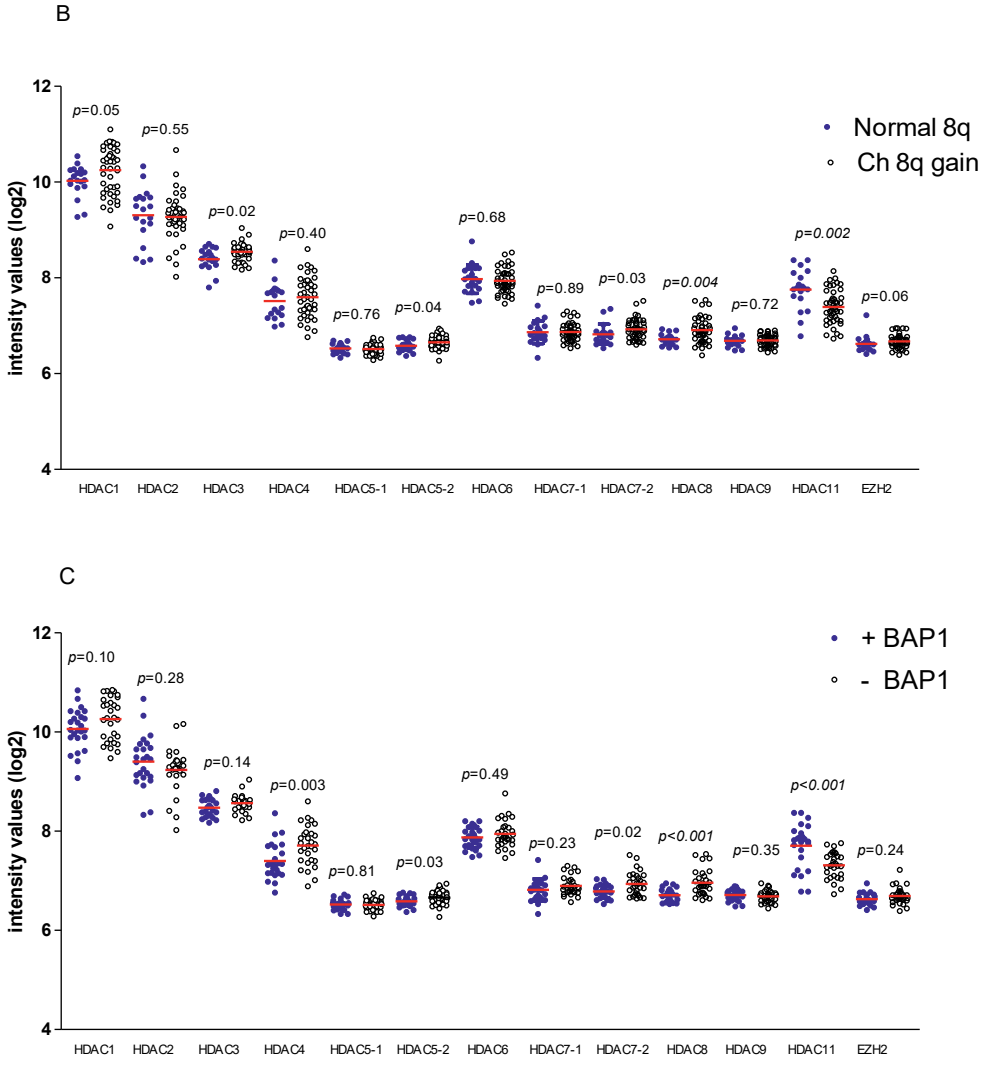


Figure 1. The expression of histone deacetylases (HDACs) in uveal melanoma in relation to the tumour's chromosome 3, chromosome 8q and BAP1 status. The expression of several HDACs and EZH2 was compared between tumours with (A) disomy for chromosome 3 (D3, n = 24) and a monosomy of chromosome 3 (M3, n = 40); (B) normal 8q (n = 19) vs. 8q gain (n = 45); (C) positive staining for BAP1 (+BAP1, n = 25) vs. negative staining for BAP1 (-BAP1, n = 30). A Mann–Whitney U test was applied. Horizontal bars indicate mean gene expression.

Expression of HDAC1 ($p = 0.02$), HDAC3 ($p = 0.04$), HDAC4 ($p = 0.001$), as well as HDAC8 ($p < 0.001$) was significantly higher in M3 tumours compared to D3 tumours. In contrast, HDAC11 had a significantly lower expression in high-risk M3 tumours ($p < 0.001$). With regard to both HDAC5 as well as HDAC7 expression, we found significant differences between D3 vs. M3 tumours for only one of the two probes that had been used (HDAC5 probe 1 $p = 0.90$, HDAC5 probe 2 $p = 0.004$, HDAC7 probe 1 $p = 0.30$, HDAC7 probe 2 $p = 0.02$).

The patterns for chromosome 8q gain (Figure 1B) and BAP1 expression (Figure 1C) resembled those of chromosome 3. HDAC1 ($p = 0.05$), HDAC3 ($p = 0.02$), the second probe of HDAC5 ($p = 0.04$) and of HDAC7 ($p = 0.03$), and HDAC8 ($p = 0.004$), were increased in tumours with 8q gain, while HDAC11 was decreased ($p = 0.002$). The loss of BAP1 expression as assessed by immunohistology was associated with an increase in HDAC4 ($p = 0.003$), the second probe of HDAC5 ($p = 0.03$) and HDAC7 ($p = 0.02$) and a decrease in HDAC11 ($p < 0.001$).

We also looked at EZH2, which is known to be higher in UM with a high mitotic count [28]. However, the EZH2 expression was not correlated with chromosome 3 or 8q status, or BAP1 status ($p = 0.24$, $p = 0.06$, $p = 0.24$).

2.2. Association between HDAC and EZH2 Expression and HLA Class I in UM

As high-risk tumours are known to have a higher HLA Class I expression than low risk tumours, we hypothesised that the expression of HLA Class I and M3-associated HDACs would be correlated. We tested this for all HDACs and EZH2 in our set of 64 tumours.

Expression of HDAC1, HDAC4, and HDAC8 was convincingly positively correlated with HLA-A and/or HLA-B (Table 1). The two HDAC7 probes showed diverging results, with the M3-associated probe being positively correlated with one HLA-A probe and with the HLA-B probe. The HDAC11 expression showed a significant negative correlation with HLA-A and -B expression (all p s < 0.001). These data indicate that the expression levels of three of the four HDACs, which are upregulated in M3 tumours, correlate with a high HLA-A and -B expression. HDAC11 shows the opposite pattern. No significant correlation was seen between the EZH2 and HLA Class I expression in this set of 64 tumours.

Table 1. Correlation between mRNA expression levels (determined by Illumina array) of different HDACs and EZH2 and the expression of HLA-A (three probes) and HLA-B. R = two-tailed Spearman correlation coefficient. Following Bonferroni correction, $p \leq 0.005$ is considered significant, and indicated in bold.

	HLA-A probe 1		**HLA-A probe 2**		**HLA-A probe 3**		**HLA-B**	
	R	***p***	**R**	***p***	**R**	***p***	**R**	***p***
HDAC1	0.367	**0.003**	0.392	**0.001**	0.380	**0.002**	0.370	**0.003**
HDAC2	−0.144	0.26	−0.167	0.19	−0.108	0.40	−0.162	0.20
HDAC3	0.262	0.04	0.241	0.05	0.293	0.02	0.318	0.01
HDAC4	0.143	0.26	0.371	**0.003**	0.354	**0.004**	0.305	0.01
HDAC5 probe 1	0.029	0.82	0.090	0.48	0.018	0.88	−0.045	0.72
HDAC5 probe 2	0.091	0.47	0.231	0.07	0.096	0.45	0.151	0.23
HDAC6	−0.179	0.16	−0.136	0.28	−0.284	0.02	−0.196	0.12
HDAC7 probe 1	−0.020	0.87	0.010	0.94	−0.014	0.91	0.108	0.39
HDAC7 probe 2	0.225	0.07	0.382	**0.002**	0.263	0.04	0.352	**0.004**
HDAC8	0.456	**<0.001**	0.588	**<0.001**	0.539	**<0.001**	0.550	**<0.001**
HDAC9	−0.066	0.60	−0.091	0.48	−0.209	0.01	−0.190	0.13
HDAC11	−0.541	**<0.001**	−0.548	**<0.001**	−0.515	**<0.001**	−0.607	**<0.001**
EZH2	0.211	0.09	0.214	0.09	0.226	0.07	0.146	0.25

2.3. In Vitro Analysis of Effect of HDAC and EZH2 Inhibition on UM Cell Lines

As mentioned earlier, high-risk M3 UMs contain more infiltrating cells, which also express HLA antigens. The correlation between the expression of certain genes and high-risk tumours is, therefore, difficult to interpret, as it cannot be excluded that the increased levels of, e.g., HLA Class I and certain HDACs, is caused to a significant extent by the increased number of infiltrating cells and not so much by the UM tumour cells themselves.

To be able to investigate an effect of HDAC activity on HLA Class I expression, we made use of the pan-HDAC inhibitor Quisinostat and treated the UM cell lines in vitro.

Recent studies have shown that HDAC inhibitors are able to reduce growth in UM cell lines [29,30], but to our knowledge, no one has reported on the effect of epigenetic enzyme inhibition on the expression of immune modulators, such as HLA Class I, in UMs. We also used an EZH2 inhibitor, Tazemetostat, and studied the effect of combination therapy.

2.3.1. Morphological Analysis of UM Cell Lines after 48h Treatment with Quisinostat and Tazemetostat

First of all, we looked at the morphological characteristics of the cell lines after 48h treatment with Quisinostat and Tazemetostat compared with untreated cell lines (Figure 2). In the presence of Quisinostat (Q, 40 nM), at 48h, the cell sizes of all three cell lines was reduced and more floating cells were visible. Upon treatment with Tazemetostat (T, 5 µM), at 48h, no large differences in the morphology or confluency were observed compared to the controls. Cells which were treated with a combination of the two drugs (40 nM Q + 5µM T) changed morphologically and resembled Quisinostat single treatment: plates with OMM2.5 and MP38 seemed to have fewer cells than when treated with Quisinostat alone.

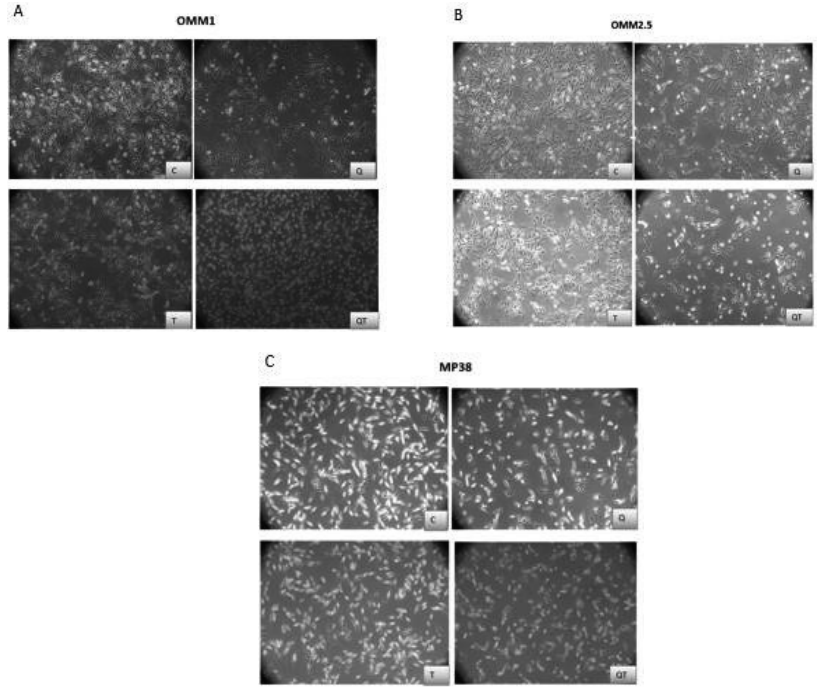


Figure 2. Photographs of uveal melanoma cell lines taken after 48h exposure to Quisinostat (Q) and/or Tazemetostat (T): the photographs (magnification 100×) show fewer and more rounded cells after incubation with 40 nM Q and after combination treatment. (A) = cell line OMM1, (B) = cell line OMM2.5, (C) = cell line MP38. C: control; Q: 40 nM Quisinostat; T: 5 µM Tazemetostat; QT: combination of 40 nM Quisinostat and 5 µM Tazemetostat.

2.3.2. Quisinostat and Cell Growth

A quantitative analysis of cell numbers after 24 and 48h using the Cell Titer-Blue Cell Viability Assay (Figure 3) confirmed these findings: Quisinostat reduced the cell numbers of OMM2.5 and MP38 cells after 24h ($p = 0.01$, $p = 0.03$), and of all three cell lines after 48h ($p < 0.001$, $p = 0.002$, $p < 0.001$). Tazemetostat by itself did not influence cell numbers. However, at 48h, the combination of both drugs (QT) decreased cell numbers significantly in two cell lines (OMM1 and MP38) compared to the Q treatment alone ($p < 0.001$ for both).

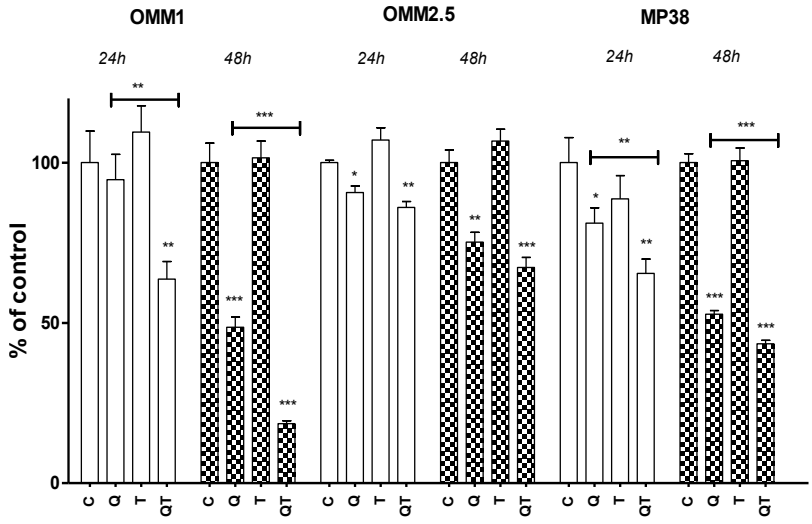


Figure 3. Cell numbers of three uveal melanoma cell lines after 48 h of treatment with Quisinostat or Tazemetostat of uveal melanoma cell lines. Cell density was determined using a cell titer blue assay. C: control; Q: 40 nM Quisinostat; T: 5 µM Tazemetostat; QT: combination of 40 nM Quisinostat and 5 µM Tazemetostat. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$. Error bars indicate the standard error of the mean.

2.3.3. Effect of Quisinostat and Tazemetostat on HLA Class I Cell Surface Expression

We investigated the effect of Quisinostat and Tazemetostat on HLA Class I surface expression of the UM cell lines by flow cytometry after 48h treatment. The three cell lines (OMM1, OMM2.5, and MP38) expressed HLA Class I, as determined using monoclonal antibody W6/32 (Figure 4A–C, controls). Quisinostat increased HLA Class I surface expression in all three cell lines ($p < 0.01$). Tazemetostat, on the other hand, did not affect the HLA Class I surface expression in the cell lines,

while the combination treatment resulted in a significant increase compared to Quisinostat alone in OMM2.5 and MP38 ($p = 0.003$ and $p = 0.004$).

2.3.4. Effect of Quisinostat and Tazemetostat on HLA Class I mRNA Expression by qPCR

We investigated the level of HLA Class I mRNA by qPCR after 48h of treatment with Quisinostat and/or Tazemetostat. HLA-A and HLA-B expressions were determined separately (Figure 4D–F and Figure 4G–I). In agreement with what we found regarding broad HLA Class I cell surface expression, Quisinostat increased HLA-A in all three cell lines ($p < 0.01$). Tazemetostat gave a slight increase in cell line OMM2.5, while in all three cell lines, combined treatment led to a lower expression than with Quisinostat alone. With regard to HLA-B, Quisinostat led to a higher expression in all three cell lines, while Tazemetostat by itself gave a significant but slight increase in MP38. The combination of the two drugs led to a high expression on all three cell lines, with divergent results when compared to Quisinostat alone.

3. Discussion

HDACs are a group of epigenetic regulators which have been shown to regulate inflammation in some malignancies [31,32]. Moreover, elevated HDAC expression has been associated with other inflammation-related diseases such as chronic hepatitis B [33] and rheumatoid arthritis (RA) [34]. To our knowledge, the relationship between the expression of these epigenetic regulators and the inflammatory phenotype in UM has not been investigated. As combinations of chemotherapy and immunotherapy are being used as adjuvant treatments and to treat metastases in e.g., cutaneous melanoma, we investigated the expression of several epigenetic regulators and their potential involvement in the regulation of HLA Class I, a molecule which plays an important role in immune responses, in UM.

In a set of 64 UMs, the expression of HDACs 1, 4, and 8 was positively correlated with the expression of HLA Class I. Using malignant cell lines such as from colorectal carcinoma, e.g., HDAC1 has been shown to be related to inflammation, by inducing STAT1 activation and the transcription of downstream targets of the IFN-Y signalling pathway [35, 36]; such targets include HLA Class I components. In addition, we show that particularly HDAC1, 3, 4, and 8 are expressed at higher levels in high-risk UM.

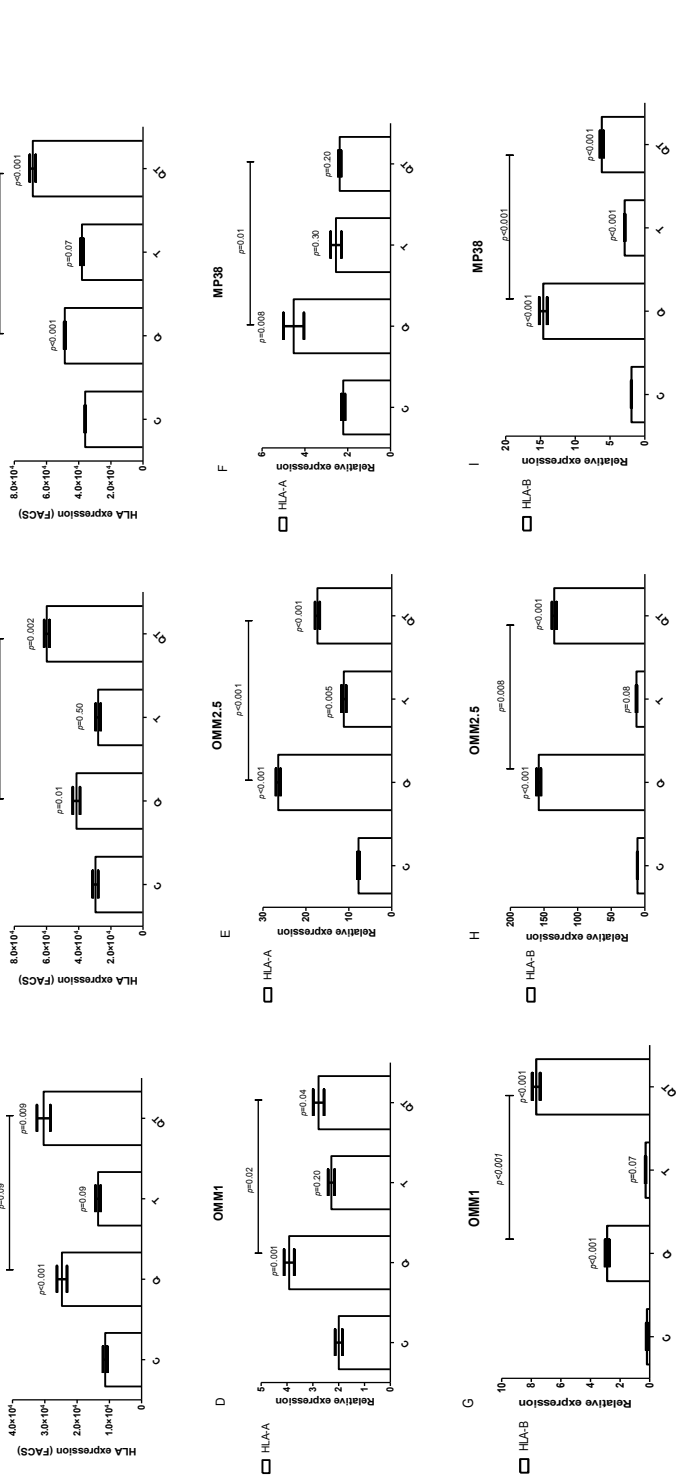


Figure 4. HLA Class I cell surface protein expression and mRNA levels after 48h of treatment with Quisinostat and/or Tazemetostat compared to the control. Cell surface expression was determined by FACS using anti-HLA W6/32 antibody (A–C). HLA-A mRNA (D–F) and HLA-B mRNA (G–I) expression was determined by qPCR. C: control; Q: 40 nM Quisinostat; T: 5 μM Tazemetostat; QT: combination of 40 nM Quisinostat and 5 μM Tazemetostat. Using an Independent t-test, $p \leq 0.05$ was considered significant. Error bars indicate the standard error of the mean of three measurements.

We tested the effects of two drugs, Quisinostat and Tazemetostat. The pan-HDAC inhibitor Quisinostat has been shown to effectively reduce UM cell viability in vitro [29, 37], and is currently being tested in clinical studies of advanced solid tumours and leukaemia [38]. Growth inhibition was accomplished by the induction of cell cycle arrest and apoptosis in other cancers such as lung cancer [39], and hepatocellular carcinoma [40]. Tazemetostat is a drug known to inhibit EZH2 and has been shown to reduce growth in medulloblastoma [41], B-cell non-Hodgkin lymphoma and epithelioid sarcoma [42]. When we applied the pan-HDAC inhibitor Quisinostat to UM cell lines, HLA Class I cell surface and mRNA expression increased, while the effect of Tazemetostat was marginal. As Quisinostat is a pan-HDAC inhibitor [30], the effect is likely due to the inhibition of deacetylation. Indeed, we have previously shown that treatment with Quisinostat strongly increased the level of acetylated Histone 3 [29]. In general, higher acetylation coincides with less chromatin compactness, higher promoter activity and increased gene transcription. We have no evidence that the effect of HDAC inhibition on HLA Class I gene transcription is caused by a direct effect on the chromatin modification around the Class I promoters. However, our results are generally in agreement with studies in other malignancies describing that HDAC inhibition will upregulate HLA Class I and some HLA regulatory components [19,20,21,22,43]. Whether increasing HLA expression in UM is helping the patient may depend on the situation: it may help to stimulate T cell-mediated cytotoxicity against metastases; however, when the primary UM is still in place, increasing HLA expression may limit the NK cell activity directed against metastatic cells which spread hematogeneously.

HDAC11 expression was significantly lower in high-risk tumours (Figure 1A–C). In 2014, Herlihy and colleagues already showed a low expression of HDAC11 in a small series of M3 UMs [23]. The gene of this HDAC is located on chromosome 3, and the loss of chromosome 3 in high-risk UMs most likely explains these findings. However, it does not necessarily mean that low HDAC11 expression has no causal relationship with an increased risk of the development of metastases. Although most studies on the function of HDAC11 suggest an oncogenic role, as in breast cancer the downregulation of HDAC11 provided the cells with an increased ability to invade from the lymph nodes to other organs [44]. It is therefore possible that the low HDAC11 expression may help the hematogenous spread to the liver.

In UMs, EZH2 plays a major role in silencing promoters and inhibiting transcription [18] and is associated with a high mitotic count [28]. Although this enzyme represses CIITA transcription by the K27m3 of histone 3, thereby silencing HLA Class II expression, we observed a high expression of EZH2 in high-risk M3 tumours, without any association to HLA Class I expression. EZH2 inhibition with Tazemetostat did not have an effect on the cell surface expression of HLA Class I, while we observed a slight increase in HLA-A mRNA in OMM2.5 and HLA-B mRNA in cell line MP38. The combination with Quisinostat led to results that varied between cell lines and HLA allele.

A Phase 1 trial is planned to use the HDAC 1, 2, and 3 inhibitor Domatinostat together with immune checkpoint inhibitors as the neo-adjuvant treatment of cutaneous melanoma patients with lymph node metastases (ClinicalTrials.gov NCT-4133048). This trial is set up under the assumption that the HDAC inhibitor will stimulate the interferon pathway, making the tumour "hot". Such tumours may contribute to a good induction of cytotoxic T cells. As already mentioned earlier, high-risk UMs already have a high HLA expression, and it is unclear whether the same approach will help to treat the metastases of UMs. While HLA expression is important for tumour cell killing by T cells, a high expression blocks recognition by NK cells. Furthermore, the expression of other cell surface molecules such as checkpoint inhibitors may also be modified by blocking epigenetic regulators.

4. Materials and Methods

4.1. Study Population

Tumours were derived from 64 eyes that underwent an enucleation for UMs between 1999 and 2008 at the Leiden University Medical Center (LUMC), Leiden, The Netherlands; 51% of the patients were male and 49% female. Their mean age at the time of enucleation was 61 years.

This work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). The project was approved by the LUMC Biobank committee and the LUMC METC committee (19 October 2016, code G16.076/NV/gk).

4.2. Chromosome Analysis

DNA was isolated using the QIAmp DNA Mini kit (Qiagen, Venlo, The Netherlands). Single-nucleotide polymorphism (SNP) analysis was performed using an Affymetrix 250K_NSP or Affymetrix SNP 6.0 array to detect chromosome 3 and 8 abnormalities [45].

4.3. Immunohistochemistry

Tissues were incubated with mouse monoclonal antibody (clone sc-28383, 1:50 dilution, Santa Cruz Biotechnology, Dallas, TX, USA) for BAP1 evaluation [46,47]. Nuclear staining was evaluated by an experienced ocular pathologist.

4.4. Tumour Gene Expression

Gene expression profiling was performed with the Illumina HT12v4 array (Illumina, Inc., San Diego, CA, USA) and the data were obtained for the expression of epigenetic regulators (HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, HDAC8, HDAC9, HDAC11, EZH2) and HLA Class I genes (HLA-A, HLA-B) as described previously [16]. Information regarding the Illumina probe numbers and gene expression levels is shown in Table S1.

4.5. Reagents

Quisinostat and Tazemetostat were purchased from Selleckchem (Houston, TX, USA). Drugs were dissolved in dimethyl sulfoxide (DMSO) to reach a stock concentration of 5 mM and diluted in the indicated fresh medium for in vitro studies.

4.6. Cell Lines and Cell Culture

The OMM1 cell line was established by Dr G.P.M. Luyten (LUMC, Leiden, The Netherlands) [48], while OMM2.5 was a gift from Dr B.R. Ksander (Schepens Eye Research Institute, Boston, MA, USA) [49]. Both were cultured in Roswell Park Memorial Institute Medium 1640 (RPMI) Dutch modified media (Life technologies, Europe bv, Bleiswijk, The Netherlands) supplemented with 10% foetal bovine serum (FBS) (Greiner Bio-one, Alphen aan den Rijn, The Netherlands), 1% GlutaMAX and 1% penicillin/streptomycin (Life technologies). MP38 was kindly provided by the Curie Institute, Paris, France [50] and cultured in Iscove's modified Dulbecco medium

(IMDM) (Life Technologies) containing 20% FBS (Greiner Bio-one) and 1% penicillin/streptomycin (Gibco). Cells were incubated in 5% CO2 at 37 °C in monolayers in tissue culture flasks in a humidified incubator

4.7. Cell Proliferation Study

Cells were seeded in triplicate in 135 µL in 96-well plates; 24 h after seeding the cells, 15 µL of 10× concentrated drug in media was added to each well. Cell viability was determined by the Cell Titer-Blue Viability Assay: after removing the medium, 100 µL of appropriately diluted CellTiter-Blue Reagent (Promega, Fitchburg, WI, USA) was added to each well and after 60 min, fluorescence was measured in a microplate reader (Victor X3, Perkin Elmer, San Jose, CA, USA).

4.8. Flow Cytometry

Cells were incubated with an optimal dilution of mouse monoclonal anti-HLA Class I antibody (W6/32, 311414, Alexa Fluor 647; Bio Legend, Amsterdam, The Netherlands) and the data were collected (10,000–15,000 cells per live gate) using the BD LSR II system (BD Biosciences, San Diego, CA, USA); results were analysed using FACSDiva software (BD Biosciences). Three independent experiments have been performed.

4.9. RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qPCR)

RNA isolation and quantitative real-time polymerase chain reaction (qPCR) have been described previously [51]. Briefly, total RNA was extracted from cell cultures using an RNeasy Mini Kit (Qiagen Benelux B.V., Venlo, The Netherlands). Using the iScript cDNA synthesis kit (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands), complementary cDNA was synthesised. Quantitative real-time polymerase chain reaction (qPCR) was performed in three independent experiments using the CFX-384 machine, with triplicates of one experiment used to make the graphs. CFX manager 3.1 (Bio-Rad) software was used to analyse the data, and the CT values of genes of interest were normalised against the geometric mean of housekeeping genes RPS11, CAPNS1, and SRPR. The sequences of primers used in the study are shown in Supplementary Table S2.

4.10. Statistical Analysis

Data were analysed with SPSS software version 22.0 (SPSS, nc. Chicago, IL, USA). Spearman correlation was performed in order to test correlations between non-parametric data. Bonferroni correction was applied for multiple testing, where appropriate. The Mann–Whitney U test was used to compare non-normal groups. Graphs were obtained using GraphPad Prism version 5.0 for Windows (GraphPad Software, La Jolla, CA, USA). An independent t-test was used to compare the difference between the means of the in vitro studies.

5. Conclusions

To the best of our knowledge, this is the first study which reports the association between HDAC epigenetic regulators and HLA Class I expression in UMs. We showed that high-risk UMs not only show a higher expression of HLA Class I antigens but also of several HDACs. The pan-HDAC inhibitor Quisinostat increases HLA Class I expression at the protein and mRNA level while EZH2 inhibition by Tazemetostat has a slight effect on HLA expression. As epigenetic anti-cancer drugs may influence the expression of immunologically relevant molecules such as HLA in UMs, they may influence the function of T cells and NK cells, and thereby the effect of immunotherapy.

Author Contributions: Conceptualisation, Z.S., M.J.J.; methodology, Z.S., A.G.J., M.V., and R.M.V.; formal analysis, Z.S.; data curation, A.P.A.W.; writing—original draft preparation, Z.S. and M.J.J.; writing—review and editing, A.G.J., M.V., A.P.A.W., F.N., P.A.v.d.V., W.G.M.K., R.M.V., G.P.M.L. and M.J.J.; supervision, A.G.J. and M.J.J.; funding acquisition, P.A.v.d.V., A.G.J. and M.J.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by a grant to Z.S. from The Iranian Ministry of Science Research and Technology and a Horizon 2020 grant from the European Community, CURE UM, nr. 667787.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Virgili, G.; Gatta, G.; Ciccolallo, L.; Capocaccia, R.; Biggeri, A.; Crocetti, E. et al. Incidence of uveal melanoma in Europe. Ophthalmology 2007, 114(12), 2309-2315.
2. Singh, A.D.; Topham, A. Incidence of uveal melanoma in the United States: 1973–1997. Ophthalmology 2003, 110(5), 956-961. Kujala, E.; Mäkitie, T.; Kivelä, T. Very long-term prognosis of patients with malignant uveal melanoma. Invest. Ophthalmol. Vis. Sci. 2003, 44(11), 4651-4659. DOI: 10.1167/iovs.03-0538.
3. Jager, M.J.; Shields, C.L.; Cebulla, C.M.; Abdel-Rahman, M.H.; Grossniklaus, H.E.; Stern, M.H. et al. Uveal melanoma. Nat. Rev. Dis. Primers 2020, 6(1), 1-25.
4. Jager, M.J.; Hurks, H.M.H.; Levitskaya, J.; Kiessling, R. HLA expression in uveal melanoma: there is no rule without some exception. Hum. Immunol 2002, 63(6), 444-451.
5. Bronkhorst, I.H.; Vu, T.H.; Jordanova, E.S.; Luyten, G.P.; Burg, S.H. van der; Jager, M.J. Different subsets of tumor-infiltrating lymphocytes correlate with macrophage influx and monosomy 3 in uveal melanoma. Invest. Ophthalmol. Vis. Sci. 2012, 53(9), 5370-5378.
6. Robertson, A.G.; Shih, J.; Yau, C.; Gibb, E.A.; Oba, J.; Mungall, K.L. et al. Integrative analysis identifies four molecular and clinical subsets in uveal melanoma. Cancer Cell 2017, 32(2), 204-220.
7. Blom, D.J.; Luyten, G.P.; Mooy, C.; Kerkvliet, S.; Zwinderman, A.H.; Jager, M.J. Human leukocyte antigen class I expression. Marker of poor prognosis in uveal melanoma. Invest. Ophthalmol. Vis. Sci. 1997, 38(9), 1865-1872.
8. Dithmar, S.; Crowder, J.; Jager, M.J.; Vigniswaran, N.; Grossniklaus, H.E. HLA class I antigen expression correlates with histological cell type in uveal melanoma. Der Ophthalmologe 2002, 99(8), 625-628.
9. Ericsson, C.; Seregard, S.; Bartolazzi, A.; Levitskaya, E.; Ferrone, S.; Kiessling, R. et al. Association of HLA class I and class II antigen expression and mortality in uveal melanoma. Invest. Ophthalmol. Vis. Sci. 2001, 42(10), 2153-2156.
10. Ma, D.; Niederkorn, J.Y. Transforming growth factor-beta down-regulates major histocompatibility complex class I antigen expression and increases the susceptibility of uveal melanoma cells to natural killer cell-mediated cytolysis. Immunology 1995, 86(2), 263-269.
11. Ma, D.; Luyten, G.P.; Luider, T.M.; Niederkorn, J.Y. Relationship between natural killer cell susceptibility and metastasis of human uveal melanoma cells in a murine model. Invest. Ophthalmol. Vis. Sci. 1995, 36(2), 435-441.

12. Niederkorn, J.Y. Immune escape mechanisms of intraocular tumors. Prog. Retin. Eye Res. 2009, 28(5), 329-347.
13. Garcia-Lora, A.; Algarra, I.; Garrido, F. MHC class I antigens, immune surveillance, and tumor immune escape. J. Cell. Physiol. 2008, 195(3), 346-355.
14. Campoli, M.; Ferrone, S. HLA antigen changes in malignant cells: epigenetic mechanisms and biologic significance. Oncogene 2008, 27(45), 5869-5885.
15. Van Essen, T.H.; van Pelt, S.I.; Bronkhorst, I.H.; Versluis, M.; Nemati, F.; Laurent, C. et al. Upregulation of HLA expression in primary uveal melanoma by infiltrating leukocytes. PLoS One 2016, 11(10), e0164392.
16. Souri, Z.; Wierenga, A.; Mulder, A.; Jochemsen, A.G.; Jager, M.J. HLA Expression in Uveal Melanoma: An Indicator of Malignancy and a Modifiable Immunological Target. Cancers 2019, 11(8), 1132.
17. Holling, T.M.; Bergevoet, M.W.; Wilson, L.; van Eggermond, M.C.; Schooten, E.; Steenbergen, R.D. et al. A role for EZH2 in silencing of IFN-γ inducible MHC2TA transcription in uveal melanoma. J. Immunol. 2007, 179(8), 5317-5325.
18. Magner, W.J.; Kazim, A.L.; Stewart, C.; Romano, M.A.; Catalano, G.; Grande, C. et al. Activation of MHC class I, II, and CD40 gene expression by histone deacetylase inhibitors. J. Immunol. 2000, 165(12), 7017-7024.
19. De Lourdes Mora-García, M.; Duenas-González, A.; Hernández-Montes, J.; De la Cruz-Hernández, E.; Pérez-Cárdenas, E.; Weiss-Steider, B. et al. Up-regulation of HLA Class-I antigen expression and antigen-specific CTL response in cervical cancer cells by the demethylating agent hydralazine and the histone deacetylase inhibitor valproic acid. J. Transl. Med. 2006, 4(1), 1-14.
20. Kitamura, H.; Torigoe, T.; Asanuma, H.; Honma, I.; Sato, N.; Tsukamoto, T. Down-regulation of HLA class I antigens in prostate cancer tissues and up-regulation by histone deacetylase inhibition. J. Urol. 2007, 178(2), 692-696.
21. Khan, A.N.; Gregorie, C.J.;, Tomasi, T.B. Histone deacetylase inhibitors induce TAP, LMP, Tapasin genes and MHC class I antigen presentation by melanoma cells. Cancer Immunol. Immunother. 2008, 57(5), 647-654.
22. Herlihy, N.; Dogrusöz, M.; van Essen, T.H.; Harbour, J.W.; van der Velden, P.A.; van Eggermond, M.C. et al. Skewed expression of the genes encoding epigenetic modifiers in high-risk uveal melanoma. Invest. Ophthalmol. Vis. Sci. 2015, 56(3), 1447-1458.
23. Li, Y.; Jia, R.; Ge, S. Role of epigenetics in uveal melanoma. Int. J. Biol. Sci. 2017, 13(4), 426-433.

24. Landreville, S.; Agapova, O.A.; Matatall, K.A.; Kneass, Z.T.; Onken, M.D.; Lee, R.S. et al. Histone deacetylase inhibitors induce growth arrest and differentiation in uveal melanoma. Clin. Cancer Res. 2012, 18(2), 408-416.
25. Triozzi, P.L.; Singh, A.D. Adjuvant therapy of uveal melanoma: current status. Ocul. Oncol. Pathol. 2015, 1(1), 54-62.
26. Jespersen, H.; Bagge, R.O.; Ullenhag, G.; Carneiro, A.; Helgadottir, H.; Ljuslinder, I. et al. Concomitant use of pembrolizumab and entinostat in adult patients with metastatic uveal melanoma (PEMDAC study): protocol for a multicenter phase II open label study. BMC Cancer 2019, 19(1), 415-421.
27. Cheng, Y.; Li, Y.; Huang, X.; Wei, W.; Qu, Y. Expression of EZH2 in uveal melanomas patients and associations with prognosis. Oncotarget 2017, 8(44), 76423–76431.
28. Heijkants, R.; Willekens, K.; Schoonderwoerd, M.; Teunisse, A.; Nieveen, M.; Radaelli, E. et al. Combined inhibition of CDK and HDAC as a promising therapeutic strategy for both cutaneous and uveal metastatic melanoma. Oncotarget 2018, 9(5), 6174–6187.
29. Moschos, M.M.; Dettoraki, M.; Androudi, S.; Kalogeropoulos, D.; Lavaris, A.; Garmpis, N.; et al. The role of histone deacetylase inhibitors in uveal melanoma: current evidence. Anticancer Res. 2018, 38(7), 3817-3824.
30. Lu, J.; Sun, H.; Wang, X.; Liu, C.; Xu, X.; Li, F. et al. Interleukin-12 p40 promoter activity is regulated by the reversible acetylation mediated by HDAC1 and p300. Cytokine 2005, 31(1), 46-51.
31. Shakespear, M.R.; Halili, M.A.; Irvine, K.M.; Fairlie, D.P.; Sweet, M.J. Histone deacetylases as regulators of inflammation and immunity. Trends Immunol. 2011, 32(7), 335-343.
32. Zhang, H.; Li, X.; Zhang, Q.; Yang, F.; Chu, X.; Zhang, D. Role of histone deacetylase expression levels and activity in the inflammatory responses of patients with chronic hepatitis B. Mol. Med. Rep 2017, 15(5), 2744-2752.
33. Angiolilli, C.; Kabala, P.A.; Grabiec, A.M.; van Baarsen, I.M.; Ferguson, B.S.; García, S. et al. Histone deacetylase 3 regulates the inflammatory gene expression programme of rheumatoid arthritis fibroblast-like synoviocytes. Ann. Rheum. Dis. 2017, 76(1), 277-285.
34. Nusinzon, I.; Horvath, C.M. Interferon-stimulated transcription and innate antiviral immunity require deacetylase activity and histone deacetylase 1. Proc. Natl. Acad. Sci. USA 2003, 100(25), 14742-14747.
35. Klampfer, L.; Huang, J.; Swaby, L.A.; Augenlicht, L. Requirement of histone deacetylase activity for signaling by STAT1. J. Biol. Chem 2004, 279(29), 30358-30368.

36. Van der Ent, W.; Burrello, C.; Teunisse, A.F.; Ksander, B.R.; van der Velden, P.A.; Jager, M.J. et al. Modeling of human uveal melanoma in zebrafish xenograft embryos. Invest. Ophthalmol. Vis. Sci. 2014, 55(10), 6612-6622.
37. Venugopal, B.; Baird, R.; Kristeleit, R.S.; Plummer, R.; Cowan, R.; Stewart, A. et al. A phase I study of quisinostat (JNJ-26481585), an oral hydroxamate histone deacetylase inhibitor with evidence of target modulation and antitumor activity, in patients with advanced solid tumors. Clin. Cancer Res. 2013, 19(15), 4262-4272.
38. Bao, L.; Diao, H.; Dong, N.; Su, X.; Wang, B.; Mo, Q. et al. Histone deacetylase inhibitor induces cell apoptosis and cycle arrest in lung cancer cells via mitochondrial injury and p53 up-acetylation. Cell. Biol. Toxicol. 2016, 32(6), 469-482.
39. He, B.; Dai, L.; Zhang, X.; Chen, D.; Wu, J.; Feng, X. et al. The HDAC Inhibitor Quisinostat (JNJ-26481585) Suppresses Hepatocellular Carcinoma alone and Synergistically in Combination with Sorafenib by G0/G1 phase arrest and Apoptosis induction. Int. J. Biol. Sci. 2018, 14(13), 1845-1858.
40. Zhang, H.; Zhu, D.; Zhang, Z.; Kaluz, S.; Yu, B.; Devi, NS. et al. EZH2 targeting reduces medulloblastoma growth through epigenetic reactivation of the BAI1/p53 tumor suppressor pathway. Oncogene 2020, 39(5), 1041-1048.
41. Italiano, A.; Soria, J.C.; Toulmonde, M.; Michot, J.M.; Lucchesi, C.; Varga, A. et al. Tazemetostat, an EZH2 inhibitor, in relapsed or refractory B-cell non-Hodgkin lymphoma and advanced solid tumours: a first-in-human, open-label, phase 1 study. Lancet Oncol. 2018, 19(5), 649-659.
42. Ritter, C.; Fan, K.; Paschen, A.; Hardrup, S.R.; Ferrone, S.; Nghiem, P. et al. Epigenetic priming restores the HLA Class-I antigen processing machinery expression in Merkel cell carcinoma. Sci. Rep. 2017, 7(1), 2290-2300.
43. Leslie, P.L.; Chao, Y.L.; Tsai, Y.H.; Ghosh, S.K.; Porrello, A.; van Swearingen, A.E. et al. Histone deacetylase 11 inhibition promotes breast cancer metastasis from lymph nodes. Nat. Commun. 2019, 10(1), 1-2.
44. Versluis, M.; de Lange, M.J.; van Pelt, S.I.; Ruivenkamp, C.A.; Kroes, W.G.; Cao, J. et al. Digital PCR validates 8q dosage as prognostic tool in uveal melanoma. PLoS One 2015, 10(3), e0116371.
45. Luyten, G.P; Naus, N.C.; Mooy, C.M.; Hagemeijer, A.; Kan-Mitchell, J.; van Drunen, E. et al. Establishment and characterization of primary and metastatic uveal melanoma cell lines. Int. J. Cancer 1996, 66(3), 380-387.

46. Chen, P.W.; Murray, T.G.; Uno, T.; Salgaller, M.L.; Reddy, R.; Ksander, B.R. Expression of MAGE genes in ocular melanoma during progression from primary to metastatic disease. Clin. Exp. Metastasis 1997, 15(5), 509-518.

47. Amirouchene-Angelozzi, N.; Nemati, F.; Gentien, D.; Nicolas, A.; Dumont, A.; Carita, G. et al. Establishment of novel cell lines recapitulating the genetic landscape of uveal melanoma and preclinical validation of mTOR as a therapeutic target. J. Mol. Oncol. 2014, 8(8), 1508-1520.

48. Bronkhorst, I.H.; Jehs, T.M.; Dijkgraaf, E.M.; Luyten, G.P.; van der Velden, P.A.; van der Burg, S.H; et al. Effect of hypoxic stress on migration and characteristics of monocytes in uveal melanoma. JAMA Ophthalmol. 2014, 132(5), 614-621.

Supplementary Materials

The following are available online at https://www.mdpi.com/2072-6694/12/12/3690/s1, Table S1: Gene expression (Illumina) Probe Number in an Illumina HT12v4 array and mean expression of the genes used in this study; Table S2: Sequences of PCR primers used in this study.

Table S1. Geneexpression (Illumina) Probe Numberin an Illumina HT12v4 array and mean expression ofthe genes used in thisstudy.

Gene of interest	Illumina probe number	Mean expression± SD
HLA-A probe 1	ILMN_1671054	11.40 ± 0.90
HLA-A probe 2	ILMN_2203950	13.84 ± 0.77
HLA-A probe 3	ILMN_2186806	10.71 ± 1.45
HLA-B	ILMN_1778401	11.33 ± 1.67
HDAC1	ILMN_1727458	10.18 ± 0.45
HDAC2	ILMN_1767747	9.28 ± 0.48
HDAC3	ILMN_1772455	8.49 ± 0.20
HDAC4	ILMN_1764396	7.57 ± 0.39
HDAC5 probe 1	ILMN_2388166	6.52 ± 0.10
HDAC5 probe 2	ILMN_1810856	6.63 ± 0.13
HDAC6	ILMN_1798546	7.94 ± 0.26
HDAC7 probe 1	ILMN_3266186	6.86 ± 0.20
HDAC7 probe 2	ILMN_1728521	6.89 ± 0.21
HDAC8	ILMN_1651544	6.85 ± 0.24
HDAC9	ILMN_2408885	6.68 ± 0.12
HDAC11	ILMN_1684690	7.49 ± 0.40
EZH2	ILMN_1708105	6.65 ± 0.15

Table S2. Sequences of PCR primers used in this study.

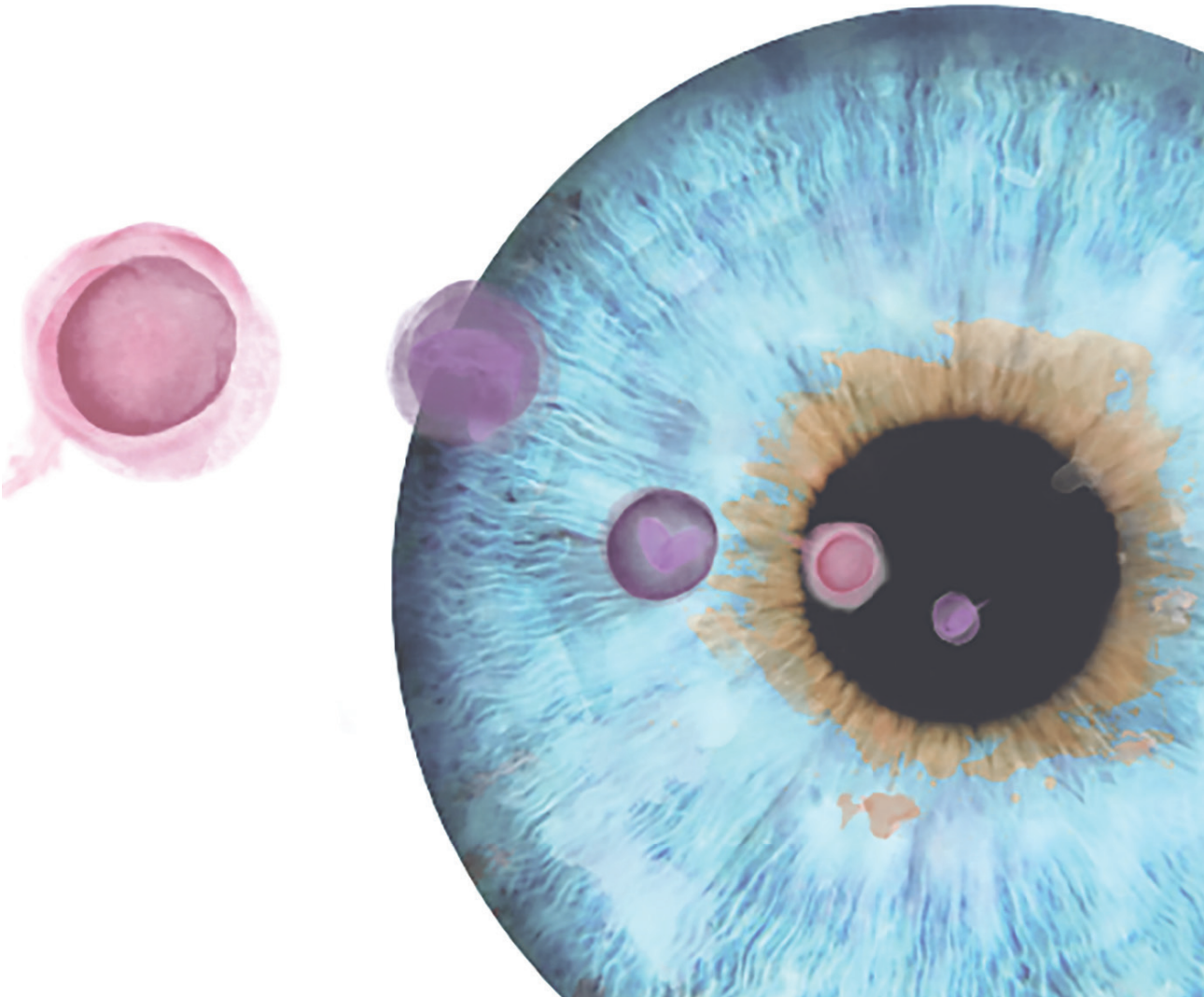
Primers	Forward	Reverse
HLA-A	5'- TGTGTTCGTGTAGGCATA	5'- TTGAGACAGAGATGGAGAC
HLA-B	5'- CTCCATCTCTGTCTCAACTT	5'- CATCAACCTCTCATAGCA
RPS11	5'- AAGCAGCCGACCATCTTTCA	5'- CGGGAGCTTCTCCTTGCC
CAPNS1	5'- ATGGTTTTGGCATTGACACATG	5'- GCTTGCCTGTGGTGTCGC
SRPR	5'- CATTGCTTTTGCACGTAACCAA	5'- ATTGTCTTGCATGCGGCC

Chapter 5

Expression of HDACs 1, 3, and 8 is upregulated in the presence of infiltrating lymphocytes in Uveal Melanoma

Z. Souri, A.G. Jochemsen, A.P.A. Wierenga, W.M.G. Kroes, R.M. Verdijk, P.A. van der Velden, G.P.M. Luyten, M.J. Jager

Cancers 2021



Simple Summary

Uveal melanoma (UM) is an ocular malignancy which is derived from melanocytes in the uveal tract. Epigenetic regulators such as Histone deacetylase enzymes (HDACs) are being tested as treatment of UM metastases. Expression of different HDACs is variable, and some are increased in high-risk tumours with loss of one chromosome 3. As this genetic abnormality is also associated with an inflammatory phenotype, we analysed whether HDAC expression was associated with inflammation. In two cohorts of UM cases, several HDACs showed a positive correlation with tumour-infiltrating leukocytes. Interferon-gamma stimulated expression of some HDACs on UM cell lines. These data suggest that cytokines produced by leukocytes may be responsible for the increased expression of some HDACs in UM with monosomy 3.

Abstract

In Uveal Melanoma (UM), an inflammatory phenotype is strongly associated with the development of metastases, and with chromosome 3/BAP1 expression loss. As expression of several Histone Deacetylases (HDACs) was associated with loss of chromosome 3, this suggested that HDAC expression might also be related to inflammation. We analysed HDAC expression and the presence of leukocytes by mRNA expression in two sets of UM and determined the T lymphocyte fraction through ddPCR. Four UM cell lines were treated with IFNɣ (50IU, 200IU). Quantitative PCR (qPCR) was used for mRNA measurement of HDACs in cultured cells.

In both cohorts (Leiden and TCGA), a positive correlation occurred between the presence of a T cell infiltrate and expression of HDACs 1, 3, and 8, while a negative correlation was observed between macrophages and HDACs 2 and 11. After stimulation of UM cell lines with IFNɣ, a slight increase of HDACs 1, 4, 5, 7, and 8 occurred in two out of four UM cell lines.

We conclude that the observed positive correlations between HDAC expression and chromosome 3/BAP1 loss may be related to the presence of infiltrating T cells.

Keywords: Uveal Melanoma, Inflammation, Metastasis, Chromosome 3, Histone deacetylase

1. Introduction

Histone Deacetylases (HDACs) are epigenetic enzymes which regulate gene expression primarily by modifying the chromatin structure through the removal of acetyl groups from histones, acting in balance with Histone Acetyl Transferases (HATs). Eighteen types of HDACs have been identified in man, classified into four classes: HDAC Class I includes HDACs 1, 2, 3, and 8, which are located in the nucleus, HDAC Class II includes HDACs 4, 5, 6, 7, 9, and 10, with both nuclear and cytoplasmic locations, HDAC Class III consists of sirtuins 1-7, and HDAC Class IV is made up by HDAC 11 [1, 2]. Several types of HDACs show overexpression in cancer cells, which has been associated with invasive behavior [3-5] and a poor clinical outcome, for instance in oral squamous cell carcinoma [6].

Inhibitors of HDACs (HDACi) are being investigated as treatment of a wide range of malignancies, one of which is Uveal Melanoma (UM), which constitutes a rare ocular tumour arising from the uveal tract. It mainly involves the choroid but may also develop in the iris or ciliary body, and gives rise to metastases in 50% of cases [7]. Specific somatic mutations and chromosome aberrations in primary UM are associated with the risk of metastases: loss of one chromosome 3 (monosomy 3) and a mutation in the BRCA1-associated protein 1 (BAP1) gene on the other chromosome 3 are associated with a very high-risk of developing metastases [8, 9]. In a recent study, we described that high-risk UM showed an elevated expression of several HDACs, which was associated with monosomy 3 [10].

It has been known for quite some time that monosomy 3 is related to an inflammatory phenotype, which is characterized by the presence of tumour-infiltrating lymphocytes (TILs), tumour-associated macrophages (TAMs) and high expression levels of HLA Class I and HLA Class II [11-15]. Infiltration of UM with immune cells is associated with an increased risk of metastasis [16-18]. Specific genetic abnormalities are associated with the development of inflammation in UM: extra copies of chromosome 8q are related to macrophage influx, while monosomy 3/BAP1 loss are also associated with the presence of lymphocytes [16, 19]. As differences in expression levels of HDACs were related to monosomy 3/BAP1 loss, we considered a relation between their expression and inflammation.

We set out to investigate the hypothesis that the variation in expression of HDACs is related to the inflammatory phenotype, due to the production of cytokines produced by the infiltrating leukocytes. We analyzed this by determining HDAC expression and the presence of an inflammatory phenotype in two cohorts of UM patients, and by treating four UM cell lines with IFNɣ.

2. Materials and Methods

2.1. Study samples

The studied tumours came from 64 eyes that underwent an enucleation for UM at the Leiden University Medical Center (LUMC), Leiden, The Netherlands, between 1999 and 2008. In this group, 51% of the patients were male and 49% female. Their mean age at the time of enucleation was 61 years. The mean follow-up time (defined as the time period between enucleation and death) was 83 months (range 2 to 229 months). Follow-up was updated in 2020. At the end of follow up, 17 patients (27%) were alive, 37 patients (58%) had died because of metastases, four (6%) had died because of other causes, while for six patients (9%) the cause of death was unknown.

MRNA levels of patients included in the TCGA database were investigated for validation of our results (n=80) [18].

This work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). The project was approved by the LUMC Biobank committee and the LUMC METC committee (19/10/2016, code G16.076/NV/gk).

2.2. Chromosome analysis

Tumour DNA was isolated using the QIAmp DNA Mini kit (Qiagen, Venlo, The Netherlands). Single-nucleotide polymorphism (SNP) analysis was performed using an Affymetrix 250K_NSP or Affymetrix SNP 6.0 array to detect chromosome 3 loss [20]. Additional copies of chromosome 8q were detected by Affymetrix SNP 6.0 array and analysed using the GISTIC 2.0 algorithm [19].

2.3. Tumour gene expression

RNA was isolated using the RNeasy mini kit (Qiagen, Venlo, The Netherlands). Gene expression levels were determined using an Illumina HT12v4 array (Illumina, Inc., San Diego, CA, USA) and data were obtained for expression of epigenetic regulators (HDAC1, HDAC2, HDAC3, HDAC4, HDAC6, HDAC7, HDAC8, HDAC9, and HDAC11) and infiltrate markers (CD3E, CD8A and CD68). Information regarding the Illumina probe numbers and gene expression levels has been published [10].

2.4. Droplet Digital PCR (ddPCR)

Droplet digital PCR (ddPCR) was used in order to measure the T cell fraction as previously described [21, 22], applying a specifically-designed probe directed at a locus of the TCR-β gene.

2.5. Cell lines and cell culture

Four UM Cell lines were used in this study: the OMM1 cell line was previously established by Dr G.P.M. Luyten (LUMC, Leiden, The Netherlands) [23]. OMM2.5 was a gift from Dr B.R. Ksander (Schepens Eye Re-search Institute, Boston, MA, USA) [24]. Both are BAP1-positive and cultured in Roswell Park Memorial Insti-tute Medium 1640 (RPMI) Dutch modified media (Life Technologies, Europe bv, Bleiswijk, The Netherlands) supplemented with 10% fetal bovine serum (FBS) (Greiner Bio-one, Alphen a/d Rijn, The Netherlands), 1% GlutaMAX and 1% penicillin/ streptomycin (Life Technologies).

Two BAP1-negative UM cell lines (MP46 and MP38) were provided by the Curie Institute, Paris, France [25] and cultured in Iscove's modified Dulbecco medium (IMDM) (Life Technologies), supplemented with 20% FBS (Greiner Bio-one) and 1% penicillin/streptomycin (Life Technologies).

2.6. Quantitative Real-Time Polymerase Chain Reaction (qPCR)

The procedure for RNA isolation and quantitative real-time polymerase chain reaction (qPCR) has been described previously [26]. In summary, total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen Benelux B.V., Venlo, The Netherlands).

The IScript cDNA synthesis kit (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands) was used for complementary cDNA synthesis according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qPCR) was performed in three independent experiments using the CFX-384 machine (Bio-Rad), with triplicates. Data were analysed using CFX manager 3.1 (Bio-Rad). CT values of genes of interest were normalized against the geometric mean of housekeeping genes *RPS11* and *CAPNS1*. The sequences of primers used in the study are shown in Table 1.

Table 1. Sequences of primers used in the qPCR test.

Primers	Forward	Reverse
HDAC1	5'- CATCGCTGTGAATTGGGCTG	5'- CCCTCTGGTGATACTTTAGCAGT
HDAC2	5'- CATGGCGTACAGTCAAGGAG	5'ATAATTTCCAATATCACCGTCGTAG
HDAC3	5'- AGTTCTGCTCGCGTTACACA	5'- CCGAGGGTGGTACCTCAAAC
HDAC4	5'- TGGGAGTTTGGAGCTCGTTG	5'- AGTCCATCTGGATGGCTTTGGG
HDAC5	5'TGGTCTACGACACGTTCATGCT	5'- TCAGGGTGCACGTGTGTGTT
HDAC6	5'-GGAGAATCAGATCGCAACCGC	5'- ACTGGGGGTTCTGCCTACTT
HDAC7	5'- GACAAGAGCAAGCGAAGTGC	5'- GAGGTGTGGGGACACTGTAG
HDAC8	5'- CCAAGAGGGCGATGATGATC	5'- GTGGCTGGGCAGTCATAACC
HDAC9	5'- GAGGACGAGAAAGGGCAGTG	5'- GTACCAGAGCTTGGGATGGC
HDAC11	5'- TGTCTACAACCGCCACATCT	5'- GGTGCCTGCATTGTATACC
RPS11	5'- AAGCAGCCGACCATCTTTCA	5'- CGGGAGCTTCTCCTTGCC
CAPNS1	5'ATGGTTTTGGCATTGACACATG	5'- GCTTGCCTGTGGTGTCGC

2.7. Statistics

Data were analyzed with SPSS software version 22.0 (SPSS, nc., Chicago, Il, USA). Spearman correlation was performed in order to test correlations between non-parametric data. Pearson's chi square test was used for categorical data analysis. Graphs were obtained using GraphPad Prism version 5.0 for Windows (GraphPad Software, La Jolla, CA, USA). An Independent t test was used to compare qPCR data.

3. Results

3.1. HDAC expression is related to clinical and genetic tumour characteristics

When we looked at the mRNA levels of the different HDACs, we noticed a moderate variable expression in the Leiden cohort (Figure 1A) as well as in the TCGA panel (Figure 1B).

We already knew that expression of some of the HDACs was related to the tumour's chromosome 3 status, but now also investigated the possible association between HDACs and other high-risk tumour characteristics, such as cell type, tumour location (involvement of the ciliary body), tumour size (indicated as cTNM stage), BAP1 expression as determined by immunohistochemical staining in the Leiden cohort of 64 cases as well as in the TCGA cohort (mRNA expression) of 80 cases.

Increased expression of HDACs 1 and 8 was associated with the presence of epithelioid cells (p= 0.002 and p = 0.005), increased HDAC4 expression was associated with ciliary body involvement (p = 0.04) and high cTNM stage (p = 0.04). Expression of HDACs 1, 4, and 8 was higher in tumours with monosomy 3 (p = 0.002, p = 0.01, and p < 0.001, respectively), while HDAC11 showed a lower expression in cases with monosomy 3 (p < 0.001). Increased expression of HDAC4 and HDAC8 was associated with loss of BAP1 (p = 0.004, p = 0.001 respectively), while the opposite relation was observed for HDAC11 (p = 0.004) (Table 2). When we looked at disomy 3 tumours only, HDAC4 and HDAC7 were associated with chromosome 8q gain (p = 0.01, p = 0.007). As expected, when we looked at the TCGA cohort, HDACs 4 and 8 but also HDAC3 showed a negative correlation with the BAP1 mRNA expression level (p < 0.001, p < 0.001, p = 0.02), while HDACs 6 and 11 (p < 0.001, p < 0.001) showed a positive correlation (Supplementary Table 1).

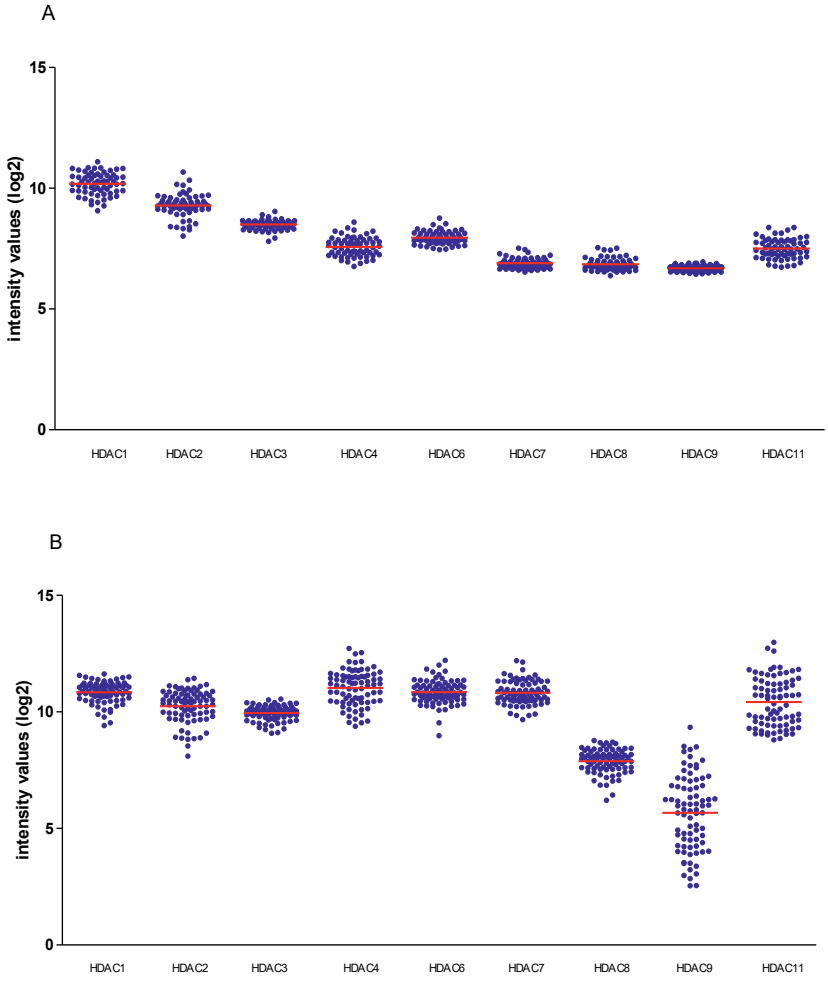


Figure 1. Distribution of HDAC mRNA in A) 64 UM from Leiden; B) 80 UM from the TCGA cohort. Horizontal bars indicate mean gene expression.

Table 2. Clinico-pathological characteristics according to low and high HDAC expression. Groups were separated according to the median into low (L) expression and high expression (H). Using Pearson's Chi Square test, $p \leq 0.05$ is considered significant. Numbers in brackets represent percentages.

	HDAC1			HDAC2			HDAC3			HDAC4			HDAC6			HDAC7			HDAC8			HDAC11		
	L	H	*p*	L	H	*p*	L	H	*p*	L	H	*p*	L	H	*p*	L	H	*p*	L	H	*p*	L	H	*p*
Cell Type (n=64)																								
Spindle	17(27)	5(8)	**0.002**	9(14)	13(20)	0.49	14(22)	8(12)	0.08	13(20)	9(14)	0.30	9(14)	13(20)	0.30	12(19)	10(16)	0.73	17(27)	5(8)	**0.005**	9(14)	13(20)	0.38
Mixed/epithelioid	15(23)	27(42)		21(33)	21(33)		17(27)	25(39)		19(30)	23(36)		23(36)	19(30)		21(33)	21(33)		17(27)	25(39)		22(34)	20(31)	
CiliaryBody Involvement (n=64)																								
Not involved	22(34)	18(28)	0.30	17(27)	23(36)	0.36	19(30)	21(33)	0.85	24(37)	16(25)	**0.04**	23(36)	17(27)	0.12	23(36)	17(26)	0.22	22(34)	18(28)	0.70	19(30)	21(33)	0.85
Involved	10(16)	14(22)		13(20)	11(17)		12(19)	12(19)		8(12)	16(25)		9(14)	15(23)		10(16)	14(22)		12(19)	12(19)		12(19)	12(19)	
cTNM Stage (n=62)																								
cTNM Stage I-IIB	19(31)	18(29)	0.57	17(27)	20(32)	0.64	16(26)	21(34)	0.50	23(37)	14(23)	**0.04**	20(32)	17(27)	0.44	22(35)	15(24)	0.07	20(32)	17(27)	0.87	16(26)	21(34)	0.50
cTNM Stage IIIA-IIIC	11(18)	14(22)		13(21)	12(19)		13(21)	12(19)		9(14)	16(26)		11(18)	14(23)		9(14)	16(26)		13(21)	12(19)		13(21)	12(19)	
Chromosome 3 Status (n=64)																								
Disomy 3	18(28)	6 (9)	**0.002**	13(20)	11(17)	0.36	15(23)	9(14)	0.08	17(26)	7(11)	**0.01**	10(16)	14(22)	0.30	16(25)	8(12)	0.06	21(33)	3 (5)	**<0.001**	4(6)	20(31)	**<0.001**
Monosomy 3	14(22)	26(41)		17(27)	23(36)		16(25)	24(37)		15(23)	25(40)		22(34)	18(28)		17(26)	23(36)		13(20)	27(42)		27(42)	13(20)	
BAP1 staining (n=55)																								
Bap1-positive	16(29)	9(16)	0.08	11(20)	14(25)	0.96	15(27)	10(18)	0.08	18(33)	7(13)	**0.004**	14(25)	11(20)	0.84	18(33)	7(13)	0.06	20(36)	5(9)	**0.001**	7(13)	18(33)	**0.004**
Bap1-negative	12(22)	18(33)		13(24)	17(31)		11(20)	19(34)		10(18)	20(36)		16(29)	14(25)		14(25)	16(29)		10(18)	20(36)		20(36)	10(18)	
Chromosome 8q Status in Disomy 3 tumours (n=24)																								
Normal 8q	11(46)	4(16)	0.80	8(33)	7(29)	0.92	8(33)	7(29)	0.23	8(33)	7(29)	**0.01**	7(29)	8(33)	0.52	13(54)	2 (8)	**0.007**	13(54)	2 (8)	0.87	1(4)	14(58)	0.09
Gained 8q	7(29)	2 (8)		5(21)	4(16)		7(29)	2 (8)		9(37)	0(0)		3(12)	6(25)		3(13)	6(25)		8(33)	1 (4)		3(12)	6(25)	

3.2. HDACs and relation with infiltrating leukocytes

As already observed [10] and confirmed (Table 2), expression levels of some of the HDACs were related to the tumour's chromosome 3/BAP1 status [10]. As monosomy 3 is associated with the presence of an inflammatory phenotype, we speculated that the epigenetic enzymes might be upregulated due to the presence of infiltrating leukocytes which produce cytokines that stimulate HDAC expression. We set out to test this hypothesis (Table 3).

Expression of HDACs 1, 3, 7, and 8 was positively correlated with the expression of the T cell markers CD3E and CD8A, while these correlations were negative for HDAC11. HDAC2 and 11 were inversely correlated with macrophage marker CD68 ($p < 0.001$, $p = 0.001$).

When performing the same analysis using the TCGA cohort, the results showed that HDACs 1, 3, and 8 were (again) positively correlated with to the presence of CD3E and CD8A TILs, while HDACs 2 and 11 were (again) negatively correlated with CD3E ($p = 0.003$, $p = 0.002$), and HDAC2 and HDAC9 with CD68 ($p < 0.001$, $p < 0.001$) (Table 4).

Table 3. Correlation between mRNA expression levels (determined by Illumina array) of different HDACs and expression of TILs (CD3E and CD8A) and TAMs (CD68) in the LUMC cohort (n=64). R = two-tailed Spearman correlation coefficient. $p \leq 0.05$ is considered significant (indicated in bold).

	CD3E		**CD8A**		**CD68**	
	R	***p***	**R**	***p***	**R**	***p***
HDAC1	.340	**.006**	.407	**.001**	.082	.52
HDAC2	-.197	.12	-.153	.23	-.430	**< 0.001**
HDAC3	.256	**.04**	.315	**.01**	.129	.31
HDAC4	.074	.56	.217	.08	.029	.82
HDAC6	-.109	.39	-.228	.07	.017	.89
HDAC7	.329	**.01**	.395	**.001**	.260	**.04**
HDAC8	.350	**.005**	.429	**< 0.001**	.241	**.05**
HDAC9	-.136	.28	-.168	.18	-.034	.79
HDAC11	-.259	**.04**	-.254	**.04**	-.415	**.001**

Table 4. Correlation between mRNA expression levels of different HDACs and expression of TILs (CD3E and CD8A) and TAMs (CD68) in the TCGA cohort (n=80). R = two-tailed Spearman correlation coefficient. $p \leq 0.05$ is considered significant (indicated in bold).

	CD3E		**CD8A**		**CD68**	
	R	***p***	**R**	***p***	**R**	***p***
HDAC1	.409	**< 0.001**	.446	**< 0.001**	-.124	.27
HDAC2	-.329	**.003**	-.143	.20	-.445	**< 0.001**
HDAC3	.323	**.003**	.373	**.001**	.201	.07
HDAC4	.076	.50	.229	**.04**	.076	.50
HDAC6	-.211	.06	-.270	**.01**	.074	.51
HDAC7	.003	.98	-.089	.43	.060	.59
HDAC8	.364	**.001**	.478	**< 0.001**	-.202	.07
HDAC9	-.098	.39	.020	.86	-.396	**< 0.001**
HDAC11	-.339	**.002**	-.452	**< 0.001**	-.169	.13

In order to obtain the real percentage of T cells as part of the tumour, we used a ddPCR technique to quantify the T cell infiltrate (Supplementary Table 2). A comparison of the T cell fraction versus the expression of HDACs 1, 4, 8, and 11 is provided in Figure 2. Significant positive correlations were observed between T cell fraction and HDACs 1, 3, and 8, and a negative correlation with HDACs 6 and 11.

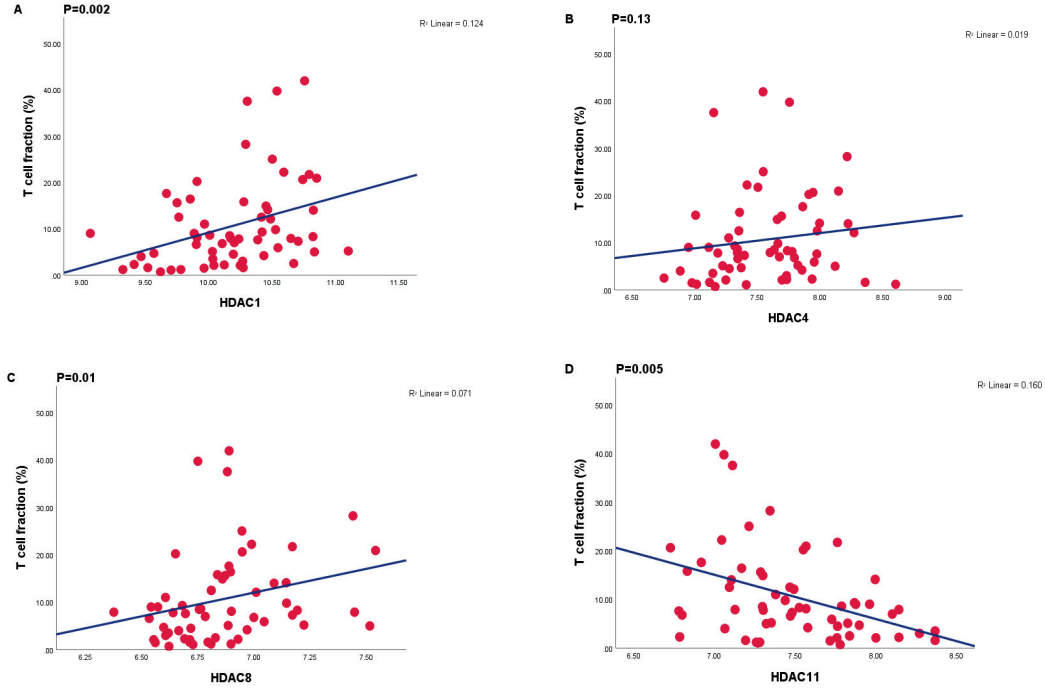


Figure 2. Correlations between mRNA expression levels (determined by Illumina array) of different HDACs and T cell fraction (%) (Determined by ddPCR) (n=59). HDAC1 $R^2 = 0.124$, $p = 0.002$, HDAC4 $R^2 = 0.019$, $p = 0.13$, HDAC8 $R^2 = 0.071$, $p = 0.01$, HDAC11 $R^2 = 0.160$, $p = 0.005$.

3.3. HDAC expression in UM cell lines

The correlation between some infiltrate markers and expression levels of HDACs 1, 3, 4, 6, and 8 could suggest that their expression levels are influenced by the presence of infiltrating cells; this may be due to the production of cytokines such as interferon ɤ (IFNɤ).

To test this option, we looked at the level of expression of a range of HDACs in four UM cell lines and exposed them to two different doses of IFNɣ for 48 hrs (Figure 3). HLA-A and HLA-B mRNA levels were measured as positive controls, and show a strong response to IFNɣ exposure (Supplementary Figure 1).

We observed low levels of HDACs 3, 6, and 7 in all cell lines, with slightly higher levels of HDACs 8 and 11. IFNɣ induced a slight but significant increase in HDACs 1, 4, 5, 7, and 8 in cell lines OMM2.5 and MP38 cell lines, and of HDAC11 in MP38. No significant changes were observed in cell lines OMM1 and MP46.

4. Discussion

Previously, we reported that expression of several HDACs was increased in high-risk UM with monosomy 3/loss of BAP1 and gain of 8q [10]. However, we observed variation in expression of several HDACs. As we wanted to get a better insight into the cause of this variable expression, we determined whether expression was related to any specific histological or genetic tumour characteristics. For this, we first used the set of 64 primary UM from Leiden which had been analysed for their chromosome 3 and 8q status, their BAP1 staining and their mRNA expression levels. Expression levels of two HDACs (1 and 8) were higher in case of epithelioid cells, and three HDACs (1, 4, 8) were increased in tumours with monosomy 3, an indicator of bad prognosis in this disease; as monosomy 3 is related to the presence of an inflammatory phenotype with increased levels of HLA Class I expression and the presence of TIL and TAM, we considered the option that the inflammatory microenvironment may be responsible for the upregulation of HDACs in UM. In the Leiden cohort, we observed that four of the HDACs (HDACs 1, 3, 7, and 8) showed a positive correlation between expression levels and TIL, while this was the case for three of the HDACs in the TCGA study (HDACs 1, 3, and 8). HDAC11 showed a consistently negative association with TIL as well as TAM. In order to test our hypothesis that the presence of infiltrating leukocytes led to HDAC expression through the production of cytokines, we treated four UM cell lines (two BAP1 positive ones: OMM1 and OMM2.5, and two BAP1-negative ones: MP46 and MP38) with two different doses of IFNɣ, an inflammatory cytokine normally expressed by immune cells such as CD8. After 48hrs we found that IFNɣ led to some induction of HDAC expression in two out of four treated cell lines (OMM2.5 and MP38).

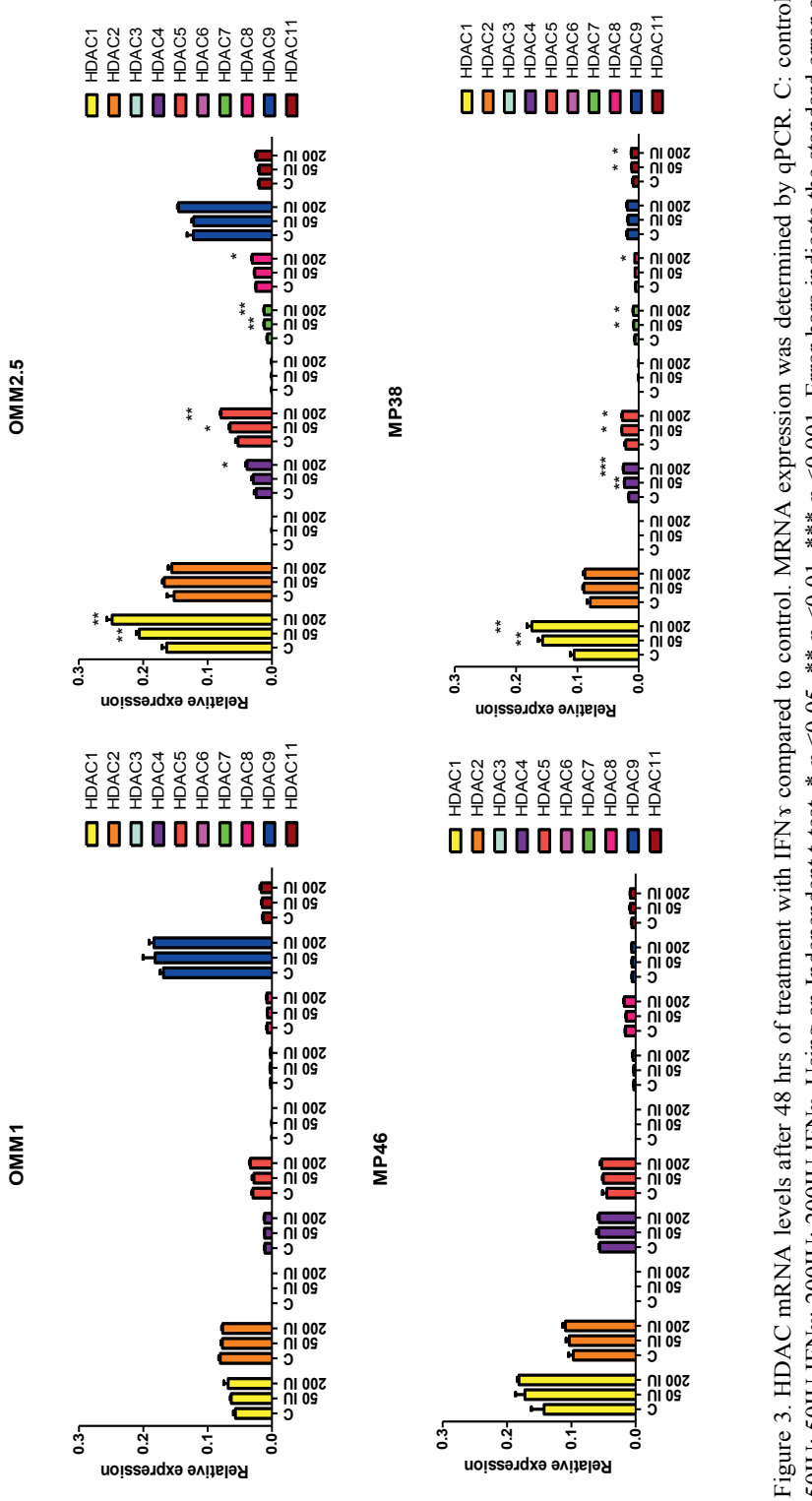


Figure 3. HDAC mRNA levels after 48 hrs of treatment with IFNs compared to control. MRNA expression was determined by qPCR. C: control; 50IU: 50IU IFNs; 200IU: 200IU IFNs. Using an Independent t-test, * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$. Error bars indicate the standard error of the mean.

This suggests a potential difference in the regulation of HDACs between individuals in response to IFNɣ. HDACs contribute towards malignancy: they block the activity of cell cycle inhibitors, inhibit differentiation and apoptosis and thereby enhance uncontrolled proliferation and survival of cancer cells. HDAC expression might be related to invasive and stem cell behavior of UM cells: a change in epigenetic regulation has been proposed by Landreville in 2012, who noticed that loss of melanocytic behavior and a shift toward stem cell behavior occurred during BAP1 inactivation [27]; this could be relevant to the increase of several HDACs after chromosome 3 loss. However, we did not notice a basic difference in expression between the BAP1-positive and BAP1-negative cell lines. Beside their role in the induction of malignancy and invasive behavior, HDACs are also involved in inflammatory processes: HDACs could act as inducers of interferon-stimulated genes: inhibition of HDACs by trichostatin inhibited the recruitment of RNA poly II and expression of the ISRE element-containing genes ISG54, ISG15, and ISG56 in primary human fibroblasts; this suggests that HDACs are necessary for the expression of such genes and may be a potential regulator for inflammatory processes [28].

We may be dealing with a circular process, in which cytokines stimulate expression of some HDACs, which subsequently stimulate inflammatory cellular pathways. As we saw a negative association between HDAC2 and the presence of macrophages, some HDACs may have a similar negative immunomodulatory effect.

We previously reported that HDACs are associated with HLA expression, which is part of the inflammatory phenotype in UM: mRNA expression of HDACs (1, 4, and 8) was positively associated with HLA-A and HLA-B expression [10]. When we now look at the relations with lymphocyte markers, HDACs 1, 3, 7, and 8 show the most consistent positive association, and HDACs 2 and 11 a strong negative association. HDAC1 is involved in the expression of type I IFN-responding genes: when cells were treated with the HDAC inhibitor Sodium Butyrate, expression of interferon-stimulated genes was blocked in several human cell lines; depletion of HDAC1 by siRNA reduced the mRNA level of ISG54 [29].

HDAC8 has been shown to have increased enzymatic activity and play a pathogenic role in pulmonary asthma; when mice in a model of allergic asthma were exposed to ovalbumin (OVA), the level of HDAC8 protein expression was significantly increased in the lungs, together with high

numbers of CD68 and CD163 macrophages, while treatment with the specific HDAC8 inhibitor PCI-34051 reduced these effects. The study reported a role for HDAC8 and Galectin-3 for the development of inflammatory macrophages [30]. Another study found that the use of PCI-34051 downregulated inflammatory cytokines in peripheral blood mononuclear cells (IL-18, IL-1β, MIP-1b, MCP-1, TNFa, and IL-6) [31]. These reports demonstrate a role for HDAC8 in the induction of inflammation.

HDAC2 and HDAC11 showed a negative correlation with markers of inflammation such as the presence of lymphocytes and macrophages, both in the Leiden cohort as well as the TCGA cohort. In a study on human cervical cancer cell lines, HDAC2 was found to inhibit transcription of MHC genes, which are associated with the inflammatory phenotype in UM [32]. HDAC2 has been studied extensively in relation to pulmonary inflammation: HDAC2 mRNA and protein expression were reduced in lung epithelial cells and macrophages after exposure to hypoxia [33]. We have previously shown that high-risk UM are characterized by a hypoxic environment [34]. It has been similarly shown that HDAC11 has a low expression in high-risk UM [35, 10]. The best explanation for this phenomenon is that HDAC11 is located on chromosome 3 and expression is decreased after loss of one of the two chromosomes 3. We previously published that monosomy 3 is associated with tumour inflammation: here, we observe a negative correlation between HDAC11 expression and inflammatory TIL and TAM markers. However, a low HDAC11 level may still contribute to the invasiveness of malignant cells: low levels of this HDAC have been reported to increase the risk of metastasis in breast cancer [36]. The expression of some of the HDACs not only shows a correlation with infiltrating lymphocytes, but has also been shown to associate with immune checkpoint expression, suggesting a possible role for these HDACs in the immune evasion of tumour cells: high levels of HDACs 1, 3, 6, and 8 were positively correlated with expression of the B7 homolog 1 checkpoint inhibitor (B7-H1) in gastric cancer. When gastric cell lines were treated with the HDAC inhibitor vorinostat after IFNɣ induction, B7-H1 was reduced, showing that HDACs play a role in the IFNɣ enhancement of B7-H1 in gastric cancer and are involved in the evasion phenotype of these malignant cells [37].

5. Conclusion

We report that expression of several HDACs is related to high-risk characteristics in UM and to inflammation, and is possibly regulated by inflammatory cytokines produced by the infiltrated immune cells. The upregulation of HDACs might contribute towards the progression of inflammation and subsequently increase the malignant behavior in this disease.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Correlation between mRNA expression levels (determined by Illumina array) of different HDACs and expression of BAP1 in the TCGA cohort (n=80). R = two-tailed Spearman correlation coefficient. $p \leq 0.05$ is considered significant (indicated in bold). Table S2: Correlation between mRNA expression levels (determined by Illumina array) of different HDACs and T cell fraction (%) as determined by ddPCR (n=59). R = two-tailed Spearman correlation coefficient. $p \leq 0.05$ is considered significant (indicated in bold). Table S3: Influence of adding IFNγ on HLA-A and HLA-B mRNA expression on cultured UM cell lines.

Author Contributions: Conceptualization, Z.S, A.G.J and M.J.J; Data curation, A.P.A.W; Formal analysis, Z.S; Funding acquisition, P.v.d.V and M.J; Methodology, Z.S, A.G.J, W.G.K and R.M.V; Supervision, A.G.J and M.J.J; Writing – original draft, Z.S and M.J.J; Writing – review & editing, A.G.J, A.PA.W, W.G.K, R.M.V, P.v.d.V, G.P.L and M.J.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by a grant to Z.S. from The Iranian Ministry of Science Research and Technology, Grant UL 2011-4991 from the Dutch Cancer Society (KWF), Stichting Leids Oogheelkundig Ondersteunings Fonds LOOF and a Horizon 2020 grant from the European Community, CURE UM, nr. 667787.

Institutional Review Board Statement: The METC of the LUMC declared no objection to this study (19/10/2016, code G16.076/NV/gk). The research adhered to Dutch law ('Code for Proper Secondary Use of Human Tissue') and the tenets of the Declaration of Helsinki (World Medical Association of Declaration 2013; ethical principles for medical research involving human subjects).

Informed Consent Statement: Informed consent was not needed for this analysis.

Data Availability Statement: Data from the Leiden cohort are accessible through GEO Series accession num-ber GSE84976 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE84976).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Moschos, M.M.; Dettoraki, M.; Androudi, S.; et al. The role of histone deacetylase inhibitors in uveal melanoma: current evidence. Anticancer Res. 2018, 38(7), 3817-3824.
2. Ruijter, A.J.; Gennip, A.H.; Caron, H.N.; et al. Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem. J. 2003, 370(3), 737-749.
3. Fritzsche, F.R.; Weichert, W.; Röske, A.; et al. Class I histone deacetylases 1, 2 and 3 are highly expressed in renal cell cancer. BMC Cancer 2008, 8(1), 381-390.
4. Niegisch, G.; Knievel, J.; Koch, A.; et al. Changes in histone deacetylase (HDAC) expression patterns and activity of HDAC inhibitors in urothelial cancers. Urol. Oncol. 2013, 31(8), 1770-1779.
5. Yang, H.; Salz, T.; Zajac-Kaye, M.; et al. Overexpression of histone deacetylases in cancer cells is controlled by interplay of transcription factors and epigenetic modulators. FASEB J. 2014, 28(10), 4265-4279.
6. Chang, H.H.; Chiang, C.P.; Hung, H.C.; et al. Histone deacetylase 2 expression predicts poorer prognosis in oral cancer patients. Oral Oncol. 2009, 45(7), 610-614.
7. Jager, M.J.; Shields, C.L.; Cebulla, C.M.; et al. Uveal melanoma. Nature Reviews Disease Primers 2020, 6(1), 1-25.
8. Harbour, J.W.; Onken, M.D.; Roberson, E.D.; et al. Frequent mutation of BAP1 in metastasizing uveal melanomas. Science 2010, 330(6009), 1410-1413.
9. Smit, K.N.; van Poppelen, N.M.; Vaarwater, J.; et al. Combined mutation and copy-number variation detection by targeted next-generation sequencing in uveal melanoma. Mod. Pathol. 2018, 31(5), 763-771.
10. Souri, Z.; Jochemsen, A.G.; Versluis, M.; et al. HDAC inhibition increases HLA class I expression in uveal melanoma. Cancers 2020, 12(12), 3690-3704.
11. Durie, F.H.; Campbell, A.M.; Lee, W.R.; et al. Analysis of lymphocytic infiltration in uveal melanoma. Invest. Ophthalmol. Vis. Sci. 1990, (10), 2106-2110.
12. De Waard-Siebinga, I.; Hilders, C.G.; Hansen, B.E.; et al. HLA expression and tumor-infiltrating immune cells in uveal melanoma. Graefe's Arch. Clin. Exp. Ophthalmol. 1996, 234(1), 34-42.
13. Blom, D.J.; Luyten, G.P.; Mooy, C.; et al. Human leukocyte antigen class I expression. Marker of poor prognosis in uveal melanoma. Invest. Ophthalmol. Vis. Sci. 1997, 38(9), 1865-1872.
14. Souri, Z.; Wierenga, A.; Mulder, A.; et al. HLA expression in uveal melanoma: an indicator of malignancy and a modifiable immunological target. Cancers 2019 11(8), 1132-1150.

15. Maat, W.; Ly, L.V.; Jordanova, E.S.; et al. Monosomy of chromosome 3 and an inflammatory phenotype occur together in uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2008, 49, 505-510.
16. Bronkhorst, I.H.; Vu, T.H.; Jordanova, E.S.; et al. Different subsets of tumor-infiltrating lymphocytes correlate with macrophage influx and monosomy 3 in uveal melanoma. Invest. Ophthalmol. Vis. Sci. 2012, 53, 5370-5378.
17. Robertson, A.G.; Shih, J.; Yau, C.; et al. Integrative analysis identifies four molecular and clinical subsets in uveal melanoma. Cancer Cell 2017, 32(2), 204-220.
18. Gezgin, G.; Dogrusöz, M.; van Essen, T.H.; et al. Genetic evolution of uveal melanoma guides the development of an inflammatory microenvironment. Cancer Immunol. Immunother. 2017, 66(7), 903-912.
19. Luyten, G.P.M.; Naus, N.C.; Mooy, C.M.; et al. Establishment and characterization of primary and metastatic uveal melanoma cell lines. Int. J. Cancer 1996, 66, 380-387.
20. Chen, P.W.; Murray, T.G.; Uno, T.; et al. Expression of MAGE genes in ocular melanoma during progression from primary to metastatic disease. Clin. Exp. Metastasis. 1997, 15(5), 509-518.
21. Amirouchene-Angelozzi, N.; Nemati, F.; Gentien, D.; et al. Establishment of novel cell lines recapitulating the genetic landscape of uveal melanoma and preclinical validation of mTOR as a therapeutic target. Mol. Oncol. 2014, 8, 1508-1520.
22. Bronkhorst, I.H.G.; Jehs, T.M.L.; Dijkgraaf, E.M.; et al. Effect of hypoxic stress on migration and characteristics of monocytes in uveal melanoma. JAMA Ophthalmol. 2014, 132, 614–621.
23. Zoutman, W.H.; Nell, R.J.; Versluis, M.; et al. Accurate quantification of T cells by measuring loss of germline T-cell receptor loci with generic single duplex droplet digital PCR assays. J. Mol. Diagn. 2017, 19(2), 236-243.
24. De Lange, M.J.; Nell, R.J.; Lalai, R.N.; et al. Digital PCR-Based T-cell Quantification-Assisted deconvolution of the microenvironment reveals that activated macrophages drive tumor inflammation in Uveal Melanoma. Mol. Cancer Res. 2018, 16(12), 1902-1911.
25. Landreville, S.; Agapova, O.A; Matatall, K.A.; et al. Histone deacetylase inhibitors induce growth arrest and differentiation in uveal melanoma. Clin. Cancer Res. 2012, 18(2), 408-416.
26. Sakamoto, S.; Potla, R.; Larner, A.C. Histone deacetylase activity is required to recruit RNA polymerase II to the promoters of selected interferon-stimulated early response genes. J. Biol. Chem. 2004, 279(39), 40362-40367.
27. Nusinzon, I.; Horvath, C.M. Interferon-stimulated transcription and innate antiviral immunity require deacetylase activity and histone deacetylase 1. Proc. Natl. Acad. Sci. 2003, 100(25), 14742-14747.

28. Lu, Y.; Stuart, J.H.; Talbot-Cooper, C.; et al. Histone deacetylase 4 promotes type I interferon signaling, restricts DNA viruses, and is degraded via vaccinia virus protein C6. PNAS 2019, 116(24), 11997-2006.
29. Zeng, L.S.; Yang, X.Z.; Wen, Y.F.; et al. Overexpressed HDAC4 is associated with poor survival and promotes tumor progression in esophageal carcinoma. Aging 2016, 8(6), 1236-1248.
30. Li, M.L.; Su, X.M.; Ren, Y.; et al. HDAC8 inhibitor attenuates airway responses to antigen stimulus through synchronously suppressing galectin-3 expression and reducing macrophage-2 polarization. Respiratory Res. 2020, 21(1), 1-4.
31. Balasubramanian, S.; Steggerda, S.; Sirisawad, M.; et al. The histone Deacetylase-8 (HDAC8) selective inhibitor PCI-34051 decreases Interleukin-1 Beta secretion in vitro and reduces inflammation in vivo. Blood 2008, 2581-2581.
32. Herlihy, N.; Dogrusöz, M.; Van Essen, T.H.; Harbour, J.W.; Van Der Velden, P.A.; Van Eggermond, M.C.J.A.;Haasnoot, G.W.; Elsen, P.J.V.D.; Jager, M.J. Skewed expression of the genes encoding epigenetic modifiers in high-risk Uveal Melanoma. Investig. Ophthalmol. Vis. Sci. 2015, 56, 1447–145
33. Leslie, P.L.; Chao, Y.L.; Tsai, Y.-H.; et al. Histone deacetylase 11 inhibition promotes breast cancer metastasis from lymph nodes. Nat. Commun. 2019, 10, 1–12.
34. Deng, R.; Zhang, P.; Liu, W.; et al. HDAC is indispensable for IFN-γ-induced B7-H1 expression in gastric cancer. Clin. Epigenetics 2018, 10(1), 1-4.

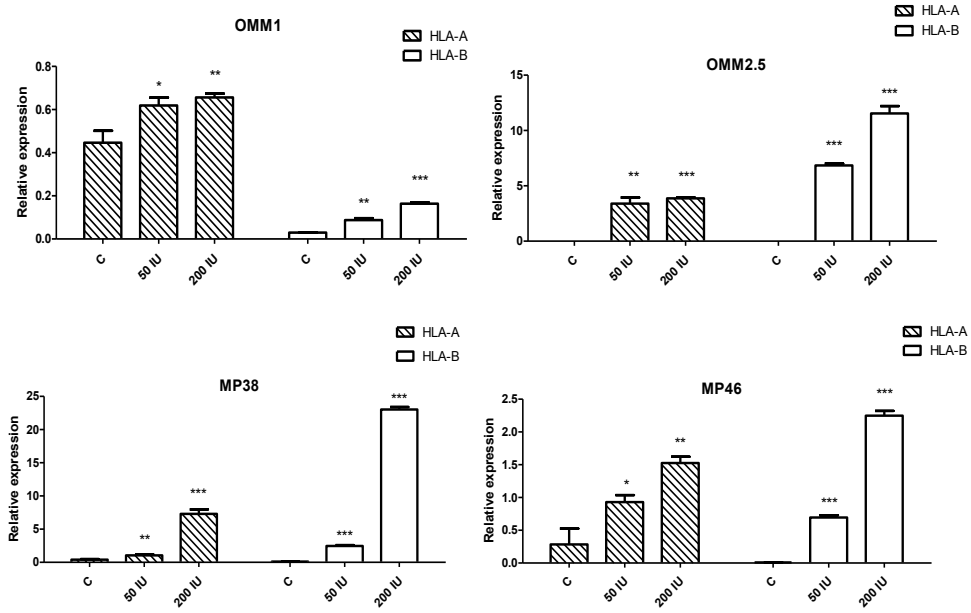
Supplementary Table 1. Correlation between mRNA expression levels (determined by Illumina array) of different HDACs and expression of BAP1 in the TCGA cohort (n=80). R = two-tailed Spearman correlation coefficient. $p \leq 0.05$ is considered significant, and indicated in bold.

	BAP1	
	R	***p***
HDAC1	-.201	.07
HDAC2	-.010	.93
HDAC3	-.268	**.02**
HDAC4	-.625	**<0.001**
HDAC6	.427	**<0.001**
HDAC7	-.022	.84
HDAC8	-.560	**<0.001**
HDAC9	-.069	-.069
HDAC11	.739	**<0.001**

Supplementary Table 2. Correlation between mRNA expression levels (determined by Illumina array) of different HDACs and T cell fraction (%) as determined by ddPCR (n=59). R = two-tailed Spearman correlation coefficient. $p \leq 0.05$ is considered significant, and indicated in bold

	T cell fraction	
	R	***p***
HDAC1	.399	**.002**
HDAC2	-.232	.08
HDAC3	.319	**.01**
HDAC4	.200	.13
HDAC6	-.314	**.02**
HDAC7	.183	.16
HDAC8	.357	**.01**
HDAC9	-.122	.36
HDAC11	-.363	**.005**

Supplementary Figure 1: Influence of adding IFNγ on HLA-A and HLA-B mRNA expression on cultured UM cell lines. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$.

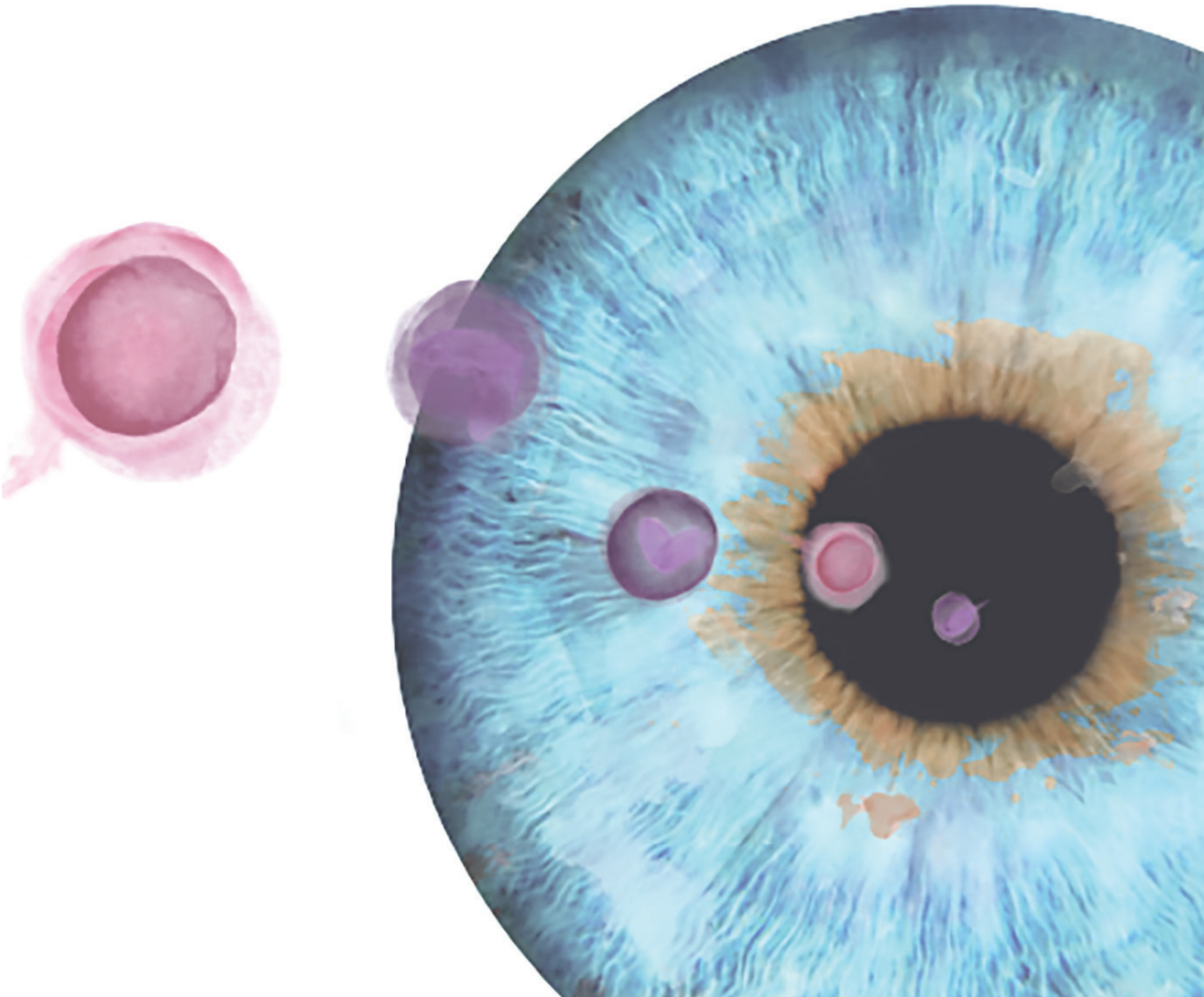


Chapter 6

MiRNAs correlate with HLA expression in Uveal Melanoma: both up- and downregulation are related to Monosomy 3

Zahra Souri, Annemijn P. A. Wierenga, Emine Kiliç, Erwin Brosens, Stefan Böhringer, Wilma G.M. Kroes, Robert M. Verdijk, Pieter A. van der Velden, Gregorius P. M. Luyten and Martine J. Jager

Cancers 2021



Simple Summary

Uveal melanoma (UM) is a rare ocular malignancy that often gives rise to metastases. Tumours with an inflammatory phenotype have an especially worse prognosis. As an increased HLA expression and the presence of tumour-infiltrating lymphocytes and macrophages may be regulated by miRNAs, we set out to investigate whether any miRNAs are associated with inflammatory parameters. Some miRNAs were increased in UM with a high HLA expression and high T cell numbers, while others were decreased, showing two opposing patterns; however, both patterns were related to chromosome 3 status. We conclude that specific miRNAs are related to the inflammatory phenotype and that these are differentially expressed between disomy 3/BAP1-positive versus monosomy 3/BAP1-negative UM

Abstract

MicroRNAs are known to play a role in the regulation of inflammation. As a high HLA Class I expression is associated with a bad prognosis in UM, we set out to determine whether any miRNAs were related to HLA Class I expression and inflammation. We also determined whether such miRNAs were related to the UM's genetic status.

The expression of 125 miRNAs was determined in 64 primary UM. Similarly, the mRNA expression of HLA-A, HLA-B, TAP1, BAP1, and immune cell markers was obtained from the array. Expression levels of 24 of the 125 miRNAs correlated with at least three out of four HLA Class I probes. Four showed a positive correlation with HLA expression and infiltration with leukocytes, 20 a negative pattern. High miRNA levels in the first group correlated with chromosome 3 loss/reduced BAP1 mRNA expression.The positive associations between miRNA-22 and miRNA-155 with HLA Class I were confirmed in the TCGA study, with TAP1 in the Rotterdam data set, the negative one between HLA-A, TAP1 and CD4 with miRNA-125b2 and miRNA-211 in the Rotterdam set. We demonstrate two patterns: miRNAs can either be related to a high or a low HLA Class I and TAP1 expression and the presence of infiltrating lymphocytes and macrophages.

However, both patterns are associated with chromosome 3/BAP1 status, which suggests a role for BAP1 loss in the regulation of HLA expression and inflammation in UM through miRNAs.

Keywords: Eye disease, Uveal Melanoma, Oncology, Inflammation, miRNA, HLA, TAP1, Monosomy3, BAP1

Introduction

Uveal melanoma (UM) is the most common type of primary intraocular malignancy in adults and carries a high risk of metastases. Tumours with monosomy of chromosome 3 (M3), gain of 8q [1–4], a mutation in the BAP1 (BRCA1-associated protein-1) gene [5,6], or a class 2 gene expression profile [7–9] are more prone to develop metastases. Tumours that have these high-risk characteristics often show an inflammatory phenotype [10,11], characterized by the presence of high numbers of tumour-infiltrating lymphocytes and macrophages [12,13], and a high expression of the HLA Class I and II antigens [14–16]. HLA Class I molecules are essential for the presentation of processed tumour antigens to cytotoxic T cells and are therefore important for immunotherapeutic approaches. However, while a high HLA expression might theoretically make the tumour cells more susceptible to killing by cytotoxic T cells, it may decrease their susceptibility to NK (natural killer) cell-mediated lysis, which is probably especially relevant during hematogeneous tumour cell migration from the eye to the liver [17,18].

Although there are effective treatments available for primary UM (radioactive plaque, proton beam therapy, stereotactic irradiation), a treatment to cure metastases is still missing and survival of high-risk cases has not improved during the last 50 years [19–22]; it is therefore important to identify new potential therapies to treat metastases of this disease.

MiRNAs are short noncoding RNAs, usually with a length of 17–22 base pairs. By binding to 3´UTR regions of complimentary mRNAs, they either stimulate or delay translation of specific genes and thereby play an important role in gene regulation [23]. In recent years, miRNAs have been identified as new players in tumorigenesis and metastases formation, and they have been associated with angiogenesis [24,25], the induction of epithelial-to-mesenchymal transition [26] and increased proliferation [27–29]. Several miRNAs have been identified to play a role in tumorigenesis of UM [30], and differences in miRNA expression occur between UM tumors with

a low or high metastatic risk [31–34]. Smit reported that miRNAs were potential actors for the progression of metastasis in UM: 13 out of 423 mature miRNAs, which were expressed in UM samples, were differentially expressed between low, intermediate, and high-risk UM [35]. MiRNAs may act as oncomirs leading to cell growth and invasion [36,37], or as tumour suppressors, having antitumor activity [38].

Inflammation is a bad prognostic sign in UM, but it is as yet unclear how this inflammation is regulated. The presence of tumour-associated macrophages (TAMs) has been associated with loss of one chromosome 3 (M3, monosomy 3) and gain of chromosome 8q, while both TAMs as well as tumour-infiltrating lymphocytes (TILs) are increased in UM with loss of chromosome 3/BAP1 [10,13,39].

As miRNAs may influence inflammation, we analysed 125 miRNAs in 64 UM samples and determined any association with HLA Class I expression and the presence of an infiltrate; we furthermore investigated whether the levels of inflammation-associated miRNAs were related to the tumour genetic status. Outcomes were compared with data from the Department of Ophthalmology, ErasmusMC, Rotterdam, The Netherlands [35], and TCGA data [11] (See Schedule in Figure 1).

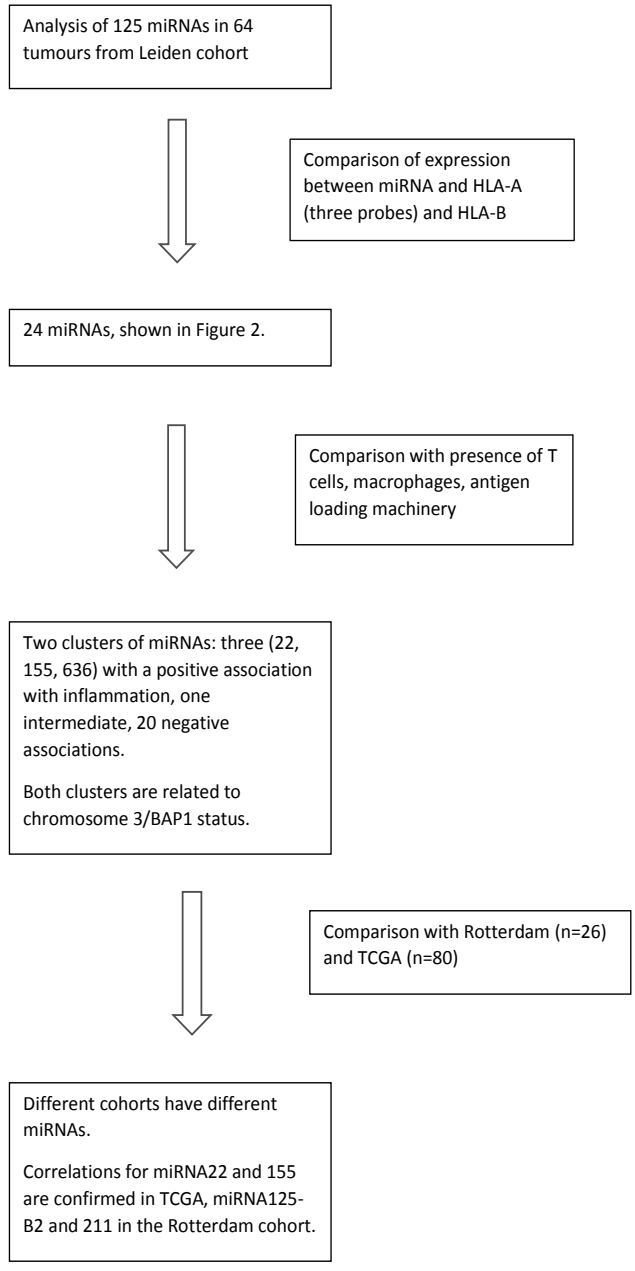


Figure 1. Schedule for analyses.

2. Materials and Methods

2.1. Study Population Leiden Cohort

Tumour material was obtained from 64 eyes that underwent an enucleation for UM between 1999 and 2008 at the Leiden University Medical Center (LUMC) in Leiden, The Netherlands. Characteristics of the cohort have been added as Table S1. 51% of the patients were male and 49% were female. The mean age at the time of enucleation was 61 years. The mean follow-up time (defined as the time period between enucleation and either date of last follow-up or death) was 83 months (range 2 to 229 months). Follow-up was updated in 2020. At the end of follow up, 17 (27%) patients were alive, 37 (58%) patients had died because of metastasis, four (6%) had died because of other causes and six (9 %) had died but the cause of death was unknown.

2.2. Chromosome Status

DNA isolation was performed on frozen tumour tissue using the QIAmp DNA Mini kit for single nucleotide polymorphism (SNP) detection (Qiagen, Venlo, The Netherlands). Chromosome 3 abnormality was detected by SNP microarray analysis using an Affymetrix 250K_NSP (Affymetrix, Santa Clara, CA, USA) array as described previously [4, 13].

2.3 Gene expression

RNA isolation was performed on frozen tumour tissue using the RNeasy mini kit (Qiagen, Venlo, The Netherlands). Gene expression was determined with the Illumina HT12v4 array (Illumina, Inc., San Diego, CA, US) for 125 miRNA, HLA Class I (HLA-A, HLA-B), HLA Class I Regulatory Factors and the Antigen-Loading Machinery (CIITA, NLRC5, IRF1, IRF8 and TAP1), Immune markers (CD3, CD4, CD8, CD68, CD163), and BAP1 as described previously [40].

2.4 Rotterdam Cohort

The 26 cases analysed from the ErasmusMC were described by Smit [35]. Both miRNA and mRNA libraries were sequenced with the Ion Proton sequencer.

Readtrimming using Cu-tadapt Version 3.4, alignment to the hg19 reference genome using HISAT2Version 2.1.0. Aligned reads were counted using htseq-count Version 0.9.1 (mirBasev20, Ho-mo_Sapiens.GRCh37.75) and normalised using DESeq2.

2.5 TCGA data

MiRNA expression data of 80 UM samples were retrieved from the repository of the Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov) [11].

2.6 Statistical analysis

Data were analysed with SPSS software version 22.0 (SPSS, nc, Chicago, Il, USA). Graphs were obtained using GraphPad Prism version 5.0 for windows (GraphPad Software, La Jolla, California, USA). Spearman correlation was performed in order to make correlations between non-parametric data. The Mann-Whitney U test was used to compare non-normal groups. Kaplan-Meier survival curves were made and the log rank test was used to determine significance.

We used R-package heatmaply to produce the heatmaps. We use 1 - cor(X, Y) to define the distance between mRNA X and Y to be used for the hiarchical clustering. The linkage function was average linkage. R version 3.6.1 was used.

2.7. Institutional Review Board Statement and informed consent

This project was approved by the METC of the LUMC (B14.003/SH/sh Approval Biobank OOG-2 “Oogtumoren (of een verdenking hierop)”). The research adhered to Dutch law and the tenets of the Declaration of Helsinki (World Medical Association of Declaration 2013; ethical principles for medical research involving human subjects). Informed consent was obtained from all subjects involved in the study.

3. Results

3.1. MiRNAs and HLA Class I

As HLA antigens are one of the characteristics of high-risk UM and may play an important role in immunotherapy, it is important to know how their expression is regulated. As a relation between miRNAs and HLA expression has been observed in other malignancies, we determined whether we could identify miRNAs that were related to HLA expression, one of the markers of the inflammatory phenotype of UM. Using a set of 64 UM from the LUMC, we compared the expression levels of miRNAs with expression of HLA Class I, using four HLA Class I probes (three for HLA-A, one for HLA-B). Of the 125 studied miRNAs, 24 showed a correlation with at least three of the four HLA probes (Table S2).

Two different patterns were observed: four miRNAs (miR-155, miR-22, miR-635 and miR-1276) showed positive correlations with at least three of the four HLA Class I probes and 20 miRNAs showed negative correlations with at least three of the four HLA Class I probes. For these 24 miRNAs, we compared the miRNA expression levels not only with HLA-A and -B expression, but also with the expression of probes for HLA regulators (TAP1, TAP2, IRF1, IRF8, CIITA, NLRC5) [40], and markers of infiltrating T cells and macrophages (see Figure 2 and Figure S1 for TAP1). A clear pattern emerged, with the same four miRNAs showing positive correlations with most markers, and the other twenty mainly showing negative correlations. In order to validate our findings, we investigated the association between miRNAs, HLA Class I and TAP1 expression in a cohort of 26 UM from the Rotterdam ErasmusMC (Table S4). Of the 47 miRs, only eleven showed a high enough expression to be able to use them in a comparison with HLA mRNA expression data from the same cohort. MiR-155 showed a positive correlation with TAP1 ($R = 0.502$, $p = 0.02$). MiR-211 showed a negative correlation with HLA-A, and TAP1, and miR-125 with HLA-A, HLA-B and TAP1. MiR-635 and miR-1276 were not available in this set.

Of the other miRs that were in both sets, miR-18B, miR-31, miR-98, miR-361, miR-454, and miR-507 did not show any significant correlations with HLA-A, HLA-B, or TAP1 expression in the Rotterdam data. We then screened the data of the TCGA, where only three of the 24 miRNA of interest were identified: miR-155 and miR-22 were positively correlated with HLA-A ($R = 0.630$, $p < 0.001$; $R = 0.328$, $p = 0.003$, respectively) and HLA-B ($R = 0.694$, $p < 0.001$; $R = 0.396$, $p < 0.001$), while miR-98 did not show a significant association with HLA-A nor HLA-B ($R = 0.113$, $p = 0.32$; $R = 0.054$, $p = 0.63$).

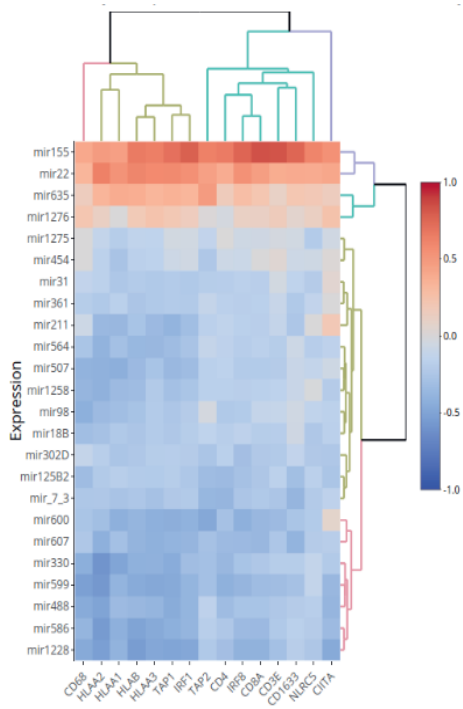


Figure 2. Heat map of the correlations between the expression levels of 24 miRNA expression with the mRNA expression of three HLA-A probes (probes HLA-A1, HLA-A2 and HLA-A3) and HLA-B, several regulators of HLA, members of the Antigen Loading Machinery, and T cell and macrophage markers. An analysis of 64 Uveal Melanoma was used to obtain the correlations. Red indicates a positive correlation and blue a negative correlation.

3.2. MiRNA expression and correlation with TILs and TAMs in Uveal Melanoma

As not only HLA expression but also the presence of an infiltrate is an important characteristic of an inflammatory phenotype, we analyzed whether the association was not only with HLA expression but also with the presence of TILs and TAMs. Out of the 101 miRNAs that were not associated with HLA expression, only three showed significant correlations with the presence of TILs and TAMs, indicating that HLA expression and a leukocyte infiltrate are strongly related (Table S3).

We then focused on the 24 miR-NAs which were correlated with HLA Class I expression.

When looking at the correlation matrix, all four miRNAs (miR-22, miR-155, miR-635, miR-1276) that had shown a positive correlation with at least three of the four HLA probes, cluster together with the HLA probes, the T lymphocyte markers (CD3, CD4 or CD8) and the macrophage markers (CD68 and CD163), while this is not the case for the negatively-associated miRs; they cluster in an opposite pattern (Figure 3).

The two opposing patterns are clearly visible in the correlation between the miRs with HLA-B (Figure 4), and macrophage marker CD68 (Figure 5): MiR-22, miR-155, and miR-635 show a positive correlation with HLA-B (p values ≤ 0.003) and MiR-22 and miR-155 with CD68 (both p values $= 0.007$), while miR-330, miR-599, and miR-1228 show negative correlations with HLA-B and CD68 (all p values ≤ 0.001).

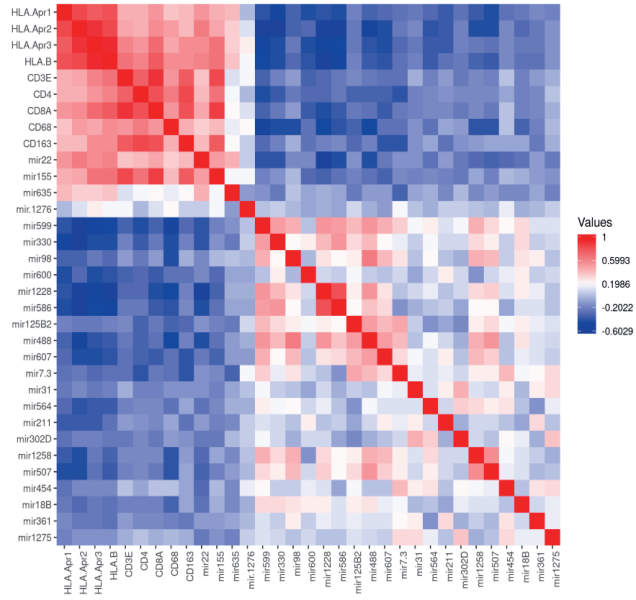


Figure 3. Expression of miRNAs was compared to the mRNA expression of HLA Class I and markers of infiltrate. Relation between miRNAs and mRNA levels from 64 Uveal melanoma are shown in a pairwise correlation matrix. Red indicates a high correlation, blue a low correlation.

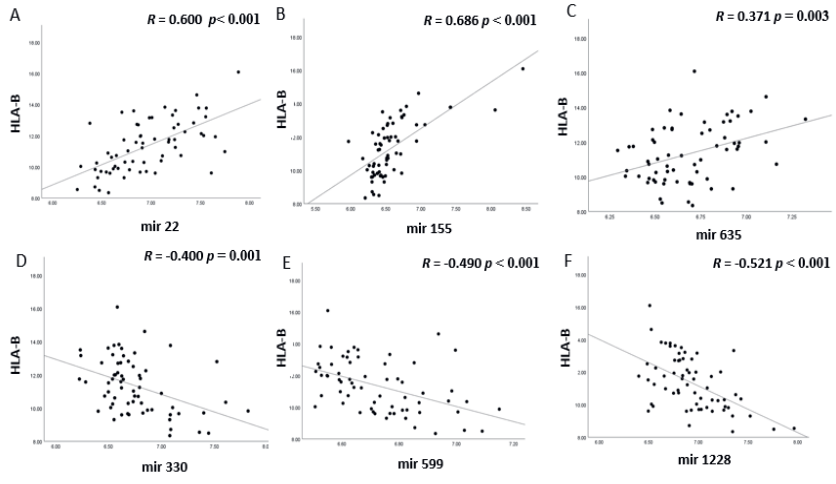


Figure 4. The expression levels of six different miRNAs (A-F) were compared to HLA-B mRNA levels in 64 Uveal Melanoma from Leiden. Positive and negative patterns are observed. *p* values were determined by Spearman correlation. $p \leq 0.05$ was considered significant. A: miR-22, B: miR-155, C: miR-635, D: miR-330; E: miR-599; F: miR-1228 versus HLA-B expression.

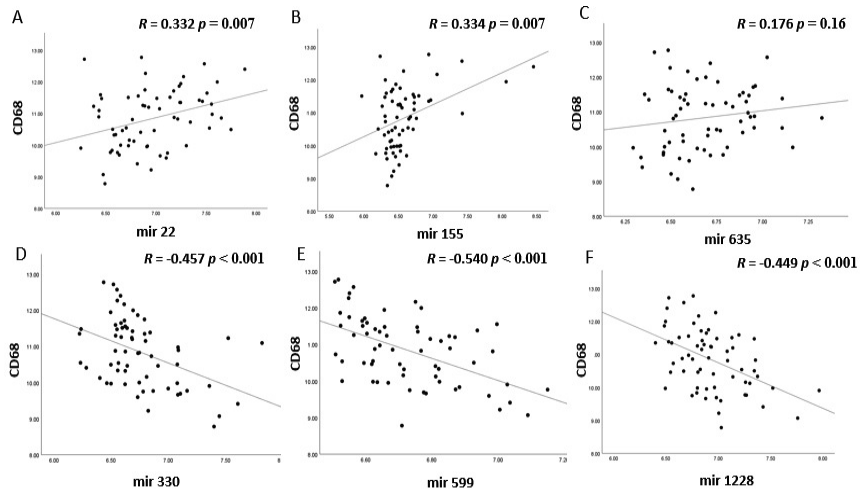


Figure 5. The expression levels of six different miRNAs (A-F) were compared to the mRNA levels of CD68, a macrophage marker, in 64 Uveal Melanoma from Leiden. *p* values were determined using Spearman correlation. $p \leq 0.05$ was considered significant. A: miR-22, B: miR-155, C: miR-635, D: miR-330; E: miR-599; F: miR-1228 versus CD68 expression.

As a control, we looked at the association between miRs of the Rotterdam cohort of 26 UM and the mRNA levels of infiltrating cells: in agreement with the prior findings, miR-155 was positively correlated with CD4 ($p = 0.02$), while miR-211 was negatively correlated with CD4 and CD8 and miR-125b2 with CD4 and CD163 (Table S4).

Of the four miRs in the Leiden cohort that had shown a positive correlation with HLA Class I and infiltrating leukocytes, three were present in the TCGA cohort: miR-155 was positively associated with CD4 (R = 0.336, $p = 0.002$), CD8 (R = 0.563, $p < 0.001$), CD68 (R = 0.277, $p = 0.01$) and CD163 (R = 0.365, $p = 0.001$); miR-22 was positively associated with CD8 (R = 0.296, $p = 0.01$) and CD163 (R = 0.254, $p = 0.02$) and not with CD4 (R = 0.142, $p = 0.21$) or CD68 (R = −0.105, $p = 0.35$), while miR-98 was only correlated with CD163 (R = 0.246, $p = 0.03$) and not with other markers: CD4 (R = 0.056, $p = 0.62$), CD8 (R = 0.162, $p = 0.15$), CD68 (R = −0.103, $p = 0.36$).

3.3. MiRNA expression and HLA Class I Regulatory Factors and the Antigen-Loading Machinery

HLA expression is influenced by regulatory factors such as IRF1, IRF8, NLRC5, and CIITA, but also through the Antigen-Loading Machinery (ALM). We investigated whether there was an association between the miRNAs that were associated with HLA Class I and these regulatory factors (Figure 6). Indeed, miR-22 and miR-155 were positively associated to the ALM components TAP1 and TAP2, and the HLA regulatory factors CIITA, NLRC5, IRF1, and IRF8, while the opposite was found for miR330, miR-599, and miR-1228.

	CIITA	*NLRC5*	*IRF1*	*IRF8*	*TAP1*	*TAP2*	*mir22*	*mir155*	*mir635*	*mir330*	*mir599*	*mir1228*
CIITA	1											
NLRC5	0.35	1										
IRF1	0.43	0.48	1									
IRF8	0.47	0.45	0.8	1								
TAP1	0.4	0.43	0.93	0.74	1							
TAP2	0.26	0.37	0.63	0.6	0.62	1						
mir22	0.42	0.34	0.55	0.49	0.58	0.36	1					
mir155	0.36	0.51	0.7	0.58	0.67	0.4	0.45	1				
mir635	0.2	0.19	0.32	0.24	0.35	0.44	0.42	0.18	1			
mir330	-0.27	-0.09	-0.44	-0.34	-0.51	-0.34	-0.34	-0.2	-0.17	1		
mir599	-0.41	-0.1	-0.47	-0.46	-0.52	-0.31	-0.4	-0.2	-0.13	0.51	1	
mir1228	-0.42	-0.25	-0.55	-0.4	-0.54	-0.21	-0.56	-0.33	-0.1	0.31	0.48	1

Figure 6. Correlation coefficients of the relation between several miRNAs and regulators of HLA expression and of members of the Antigen-Loading Machinery. The expression of three miRNAs (miR-22, 155, 635) that are positively associated with HLA expression and of three (miR-330, 599, 1228) that are negatively associated with HLA expression were compared to Class I Regulatory Factors and members of the Antigen-Loading Machinery. The correlation coefficients based on Spearman correlations (n=64) are shown. The intensity of colors indicates the strength of the correlation.

3.4. MiRNA expression and chromosome 3 status

As we and others [10–12] have shown that an inflammatory infiltrate is associated with M3, we subsequently determined whether the differential expression of miRNAs was related to the tumour chromosome 3 status. Of the four miRNAs which were high in tumours with a high HLA expression and infiltrate, three (miR-22, miR-155, and miR-635) were significantly higher in M3 tumours than D3 tumours (Figure 7A). Of the 20 miRs that were negatively correlated to HLA Class I, ten showed a lower mean expression in M3 tumours than in D3 tumours (Figure 7B).

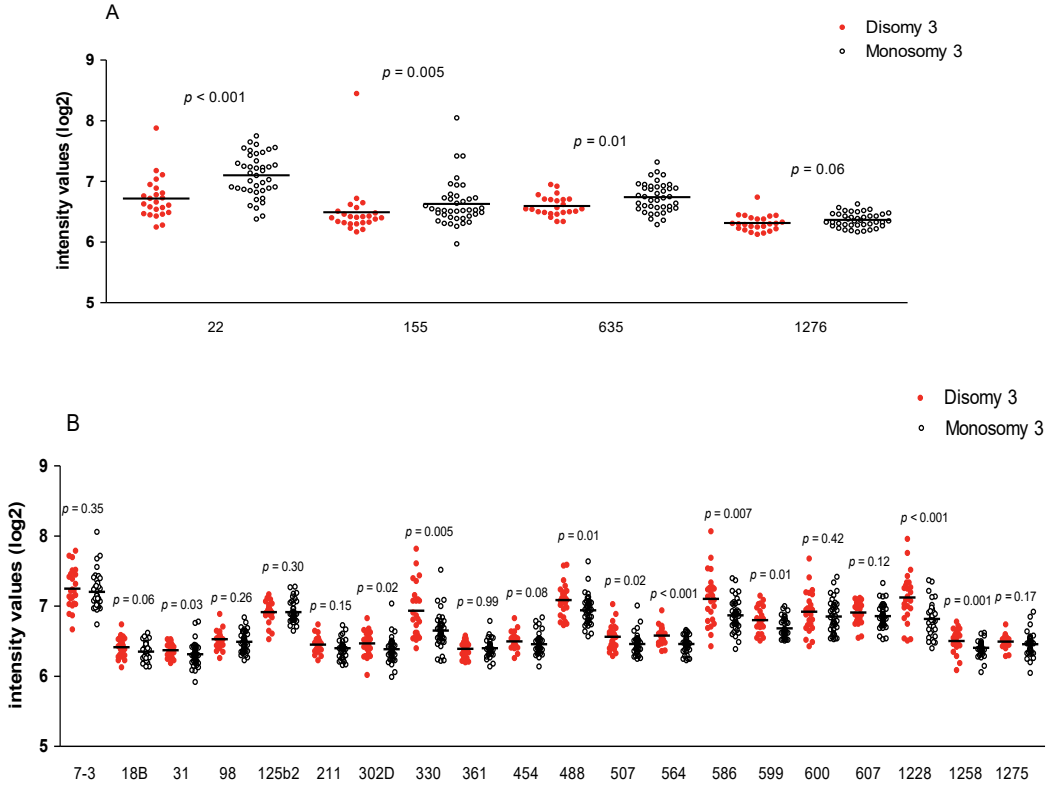


Figure 7. The expression of miRNAs in D3 (n = 24) and M3 (n = 40) tumours was compared. MiRNAs that are positively (A) or negatively (B) associated to HLA Class I are shown. Using a Mann-Whitney U test, $p \leq 0.05$ was considered significant. MiRNA name is indicated as numbers. Horizontal bars indicate mean expression.

3.5. MiRNA expression and BAP1 expression

In addition to loss of chromosome 3, loss of expression of BAP1 is associated with increased inflammation in UM [13]. We compared mRNA expression levels of BAP1 with HLA expression and the presence of TAMs; BAP1 expression was negatively correlated with HLA-B ($p < 0.001$) and CD68 ($p = 0.006$) (see Figure 8).

Moreover, BAP1 expression was negatively correlated to the pro-inflammatory miR-22, miR-155, and miR-635 ($p < 0.001$, $p < 0.001$, $p = 0.01$, respectively), and had a positive correlation with the anti-inflammatory miR-330, miR-599, and miR-1228 ($p = 0.05$, $p = 0.01$, and $p = 0.001$, respectively).

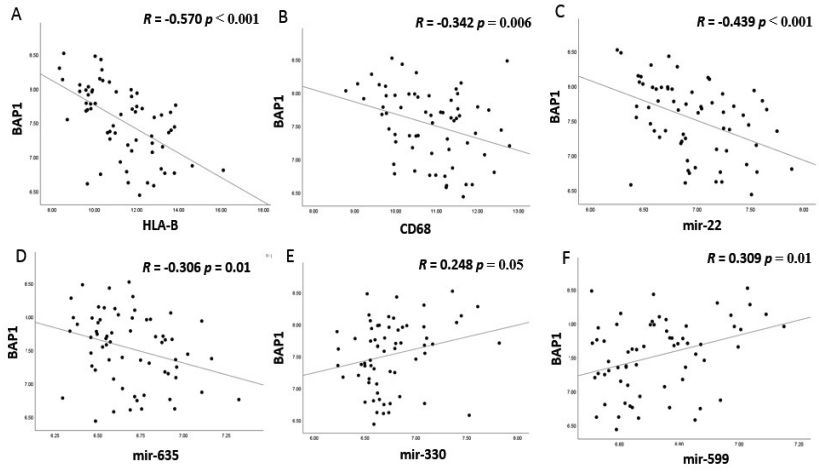


Figure 8. Correlation between BAP1 mRNA expression and HLA-B (A), CD68 (B), and examples of the two groups of miRNAs (C-F). *p* values were determined by Spearman correlation. $p \leq 0.05$ was considered significant. A: HLA-B, B: CD68, C: miR-22, D: miR-635; E: miR-330; F: miR-599 versus BAP1 expression.

3.6. MiRNA expression and survival

As we found associations between miRNA expression and loss of chromosome 3/BAP1 mRNA expression, we checked whether the levels of miRNAs were related to survival in UM patients (Figure 9).

According to Kaplan-Meier survival curves, high levels of miR-22 ($p = 0.02$) and miR-155 ($p = 0.04$) were related to decreased survival in UM patients while the opposite was found for miR-330 ($p = 0.04$) and miR-599 ($p = 0.009$). Differences with regard to miR-635 ($p = 0.09$) and miR-1228 ($p = 0.08$) did not reach statistical significance. When we looked at the miRNA from the TCGA, we found that high levels of miR-22 ($p = 0.003$) and miR-155 ($p = 0.004$) were related to a decreased survival while this was not the case for miR-98 ($p = 0.26$).

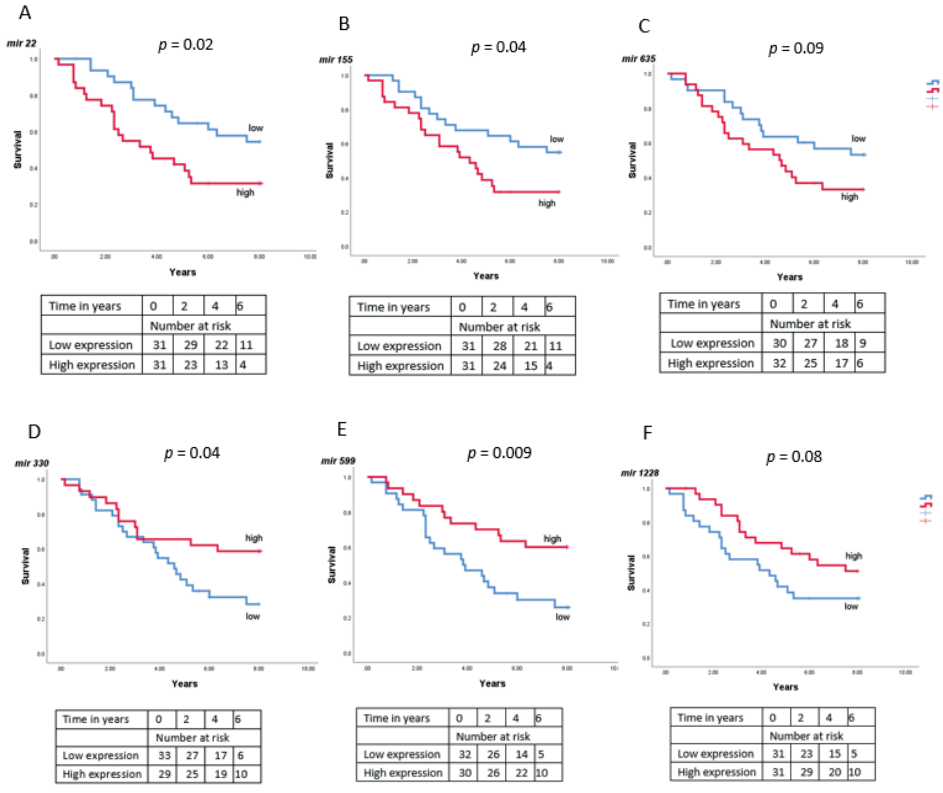


Figure 9. The relation between low and high miRNA expression and survival was determined in a group of 64 Uveal Melanoma patients from Leiden, using Kaplan-Meier survival curves (A–F). A log-rank test was used to determine significance. $p \leq 0.05$ was considered significant. A: miR-22; B: miR-155; C: miR-635; D: miR-330; E: miR-599; F: miR-1228.

4. Discussion

UM are defined by mutations which carry different risks of metastases. Most UM carry a mutation in the GNAQ or GNA11 gene, which do not contribute to prognostication [41–43]. EIF1AX and SF3B1 mutations occur especially in low-risk D3 tumours [44], while BAP1 mutations are observed in most high-risk M3 UM [13]. The tumours with M3/loss of BAP1 often show an inflammatory phenotype. However, despite all the different investigations regarding inflammation as a contributor towards metastasis in UM, it is still unclear how inflammation is regulated and how immune cells are recruited [39,45].

We therefore set out to explore which miRNAs are associated with inflammation in UM and whether their expression is related to chromosome 3/BAP1 loss. Robertson et al. previously identified different miRNA clusters in UM which were associated with chromosome 3 status and also suggested that expression of some miRNAs might be related to the immune environment [11]. Similar to previous studies [11,31–35], we observed a difference in miRs between low and high-risk UM.

One of the problems when looking at miRs is that different studies identify different miRs as being relevant: as stated in an extensive review by Aughton et al. [33], it should be noted that many studies looked at miRNAs in UM and hardly ever were the same miRNAs reported as significantly associated with an increased metastatic risk in UM. As they stated, differences may be due to differences in tissue sampling, classifications or miRNA detection systems. As we were interested in relations between miRs and inflammation, we first tried to identify patterns. HLA Class I expression is a key component in the development of inflammation in the eye, an immune-privileged organ. In spite of this immune privilege, inflammation occurs in many intraocular UM. A study from our laboratory in 1996 already described positive correlations between HLA expression and the presence of infiltrating leukocytes such as CD3 and CD4 lymphocytes, monocytes/macrophages and NK cells in UM [46]. In addition, van Essen in 2016 found a positive association between HLA Class I, members of the Antigen Processing Machinery, such as TAP1, and the amount of lymphocyte and macrophage infiltration in UM [40]. He showed that mRNA expression correlated with immune-histochemical staining for HLA Class I, and we therefore used mRNA expression with four different HLA Class I probes to compare HLA expression with miRNA levels in the same tumours. Out of the 125 miRNAs for which we had information from the Leiden cohort, we identified 24 that were associated with HLA Class I expression. Among these 24 miRNAs, we identified two clusters, one with a positive association with HLA Class I and infiltrating leukocytes, and the other cluster showing negative associations. Three of the four miRNAs (miR-22, 155, 635, and 1276) that were associated with a high HLA expression, were also related to high numbers of TILs and/or TAMs and were increased in tumours with M3.

Most of the 20 miRNAs that showed an inverse correlation with HLA Class I expression also showed an inverse association with infiltrating leukocytes; ten of these miRNAs were decreased in tumours with M3. Several of the miRs have previously been associated with HLA expression or with inflammation. Aughton et al. [33] compared the information from several papers on miRNAs in uveal melanoma and only found a few that had been reported in multiple papers. They described that miRNA that were associated with an increased metastatic risk could either be upregulated or downregulated. Earlier, Worley et al. [31] had also shown that miRNA clustered into an upregulated and downregulated group, both related to prognosis. One of the upregulated miRs was miR-155 [33]. This miR is not only upregulated in UM, it is also increased in plasma of UM patients at the time of metastasis [47]. One possibility is that miR-155 functions as an immune stimulator: Mir-155 is known as an important regulator of the NFkB pathway in macrophages and thereby may modulate inflammatory responses [48]. We previously described that NFkB activity, one of the most important signaling pathways in inflammation, is increased in BAP1-negative UM [49]; here we show that miR-155 is elevated in M3 tumours which we know are mostly BAP1-negative. Further studies are needed in order to determine whether miR-155 plays a role in the regulation of NFkB in UM. Our data do not exclude the possibility that we are looking at the presence of miR-155 containing macrophages, as it is known that this miR is expressed in monocytes in peripheral blood and in CD68+ cells obtained from healthy brain tissue [50]. The association of miR-155 with TAM may therefore be due to its presence within these cells, thereby explaining its high expression in M3 UM, which are known to carry many macrophages [10]. The presence of infiltrating cells may influence the tumour cells, HLA expression, as we previously described [40]. In a study involving epithelial ovarian cancer (another immune-privileged organ), miR-22 was similarly associated with the presence of an intra-tumoural immune infiltrate [51]. When we analysed which miRNAs were correlated with HLA expression, several strong positive correlations were observed, but, in addition, many other miRs showed a negative correlation. This group of miR also showed a negative correlation with the presence of TILs and TAMs, and with members of the Antigen Processing Machinery, such as TAP1. One of the downregulated miRNAs in high-risk M3 UM was miR-599. One study induced inflammation in endothelial cells by lipopolysaccharide (LPS) and found down-regulation of mir-599 in endothelial cell injury, with upregulation of TNF, IL-6, ICAM-1, and VCAM-1 [52]. Overexpression of miR-599 led to the

suppression of the inflammatory factors and downregulation of the JAK-STAT pathway known to be important in migration, proliferation, and inflammation by targeting ROCK1, a suppressor of inflammatory processes. Another miR with potential immunosuppressive capacity was miR-1228. In gastric cancer, miR-1228 was decreased in cell lines, and its induction led to a decrease of mesenchymal markers and invasiveness [53]. This decrease of miR-1228 was held responsible for NFkB upregulation.That miR expression may play a role in HLA expression was demonstrated by the group of Seliger, which observed that in renal cell carcinoma, overexpression of specific miRNAs led to a reduction of HLA-G, enhancing NK-cell mediated cytotoxicity in vitro. Tumours expressing HLA-G had a significantly higher frequency of CD3+ and CD8+ T cells [54]. In cutaneous melanoma, TAP1 was the target of regulation by miRs. MiR-200a-5p was found to bind to the 3′untranslated region (UTR) of TAP1, and overexpression in a cell line was accompanied by a decrease in HLA-Class I surface expression, more so of HLA-B/C than HLA-A [55]. High levels of miR-200a-5p were associated with a shorter overall survival of the cutaneous melanoma patients. Mari et al. (2018) observed that in esophageal adenocarcinoma, miRs were able to regulate expression of TAP1 and HLA-Class I [56]. It looks as if a similar mechanism may play a role in a range of malignancies, including UM, but every malignancy may involve different miRs, and that some may be immune-suppressive, while others may have the opposite effect. We not only observed strong positive correlations between several miRNA and TAP1 (with miR-22, miR-155, and miR-635), but also identified many negative correlations, such as, e.g., with miR-330, miR-599, and miR-1228 (Table 1, Figure S1).

Table 1. Overview of correlations between different miRNAs and HLA-A, HLA-B and TAP1 in the Leiden cohort (n = 64), TCGA cohort (n = 80) and the Rotterdam cohort (n = 26). r = two-tailed Spearman correlation coefficient. $p \leq 0.05$ considered significant. Significant correlations are indicated as bold. Negative associations are underlined.

miRNA	HLA-A			HLA-B			TAP1		
	Leiden (*n* = 64)	**TCGA (*n* = 80)**	**Rotterdam (*n* = 26)**	**Leiden (*n* = 64)**	**TCGA (*n* = 80)**	**Rotterdam (*n* = 26)**	**Leiden (*n* = 64)**	**TCGA (*n* = 80)**	**Rotterdam (*n* = 26)**
miRNA-22	**<0.001**	**0.003**	0.17	**<0.001**	**<0.001**	0.24	0.06	**0.002**	0.74
miRNA-155	**<0.001**	**<0.001**	0.08	**<0.001**	**<0.001**	0.07	**<0.001**	**<0.001**	**0.02**
miRNA-125-B2	**0.04**		**0.007**	**0.02**		**0.05**	0.15		**0.004**
miRNA-211	**0.004**		**0.05**	**0.04**		0.11	**0.002**		**0.005**

In UM, the group of three miRs were not only positively related to HLA Class I and TAP1 expression, but also with the HLA-transcription factors CIITA, NLRC5, IRF1, and IRF8. We suggest that a group of miRs suppress HLA expression and that during tumour progression (associated with addition of copies of chromosome 8q and loss of one chromosome 3 and loss of BAP1 expression) the HLA-suppressive miRNAs are lost and the stimulatory ones get expressed. While affecting HLA expression, miRNAs may also function as oncogenes, affecting proliferation, and as regulators of inflammation, influencing the influx of inflammatory cells during tumour progression. Such a function has been attributed to eg. miR-330, which has a tumour suppressive role in ovarian cancer, being able to downregulate MAPK signaling and ERK proteins [57], but also influencing inflammation: a forced expression of miR-330 led to the inhibition of oxidative stress and inflammation in macrophages [58]. Overall, we report two patterns for the expression of miRNA in UM, both related to the tumour chromosome/BAP1 expression status: one showing a relation between an increase in specific miRNAs and infiltrate, the other an opposite relationship. When looking at the miRNAs that are increased in UM that display inflammation, two options appear: as both miRNAs as well as leukocytes are present in a higher concentration in M3 tumours, it may be that we are looking at expression of miRNAs in infiltrating leukocytes. Another option is that loss of chromosome 3/BAP1 led to an upregulation of certain miRNAs in the UM cells themselves, which subsequently influenced the influx of inflammatory cells and led to upregulation of HLA antigens. It is clear that the genetic make-up of the tumour greatly influences miR expression and may thereby regulate HLA expression and inflammation. Using the TCGA gene expression data, Sharma et al. [59] observed that a cluster of miRNAs was embedded in the

3′UTR region of the BAP1 gene. They analysed which miRNAs would best bind to BAP1 and its mutations. These BAP1-associated miRNAs affected at least 69 target genes. Among them were e.g., histone deacetylases (HDAC1, HDAC2, HDAC3 and HDAC4), and immune regulators such as TGFbeta1, TNF, and NFkB1, RELA and RELB. We have described associations between upregulation of the NFkB pathway and these molecules in UM with BAP1 loss [49]. Their miRNAs differed from the ones we identified. The loss of one chromosome 3 has for a long time been associated with a worse prognosis in UM [1]. It has been shown that a mutation in BAP1 on chromosome 3 is similarly associated with a bad prognosis, leading to loss of BAP1 expression. We previously described an association between chromosome 3/BAP1 loss and inflammatory phenotype in UM [10,12,13], which has been confirmed by others [11,60,61]. We therefore propose that BAP1 loss may influence the presence of miRs, which may regulate inflammation. We propose a functional network of miRs, some of which have a pro-inflammatory role, as seen for miRs miR-22, miR-155, and miR-635, and some which have an anti-inflammatory function, or are just bystanders. However, both groups seem to be regulated by BAP1. It may be interesting to use the identified infiltrate-associated miRNAs to reduce local inflammation and decrease the level of cytokine production at the tumour site. Furthermore, it is important to look at other types of RNA: in addition to coding genes which may contribute to the development of UM, and miRNAs, non-coding genes that express non-translating RNAs such as lncRNA's have also been shown to be involved in the pathogenesis of this disease. The long non-coding RNA *LINC00518*, controlled by MITF, has been shown to be involved in the progression of UM [62] while *RHPN1-AS1* was an inducer of migratory characteristics [63].

5. Conclusions

We have identified two patterns of miRNAs: a set of miRNAs are either related to an up- or to a downregulation of HLA Class I expression, TAP1, and an inflammatory infiltrate in UM, while both show a relation to chromosome 3 status and BAP1 expression. Specific miRNAs may therefore be regarded as immune-stimulatory miRNAs, while others act as immune-suppressants. The upregulated miRNAs may serve as therapeutic targets, while the downregulated miRs could be investigated for their potential to inhibit inflammation. Taken together, our study provides the

basis to consider the miRNAs as important regulators of inflammation in UM, under the regulation of BAP1.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: The expression levels of six different miRNAs were compared to the mRNA levels of TAP1 in 64 Uveal Melanoma in the Leiden cohort, Table S1: Characteristics of patients and tumours in the Leiden cohort of 64 uveal melanoma used in the miRNA study. Table S2: Correlation between different miRNAs and HLA Class I in UM (n = 64). r = two-tailed Spearman correlation coefficient. $p \leq 0.05$ considered significant. Shaded in blue: negative association; shaded in red: positive association. Only miRNAs were selected for further analysis that had significant associations with at least three of the four HLA probes. Table S3: Correlation between the miRNAs (which are not associated with HLA Class I) with TIL and TAM markers in UM (n = 64). r = two-tailed Spearman correlation coefficient. $p \leq 0.05$ considered significant. Table S4: Correlation between miRNA gene expression and HLA Class I and infiltrate markers in a cohort of 26 UM tumours from Rotterdam; Spearman correlation, $p \leq 0.05$ considered significant.

Author Contributions: Conceptualization, Zahra Souri and Martine Jager; Data curation, Annemijn Wierenga, Emine Kiliç and Erwin Brosens; Formal analysis, Zahra Souri; Funding acquisition, Pieter van der Velden and Martine Jager; Methodology, Zahra Souri, Wilma Kroes and Robert Verdijk; Software, Stefan Böhringer; Supervision, Martine Jager; Writing – original draft, Zahra Souri and Martine Jager; Writing – review & editing, Annemijn Wierenga, Emine Kiliç, Erwin Brosens, Stefan Böhringer, Wilma Kroes, Robert Verdijk, Pieter van der Velden, Gregorius Luyten and Martine Jager.

Funding: This research was funded by a grant to Z.S. from The Iranian Ministry of Science Research and Technology, Grant UL 2011-4991 from the Dutch Cancer Society (KWF), Stichting Leids Oogheelkundig Ondersteunings Fonds, the Nelly Reef Fund, Family G., Ms. Brouwer and a Horizon 2020 grant from the European Community, CURE UM, nr. 667787.

Institutional Review Board Statement: The METC of the LUMC declared no objection to this study (19/10/2016, code G16.076/NV/gk). The research adhered to Dutch law ('Code for Prop-er Secondary Use of Human Tissue') and the tenets of the Declaration of Helsinki (World Medical Association of Declaration 2013; ethical principles for medical research involving human subjects).

Informed Consent Statement: The METC waved the need for Informed consent for this study.

Data Availability Statement: Data from the Leiden cohort are accessible through GEO Series accession number GSE84976 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE84976). For the TCGA data see ref 11. For the Rotterdam data see ref. 35.

Conflicts of Interest: The authors declare no conflict of interest

References

1. Prescher, G.; Bornfeld, N.; Becher, R. Nonrandom chromosomal abnormalities in primary uveal melanoma. J. Natl. Cancer Inst. 1990, 82, 1765–1769, doi:10.1093/jnci/82.22.1765.
2. Horsthemke, B.; Prescher, G.; Becher, R.; Bornfeld, N. Loss of chromosome 3 alleles and multiplication of chromosome 8 alleles in uveal melanoma. Genes Chrom. Cancer 1992, 4, 217–221, doi:10.1002/gcc.2870040305.
3. Singh, A.D.; Tubbs, R.; Biscotti, C.; Schoenfield, L.; Triozzi, P. Chromosomal 3 and 8 status within hepatic metastasis of uveal melanoma. Arch. Pathol. Lab. Med. 2009, 133, 1223–1227, doi:10.1043/1543-2165-133.8.1223.
4. Versluis, M.; de Lange, M.J.; van Pelt, S.I.; Ruivenkamp, C.A.; Kroes, W.G.; Cao, J.; Jager, M.J.; Luyten, G.P.; van der Velden, P.A. Digital PCR validates 8q dosage as prognostic tool in uveal melanoma. PLoS ONE 2015, 10, e0116371, doi:10.1371/journal.pone/0116371.
5. Harbour, J.W.; Onken, M.D.; Roberson, E.D.; Duan, S.; Cao, L.; Worley, L.A.; Council, M.L.; Matatall, K.A.; Helms, C.; Bowcock, A.M. Frequent mutation of BAP1 in metastasizing uveal melanomas. Science 2010, 330, 1410–1403, doi:10.1126/science.1194472.
6. Koopmans, A.E.; Verdijk, R.M.; Brouwer, R.W.; Van den Bosch, T.P.; Van den Berg, M.M.; Vaarwater, J.; Kockx, C.E.M.; Paridaens, D.; Naus, N.C.; Nellist, M.; et al. Clinical significance of immunohistochemistry for detection of BAP1 mutations in uveal melanoma. Mod. Pathol. 2014, 27, 1321–1330.
7. Tschentscher, F.; Hüsing, J.; Hölter, T.; Kruse, E.; Dresen, I.G.; Jöckel, K.H.; Anastassiou, G.; Schilling, H.; Bornfeld, N.; Horsthemke, B.; et al. Tumor classification based on gene expression profiling shows that uveal melanomas with and without monosomy 3 represent two distinct entities. Cancer Res. 2003, 63, 2578–2584.
8. Onken, M.D.; Worley, L.A.; Ehlers, J.P.; Harbour, J.W. Gene expression profiling in uveal melanoma reveals two molecular classes and predicts metastatic death. Cancer Res. 2004, 64, 7205–7509, doi:10.1158/0008-5472.CAN-04-1750.

9. Van Gils, W.; Lodder, E.M.; Mensink, H.W.; Kiliç, E.; Naus, N.C.; Bruggenwirth, H.T.; van IJcken, W.; Paridaens, D.; Luyten, G.P.; de Klein, A. Gene expression profiling in uveal melanoma: Two regions on 3p related to prognosis. Investig. Ophthalmol. Vis. Sci. 2008, 49, 4254–4262.
10. Maat, W.; Ly, L.V.; Jordanova, E.S.; de Wolff-Rouendaal, D.; Schalij-Delfos, N.E.; Jager, M.J. Monosomy of chromosome 3 and an inflammatory phenotype occur together in uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2008, 49, 505–510, doi:10.1167/iovs.07-0786.
11. Robertson, A.G.; Shih, J.; Yau, C.; Gibb, E.A.; Oba, J.; Mungall, K.L.; Hess, J.M.; Uzunangelov, V.; Walter, V.; Danilova, L.; et al. Integrative analysis identifies four molecular and clinical subsets in uveal melanoma. Cancer Cell 2017, 32, 204–220, doi:10.1016/j.ccell.2017.07.003.
12. Bronkhorst, I.H.; Vu, T.K.; Jordanova, E.S.; Luyten, G.P.; van der Burg, S.H.; Jager, M.J. Different subsets of tumor-infiltrating lymphocytes correlate with macrophage influx and monosomy 3 in uveal melanoma. Investig. Ophthal. Vis. Sci. 2012, 53, 5370–5378, doi:10.1167/iovs.11-9280.
13. Gezgin, G.; Dogrusöz, M.; van Essen, T.H.; Kroes, W.G.; Luyten, G.P.; van der Velden, P.A.; Walter, V.; Verdijk, R.M.; van Hall, T.; van der Burg, S.H.; et al. Genetic evolution of uveal melanoma guides the development of an inflammatory microenvironment. Cancer Immunol. Immunother. 2017, 66, 903–912, doi:10.1007/s00262-017-1991-1.
14. Blom, D.J.; Luyten, G.P.; Mooy, C.; Kerkvliet, S.; Zwinderman, A.H.; Jager, M.J. Human leukocyte antigen class I expression. Marker of poor prognosis in uveal melanoma. Investig. Ophthal. Vis. Sci. 1997, 38, 1865–1872.
15. Ericsson, C.; Seregard, S.; Bartolazzi, A.; Levitskaya, E.; Ferrone, S.; Kiessling, R.; Larsson, O. Association of HLA class I and class II antigen expression and mortality in uveal melanoma. Investig. Ophthal. Vis. Sci. 2001, 42, 2153–2156.
16. Dithmar, S.; Crowder, J.; Jager, M.J.; Vigniswaran, N.; Grossniklaus, H.E. HLA class I antigen expression correlates with histological cell type in uveal melanoma. Ophthalmologe 2002, 99, 625–628, doi: 10.1007/s00347-001-0596-3.
17. Niederkorn, J.Y. Immune escape mechanisms of intraocular tumors. Prog. Retin. Eye Res. 2009, 28, 329–347, doi:10.1016/j.preteyeres.2009.06.002.
18. Jager, M.J.; Hurks, H.M.H.; Levitskaya, J.; Kiessling, R. HLA expression in uveal melanoma: There is no rule without some exception. Hum. Immunol. 2002, 63, 444–451, doi:10.1016/s0198-8859(02)00389-0.
19. Singh, A.D.; Turell, M.E.; Topham, A.K. Uveal melanoma: Trends in incidence, treatment, and survival. Ophthalmology 2011, 118, 1881–1885, doi:10.1016/j.ophtha.2011.01.040.
20.

21. Kaliki, S.; Shields, C.L. Uveal melanoma: Relatively rare but deadly cancer. Eye 2017, 31, 241–257, doi: 10.1038/eye.2016.275.
22. Jager, M.J.; Shields, C.L.; Cebulla, C.M.; Abdel-Rahman, M.H.; Grossniklaus, H.E.; Stern, M.H.; Carvajal, R.D.; Belfort, R.N.; Jia, R.; Shields, J.A.; et al. Uveal melanoma. Nat. Rev. Dis. Primers 2020, 6, 1–25, doi: 10.1038/s41572-020-0158-0.
23. Roelofsen, C.D.; Wierenga, A.P.; van Duinen, S.; Verdijk, R.M.; Bleeker, J.; Marinkovic, M.; Luyten, G.P.; Jager, M.J. Five decades of enucleations for uveal melanoma in one center: More tumors with high risk factors, no improvement in survival over time. Ocul. Oncol. Pathol. 2021, 7, 133–141.
24. Wahid. F.; Shehzad, A.; Khan, T.; Kim, Y.Y. MicroRNAs: Synthesis, mechanism, function, and recent clinical trials. Biochim. Biophys. Acta Mol. Cell Res. 2010, 1803, 1231–1243.
25. Dews, M.; Homayouni, A.; Yu, D.; Murphy, D.; Sevignani, C.; Wentzel, E.; Furth, E.E.; Lee, W.M.; Enders, G.H.; Mendell, J.T.; et al. Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster. Nat. Genet. 2006, 38, 1060, doi:10.1038/ng1855.
26. Kosaka, N.; Iguchi, H.; Hagiwara, K.; Yoshioka, Y.; Takeshita, F.; Ochiya, T. Neutral sphingomyelinase 2 (nSMase2)-dependent exosomal transfer of angiogenic microRNAs regulate cancer cell metastasis. J. Biol. Chem. 2013, 288, 10849–10859, doi:10.1074/jbc.M112.446831.
27. Ma, L.; Young, J.; Prabhala, H.; Pan, E.; Mestdagh, P.; Muth, D.; Teruya-Feldstein, J.; Reinhardt, F.; Onder, T.T.; Valastyan, S.; et al. MiR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. Nat. Cell. Biol. 2010, 12, 247–256, doi:10.1038/ncb2024.
28. Hayashita, Y.; Osada, H.; Tatematsu, Y.; Yamada, H.; Yanagisawa, K.; Tomida, S.; Yatabe, Y.; Kawahara, K.; Sekido, Y.; Takahashi, T. A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res. 2005, 65, 9628–9632, doi:10.1158/0008-5472.CAN-05-2352.
29. Chun-Zhi, Z.; Lei, H.; An-Ling, Z.; Yan-Chao, F.; Xiao, Y.; Guang-Xiu, W.; Zhi-Fan, J.; Pei-Yu, P.; Qing-Yu, Z.; Chun-Sheng, K. MicroRNA-221 and microRNA-222 regulate gastric carcinoma cell proliferation and radioresistance by targeting PTEN. BMC Cancer 2010, 10, 367, doi:10.1186/1471-2407-10-367.
30. Shi, W.; Gerster, K.; Alajez, N.M.; Tsang, J.; Waldron, L.; Pintilie, M.; Hui, A.B.; Sykes, J.; P'ng, C.; Miller, N.; et. al. MicroRNA-301 mediates proliferation and invasion in human breast cancer. Cancer Res. 2011, 71, 2926–2937, doi:10.1158/0008-5472.CAN-10-3369.
31. Falzone, L.; Romano, G.L.; Salemi, R.; Bucolo, C.; Tomasello, B.; Lupo, G.; Anfuso, C.D.; Spandidos, D.A.; Libra, M.; Candido, S. Prognostic significance of deregulated microRNAs in uveal melanomas. Mol. Med. Rep. 2019, 19, 2599–2610, doi:10.3892/mmr.2019.9949.

32. Worley, L.A.; Long, M.D.; Onken, M.D.; Harbour, J.W. Micro-RNAs associated with metastasis in uveal melanoma identified by multiplexed microarray profiling. Melanoma Res. 2008, 18, 184–190, doi:10.1097/CMR.0b013e3282feeac6.
33. Yang, C.; Wei, W. The miRNA expression profile of the uveal melanoma. Sci. China Life Sci. 2011, 54, 351–358.
34. Aughton, K.; Kalirai, H.; Coupland, S.E. MicroRNAs and Uveal Melanoma: Understanding the diverse role of these small molecular regulators. Int. J. Mol. Sci. 2020, 16, 5648, doi:10.3390/ijms21165648.
35. Wróblewska, J.P.; Lach, M.S.; Ustaszewski, A.; Kulcenty, K.; Ibbs, M.; Jagiełło, I.; Suchorska, W.M.; Marszałek, A. The potential role of selected miRNA in Uveal Melanoma primary tumors as early biomarkers of disease progression. Genes 2020, 11, 271, doi:10.3390/genes11030271.
36. Smit, K.N.; Chang, J.; Derks, K.; Vaarwater, J.; Brands, T.; Verdijk, R.M.; Wiemer, E.A.; Mensink, H.W.; Pothof, J.; de Klein, A.; et al. Aberrant MicroRNA expression and its implications for uveal melanoma metastasis. Cancers 2019, 11, 815, doi:10.3390/cancers11060815.
37. Yang, L.; Qiming, H.; Xuehui, S.; Xiang, J.; Li, S.; Xiaolin, X.; Wenbin, W. MicroRNA 145 may play an important role in uveal melanoma cell growth by potentially targeting insulin receptor substrate-1. Chin. Med. J. 2014, 127, 1410–1406.
38. Ling, J.W.; Lu, P.R.; Zhang, Y.B.; Jiang, S.; Zhang, Z.C. MiR-367 promotes uveal melanoma cell proliferation and migration by regulating PTEN. Genet. Mol. Res. 2017, 16, doi:10.4238/gmr16039067.
39. Chen, X.; He, D.; Da Dong, X.; Dong, F.; Wang, J.; Wang, L.; Wang, L.; Tang, J.; Hu, D.N.; Yan, D.; et al. MicroRNA-124a is epigenetically regulated and acts as a tumor suppressor by controlling multiple targets in uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2013, 54, 2248–2256, doi:10.1167/iovs.12-10977.
40. Bronkhorst, I.H.; Ly, L.V.; Jordanova, E.S.; Vrolijk, J.; Versluis, M.; Luyten, G.P.; Jager, M.J. Detection of M2-macrophages in uveal melanoma and relation with survival. Investig. Ophthalmol. Vis. Sci. 2011, 52, 643–650, doi:10.1167/iovs.10-5979.
41. Van Essen, T.H.; van Pelt, S.I.; Bronkhorst, I.H.; Versluis, M.; Nemati, F.; Laurent, C.; Luyten, G.P.; van Hall, T.; van den Elsen, P.J.; van der Velden, P.A. Upregulation of HLA expression in primary uveal melanoma by infiltrating leukocytes. PLoS ONE 2016, 11, doi:10.1371/journal.pone.0164292.
42. Van Raamsdonk, C.D.; Bezrookove, V.; Green, G.; Bauer, J.; Gaugler, L.; O'Brien, J.M.; Simpson, E.M.; Barsh, G.S.; Bastian, B.C. Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. Nature 2009, 457, 599–602, doi:10.1038/nature07586.

43. Van Raamsdonk, C.D.; Griewank, K.G.; Crosby, M.B.; Garrido, M.C.; Vemula, S.; Wiesner, T.; Obenauf, A.C.; Wackernagel, W.; Green, G.; Bouvier, N.; et al. Mutations in GNA11 in uveal melanoma. N. Engl. J. Med. 2010, 363, 2191–2199, doi:10.1056/NEJMoa1000584.
44. Koopmans, A.E.; Vaarwater, J.; Paridaens, D.; Naus, N.C.; Kilic, E.; de Klein, A.; Rotterdam Ocular Melanoma Study Group. Patient survival in uveal melanoma is not affected by oncogenic mutations in GNAQ and GNA11. Br. J. Cancer 2013, 109, 493–496, doi:10.1038/bjc.2013.299.
45. Martin, M.; Maßhöfer, L.; Temming, P.; Rahmann, S.; Metz, C.; Bornfeld, N.; van de Nes, J.; Klein-Hitpass, L.; Hinnebusch, A.G.; Horsthemke, B.; et al. Exome sequencing identifies recurrent somatic mutations in EIF1AX and SF3B1 in uveal melanoma with disomy 3. Nat. Genet. 2013, 45, 933–936, doi:10.1038/ng.2674.
46. Brouwer, N.J.; Gezgin, G.; Wierenga, A.; Bronkhorst, I.H.; Marinkovic, M.; Luyten, G.P.; Versluis, M.; Kroes, W.G.; van der Velden, P.A.; Verdijk, R.M.; et al. Tumour angiogenesis in uveal melanoma is related to genetic evolution. Cancers 2019, 11, 979, doi:10.3390/cancers11070979.
47. De Waard-Siebinga, I.; Hilders, C.G.; Hansen, B.E.; van Delft, J.L.; Jager, M.J. HLA expression and tumor-infiltrating immune cells in uveal melanoma. Graefe's Archi. Clin. Exp. Ophthalmol. 1996, 234, 34–42, doi: 10.1007/BF00186516.
48. Achberger, S.; Aldrich, W.; Tubbs, R.; Crabb, J.W.; Singh, A.D.; Triozzi, P.L. Circulating immune cell and microRNA in patients with uveal melanoma developing metastatic disease. Mol. Immunol. 2014, 58, 182–186, doi:10.1016/j.molimm.2013.11.018.
49. Mann, M.; Mehta, A.; Zhao, J.L.; Lee, K.; Marinov, G.K.; Garcia-Flores, Y.; Lu, L.F.; Rudensky, A.Y.; Baltimore, D. An NF-κB-microRNA regulatory network tunes macrophage inflammatory responses. Nat. Commun. 2017, 8, 851, doi:10.1038/s41467-017-00972-z.
50. Souri, Z.; Wierenga, A.; van Weeghel, C.; van der Velden, P.A.; Kroes, W.G.; Luyten, G.P.; van der Burg, S.H.; Jochemsen, A.G.; Jager, M.J. Loss of BAP1 Is associated with upregulation of the NFkB pathway and increased HLA Class I expression in Uveal Melanoma. Cancers 2019, 11, 1102, doi:10.3390/cancers11081102.
51. Moore, C.S.; Rao, V.T.; Durafourt, B.A.; Bedell, B.J.; Ludwin, S.K.; Bar-Or, A.; Antel, J.P. MiR-155 as a multiple sclerosis–relevant regulator of myeloid cell polarization. Ann. Neurol. 2013, 74, 709–720, doi:10.1002/ana.23967.
52. Korsunsky, I.; Parameswaran, J.; Shapira, I.; Lovecchio, J.; Menzin, A.; Whyte, J.; Dos Santos, L.; Liang, S.; Bhuiya, T.; Keogh, M. Two microRNA signatures for malignancy and immune infiltration predict overall survival in advanced epithelial ovarian cancer. J. Investig. Med. 2017, 65, 1068–1076, doi:10.1136/jim-2017-000457.

53. Wang, J.; Du, A.; Wang, H.; Li, Y. MiR-599 regulates LPS-mediated apoptosis and inflammatory responses through the JAK2/STAT3 signalling pathway via targeting ROCK1 in human umbilical vein endothelial cells. Clin. Exp. Pharmacol. Physiol. 2020, 47, 1420–1428, doi:10.1111/1440-1681.13316.
54. Jia, L.; Wu, J.; Zhang, L.; Chen, J.; Zhong, D.; Xu, S.; Xie, C.; Cai, J. Restoration of miR-1228* expression suppresses epithelial-mesenchymal transition in gastric cancer. PLoS ONE 2013, 8, e58637, doi:10.1371/journal.pone.0058637.
55. Jasinski-Bergner, S.; Stoehr, C.; Bukur, J.; Massa, C.; Braun, J.; Hüttelmaier, S.; Spath, V.; Wartenberg, R.; Legal, W.; Taubert, H.; et al. Clinical relevance of miR-mediated HLA-G regulation and the associated immune cell infiltration in renal cell carcinoma. Oncoimmunology 2015, 4, e1008805, doi:10.1080/2162402X.2015.1008805.
56. Lazaridou, M.F.; Massa, C.; Handke, D.; Mueller, A.; Friedrich, M.; Subbarayan, K.; Tretbar, S.; Dummer, R.; Koelblinger, P.; Seliger, B. Identification of microRNAs Targeting the Transporter Associated with Antigen Processing TAP1 in Melanoma. J. Clin. Med. 2020, 9, 2690, doi:10.3390/jcm9092690.
57. Mari, L.; Hoefnagel, S.J.; Zito, D.; van de Meent, M.; van Endert, P.; Calpe, S.; Serra, M.D.; Heemskerk, M.H.; van Laarhoven, H.W.; Hulshof, M.C.; et al. MicroRNA 125a regulates MHC-I expression on esophageal adenocarcinoma cells, associated with suppression of antitumor immune response and poor outcomes of patients. Gastroenterology 2018, 155, 784–798, doi:10.1053/j.gastro.2018.06.030.
58. Lin, M.; Xia, B.; Qin, L.; Chen, H.; Lou, G. S100A7 regulates ovarian cancer cell metastasis and chemoresistance through MAPK signaling and is targeted by miR-330-5p. DNA Cell Biol. 2018, 37, 491–500, doi:10.1089/dna.2017.3953.
59. Liu, J.; Huang, G.Q.; Ke, Z.P. Silence of long intergenic noncoding RNA HOTAIR ameliorates oxidative stress and inflammation response in ox-LDL-treated human macrophages by upregulating miR-330-5p. J. Cell. Physiol. 2019, 234, 5134–5142, doi:10.1002/jcp.27317.
60. Sharma, A.; Biswas, A.; Liu, H.; Sen, S.; Paruchuri, A.; Katsonis, P.; Lichtarge, O.; Chand Dakal, T.; Maulik, U.; Gromiha, M.M. Mutational landscape of the BAP1 locus reveals an intrinsic control to regulate the miRNA network and the binding of protein complexes in uveal melanoma. Cancers 2019, 11, 1600.
61. Figueiredo, C.R.; Kalirai, H.; Sacco, J.J.; Azevedo, R.A.; Duckworth, A.; Slupsky, J.R.; Coulson, J.M.; Coupland, S.E. Loss of BAP1 expression is associated with an immunosuppressive microenvironment in uveal melanoma, with implications for immunotherapy development. J. Pathol. 2020, 250, 420–439, doi:10.1002/path.5384.

62. Durante, M.A.; Rodriguez, D.A.; Kurtenbach, S.; Kuznetsov, J.N.; Sanchez, M.I.; Decatur, C.L.; Snyder, H.; Feun, L.G.; Livingstone, A.S.; Harbour, J.W. Single-cell analysis reveals new evolutionary complexity in uveal melanoma. Nat. Commun. 2020, 11, 496, doi:10.1038/s41467-019-14256-1.
63. Barbagallo, C.; Caltabiano, R.; Broggi, G.; Russo, A.; Puzzo, L.; Avitabile, T.; Longo, A.; Reibaldi, M.; Barbagallo, D.; di Pietro, C.; et al. LncRNA LINC00518 acts as an oncogene in uveal melanoma by regulating an RNA-based network. Cancers 2020, 12, 3867, doi:10.3390/cancers12123867.
64. Lu, L.; Yu, X.; Zhang, L.; Ding, X.; Pan, H.; Wen, X.; Xu, S.; Xing, Y.; Fan, J.; Ge, S.; et al. The Long Non-Coding RNA RHPN1-AS1 Promotes Uveal Melanoma Progression. Int. J. Mol. Sci. 2017, 18, 226, doi:10.3390/ijms18010226.

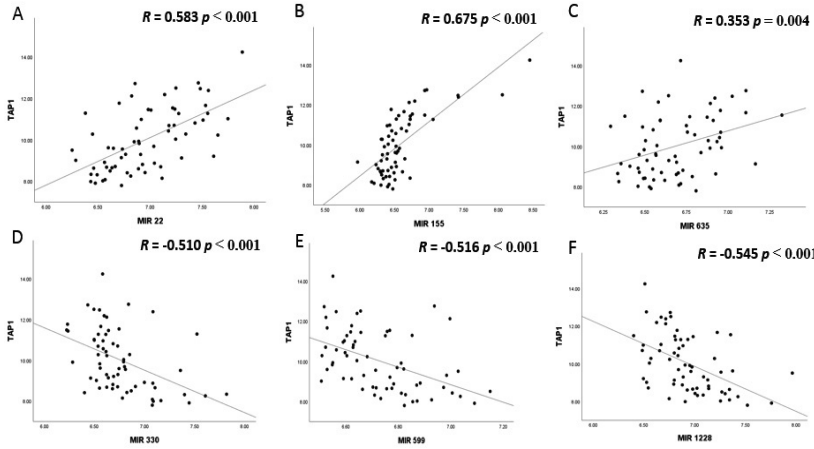


Figure S1. The expression levels of six different miRNAs (A-F) were compared to the mRNA levels of TAP1 in 64 Uveal Melanoma in the Leiden cohort. Three pro-inflammatory miRNAs (miR-22, 155 and 635) show positive as-sociations while three anti-inflammatory miRNAs (miR-330, 599, and 1228) show negative associations. Spearman cor-relation. $p \leq 0.05$ was considered significant.

Table S1. Characteristics of patients and tumours in the Leiden cohort of 64 uveal melanoma used in the miRNA study.

	Number of Cases	% of Cases
Gender		
Male	33	51%
Female	31	49%
Age (Years) at Enucleation (SD)		
≤60	30	47%
>60	34	53%
Cell Type		
Spindle	22	33%
Mixed/epithelioid	41	62%
Largest Tumour Diameter (LBD) in mm		
< 13.0 (median)	27	42%
≥ 13.0 (median)	37	58%
Tumour Prominence in mm		
< 8.0 (median)	29	45%
≥ 8.0 (median)	35	55%
Ciliary Body Involvement		
Not involved	40	62%
Involved	24	37%
cTNM Stage		
Stage I-IIB	37	58%
Stage IIIA-IIIC	25	39%
Metastasis		
No	26	41%
Yes	38	59%

Table S2. Correlation between different miRNAs and HLA Class I in UM (n = 64). r = two-tailed Spearman correlation coefficient. $p \leq 0.05$ considered significant and indicated as bold. Shaded in blue: negative association; shaded in red (*): positive association. Only miRNAs were selected for further analysis that had significant associations with at least three of the four HLA probes.

	HLA-A pr1		HLA-A pr2		HLA-A pr3		HLA-B	
	R	*p*	R	*p*	R	*p*	R	*p*
mir-let7-B-HG	-.038	.76	-.049	.70	-.102	.42	-.025	.84
mir-let7-G	-.091	.47	-.089	.48	-.090	.48	-.103	.42
mir-7-3-HG	-.302	**.01**	-.302	**.01**	-.275	**.03**	-.274	**.03**
mir-10-A	-.162	.20	-.063	.62	-.145	.25	-.176	.16
mir-17-HG	-.208	.10	-.095	.45	-.095	.46	-.070	.58
mir-18-B	-.235	.06	-.303	**.01**	-.255	**.04**	-.270	**.03**
mir-21	-.227	.07	-.194	.12	-.116	.36	-.122	.32
***mir-22-HG**	.569	**<.001**	.654	**<.001**	.565	**<.001**	.600	**<.001**
mir-25	-.191	.13	-.257	.04	-.199	.12	-.255	**.04**
mir-29-B1	-.213	.09	-.106	.41	-.181	.15	-.148	.24
mir-30-D	-.197	.12	-.153	.23	-.229	.07	-.159	.21
mir-31	-.324	**.009**	-.242	**.05**	-.367	**.003**	-.263	**.04**
mir-34-A	-.147	.24	-.095	.46	.011	.93	-.080	.53
mir-96	-.088	.49	.019	.88	-.061	.63	.028	.83
mir-98	-.339	**.006**	-.384	**.002**	-.263	**.04**	-.340	**.006**
mir-100-HG	-.296	.08	-.209	.10	-.180	.15	-.250	**.04**
mir-106-A	-.125	.32	-.104	.41	-.124	.33	-.107	.40
mir-125-B1	-.090	.48	-.042	.74	-.105	.41	-.108	.39
mir-125-B2	-.254	**.04**	-.300	**.02**	-.272	**.03**	-.288	**.02**
mir-129-2	.038	.76	-.052	.68	.007	.96	-.020	.87
mir-130-A	-.242	.05	-.201	.11	.007	.96	-.132	.30
mir-135-B	.000	1	-.008	.95	.010	.94	-.023	.86
mir-149	-.132	.30	-.073	.57	-.122	.34	-.109	.39
***mir-155-HG**	.536	**<.001**	.579	**<.001**	.619	**<.001**	.686	**<.001**
mir-181-C	-.142	.26	-.287	**.02**	-.171	.18	-.227	.07
mir-185	-.313	.01	-.217	.08	-.219	.08	-.282	**.02**
mir-194-1	-.154	.23	-.142	.26	-.159	.21	-.148	.24
mir-199-B	.025	.85	-.081	.52	-.024	.85	-.005	.97
mir-202	-.180	.15	-.128	.31	-.130	.31	-.164	.20
mir-203	.042	.74	-.079	.53	-.007	.96	-.007	.96
mir-211	-.351	**.004**	-.381	**.002**	-.354	**.004**	-.262	**.04**
mir-212	-.127	.32	-.142	.26	-.197	.12	-.133	.29
mir-215	-.085	.50	-.048	.70	-.033	.80	.048	.71
mir-218-2	.043	.74	.058	.65	.071	.58	.029	.82
mir-219-2	.363	**.003**	.229	.07	.133	.30	.250	**.05**
mir-221	-.067	.60	-.046	.72	-.089	.49	-.135	.29
mir-297	-.052	.68	-.048	.70	.017	.90	.020	.88

mir-300	-.096	.44	-.139	.27	-.293	**.02**	-.261	**.04**
mir-302-C	.157	.21	.029	.82	.100	.43	.094	.46
mir-302-D	-.322	**.01**	-.243	**.05**	-.269	**.03**	-.271	**.03**
mir-320-C1	.094	.46	.078	.54	-.003	.98	-.030	.81
mir-330	-.404	**.001**	-.541	**<.001**	-.415	**.001**	-.400	**.001**
mir-335	.011	.93	.067	.60	-.066	.60	-.081	.53
mir-342	-.012	.92	-.085	.51	-.093	.47	-.005	.97
mir-345	-.195	.12	-.198	.12	-.209	.10	-.190	.13
mir-361	-.229	.07	-.301	**.02**	-.285	**.02**	-.333	**.007**
mir-365-A	.129	.31	.065	.61	.153	.23	.037	.77
mir-369	-.094	.46	-.089	.48	-.062	.62	-.047	.71
mir-373	-.049	.70	-.149	.24	-.083	.52	-.106	.41
mir-382	.119	.35	.196	.12	.166	.19	.189	.13
mir-423	.153	.23	.192	.13	.094	.46	.172	.17
mir-429	.210	.10	.243	**.05**	.115	.36	.221	.08
mir-448	-.138	.28	-.052	.69	-.052	.69	-.058	.65
mir-450-A1	-.172	.17	-.160	.21	-.226	.07	-.209	.10
mir-454	-.357	**.004**	-.241	**.05**	-.237	.06	-.279	**.03**
mir-486	-.046	.72	-.108	.40	-.048	.70	-.161	.20
mir-488	-.343	**.005**	-.545	**<.001**	-.397	**.001**	-.377	**.002**
mir-489	.209	.10	.240	.06	.181	.15	.230	.07
mir-496	-.049	.70	-.076	.55	-.150	.24	-.121	.34
mir-504	-.084	.51	.036	.78	.022	.86	-.075	.56
mir-505	-.147	.25	-.156	.22	-.054	.67	-.049	.70
mir-507	-.415	**.001**	-.442	**<.001**	-.239	.06	-.333	**.007**
mir-518-E	-.015	.91	.101	.43	.146	.25	.121	.34
mir-525	-.098	.44	.048	.71	-.030	.81	-.028	.83
mir-526-A2	-.386	**.002**	-.214	.09	-.212	.09	-.244	**.05**
mir-539	-.272	**.03**	-.103	.42	-.229	.07	-.229	.07
mir-541	.136	.28	.161	.21	.102	.42	.154	.22
mir-551-A	-.053	.68	.001	.99	-.064	.62	-.096	.45
mir-557	-.043	.74	-.189	.13	-.135	.29	-.115	.37
mir-558	-.114	.37	-.084	.51	-.063	.62	-.065	.61
mir-559	.008	.95	.047	.71	-.063	.62	.072	.57
mir-564	-.306	**.01**	-.353	**.004**	-.308	**.01**	-.363	**.003**
mir-568	-.147	.24	-.195	.12	-.181	.15	-.133	.30
mir-574	.167	.18	.178	.16	.252	**.04**	.176	.16
mir-576	-.138	.28	-.039	.76	-.006	.96	-.006	.96
mir-577	.093	.47	.087	.49	.137	.28	.123	.33
mir-586	-.427	**<.001**	-.557	**<.001**	-.441	**<.001**	-.466	**<.001**
mir-590	-.129	.31	-.007	.96	-.070	.58	-.104	.41
mir-593	-.087	.50	-.166	.19	-.149	.24	-.204	.11
mir-599	-.386	**.002**	-.570	**<.001**	-.462	**<.001**	-.490	**<.001**
mir-600-HG	-.391	**.001**	-.310	**.01**	-.409	**.001**	-.372	**.002**
mir-604	-.139	.27	-.115	.36	-.117	.36	-.161	.20
mir-607	-.307	**.013**	-.409	**.001**	-.362	**.003**	-.352	**.004**
mir-630	.117	.36	.253	**.04**	.207	.10	.194	.12
mir-631	.030	.81	.084	.51	.056	.66	.036	.77
***mir-635**	.383	**.002**	.375	**.002**	.322	**.009**	.371	**.003**
mir-637	.080	.53	.170	.18	.075	.56	-.039	.76

mir-638	-.003	.98	.001	.10	.007	.96	.004	.97
mir-639	-.018	.89	-.026	.84	.008	.95	-.019	.88
miR-640	-.065	.61	-.144	.26	-.159	.21	-.105	.41
mir-642-A	-.141	.27	-.128	.31	-.210	.10	-.134	.29
mir-645	-.017	.89	.030	.81	.065	.61	-.070	.58
mir-657	-.074	.56	-.069	.59	-.184	.15	-.077	.54
mir-708	-.029	.82	.009	.94	-.031	.81	.016	.90
mir-759	.091	.47	.009	.94	.113	.37	.009	.94
mir-760	.155	.22	.159	.21	.145	.25	.167	.19
mir-877	-.005	.97	.110	.38	.102	.42	.092	.47
mir-933	-.158	.21	-.166	.19	-.209	.10	-.197	.12
mir-939	.140	.27	.085	.51	.172	.17	.162	.20
mir-940	.063	.62	-.017	.89	.036	.78	.037	.77
mir-943	-.086	.50	-.043	.74	-.059	.64	.031	.81
mir-1208	-.138	.28	-.183	.15	-.088	.49	-.145	.25
mir-1224	.065	.61	.198	.12	.220	.08	.138	.28
mir-1228	-.428	**<.001**	-.564	**<.001**	-.469	**<.001**	-.521	**<.001**
mir-1237	-.079	.53	.051	.69	.002	.99	.059	.64
mir-1246	-.005	.97	.077	.55	.073	.57	.024	.85
mir-1247	-.132	.30	-.109	.40	-.176	.16	-.120	.34
mir-1253	.025	.85	.111	.38	.094	.46	.109	.39
mir-1258	-.325	**.009**	-.420	**.001**	-.229	.07	-.332	**.007**
mir-1267	.041	.75	-.001	.10	-.001	.10	.023	.86
mir-1269-A	-.108	.40	.077	.54	.010	.94	-.078	.54
mir-1271	-.215	.09	-.151	.23	-.135	.29	-.180	.15
mir-1275	-.258	**.04**	-.197	.12	-.266	**.03**	-.249	**.05**
***mir-1276**	.129	.31	.242	**.05**	.299	**.02**	.249	**.05**
mir-1277	-.135	.29	-.076	.55	-.066	.60	-.108	.39
mir-1281	.081	.53	.117	.36	.140	.27	-.018	.88
mir-1282	-.078	.54	.064	.61	.009	.95	-.003	.98
mir-1323	-.059	.64	-.223	.08	-.163	.20	-.121	.34
mir-1537	-.124	.33	.073	.57	.025	.85	-.022	.86
mir-1909	.125	.32	.111	.38	.088	.49	.085	.50
mir-1914	-.123	.33	-.101	.43	-.071	.58	-.181	.15
mir-1915	-.121	.34	-.062	.63	-.019	.88	-.054	.67
mir-2115	.055	.67	-.034	.79	-.080	.53	-.061	.63
mir-2116	-.041	.75	.059	.64	-.170	.18	-.070	.58
mir-2278	-.022	.86	-.114	.37	-.075	.56	-.078	.59

Table S3. Correlation between the miRNAs (which are not associated with HLA Class I) with TIL and TAM markers in UM (n=64). r = two-tailed Spearman correlation coefficient. $p \leq 0.05$ considered significant and indicated in bold.

	CD3E		CD4		CD8A		CD68		CD163	
	R	***p***	**R**	***p***	**R**	***p***	**R**	***p***	**R**	***p***
miR-let7-B-HG	.160	.20	-.067	.60	.068	.60	.188	.14	-.121	.34
miR-let7-G	-.092	.47	.061	.63	-.025	.85	.012	.93	.048	.71
miR-10-A	-.073	.56	-.033	.79	-.080	.53	-.179	.16	-.022	.86
miR-17-HG	-.010	.94	-.098	.44	.000	.10	.087	.49	-.095	.46
miR-21	-.132	.30	.057	.65	-.015	.91	-.096	.45	.042	.74
mir-25	-.183	.15	-.159	.21	-.106	.41	-.233	.06	-.197	.12
mir-29-B1	-.078	.54	-.140	.27	-.126	.32	.042	.74	-.028	.82
mir-30-D	-.151	.23	-.169	.18	-.215	.09	-.164	.20	-.195	.12
mir-34-A	-.199	.12	-.069	.59	-.026	.84	-.032	.80	-.041	.74
mir-96	-.040	.75	.057	.66	-.022	.86	.080	.53	-.023	.86
mir-100-HG	-.176	.16	-.276	**.03**	-.191	.13	-.191	.13	-.141	.27
miR-106-A	.012	.93	-.003	.98	.095	.45	-.036	.78	-.056	.66
mir-125-B1	-.073	.57	.080	.53	-.050	.70	-.116	.36	.045	.72
miR-129-2	-.103	.42	-.131	.31	-.186	.14	-.343	**.005**	-.031	.80
mir-130-A	-.011	.93	-.016	.90	-.022	.86	-.038	.76	.077	.54
mir-135-B	-.046	.72	.091	.47	.109	.39	.032	.80	.015	.90
miR-149	-.072	.57	-.097	.44	-.113	.37	.100	.43	-.035	.78
mir-181-C	-.064	.61	-.013	.92	-.208	.10	-.223	.08	.151	.23
mir-185	-.304	**.01**	-.265	**.03**	-.302	**.01**	-.298	**.02**	-.040	.76
mir-194-1	-.010	.94	-.193	.13	-.055	.67	.047	.71	-.250	**.05**
mir-199-B	.052	.68	-.249	**.05**	-.125	.32	-.179	.16	-.252	**.04**
mir-202	-.097	.44	-.010	.94	-.146	.25	-.221	.08	.084	.51
mir-203	.201	.11	.067	.60	.064	.61	-.026	.84	-.031	.81
mir-212	-.232	.06	-.125	.32	-.160	.21	-.220	.08	-.040	.75
mir-215	-.031	.81	-.269	**.03**	-.010	.93	-.059	.64	.022	.86
miR-218-2	.200	.11	.100	.43	.171	.18	.051	.69	.184	.14
mir-219-2	.254	**.04**	.344	**.005**	.113	.38	.229	.07	.230	.07
mir-221	-.210	.10	-.238	.06	-.200	.11	-.290	**.02**	-.067	.60
mir-297	-.006	.96	-.112	.38	.029	.82	-.036	.78	-.057	.65
mir-300	-.148	.24	-.029	.82	-.223	.08	-.141	.26	-.081	.53
mir-302-C	.236	.06	.057	.65	.239	.06	.139	.27	.093	.46
mir-320-C1	-.034	.79	.075	.56	-.026	.84	.064	.61	.101	.43
mir-335	-.024	.85	-.148	.24	-.042	.74	-.140	.27	-.154	.23
mir-342	.011	.93	-.154	.22	-.044	.73	-.330	**.01**	-.182	.15
mir-345	-.114	.37	-.146	.25	-.158	.21	-.112	.38	-.197	.12
mir-365-A	.079	.53	-.219	.08	-.006	.96	-.127	.32	-.094	.46
miR-369	-.299	**.02**	-.081	.52	-.100	.43	.020	.88	-.125	.32
mir-373	-.184	.14	-.153	.23	-.207	.10	-.282	**.02**	-.088	.49
mir-382	.401	**.001**	.157	.21	.326	**.01**	.270	**.03**	.138	.28
mir-423	.197	.12	.165	.19	.157	.22	.155	.22	-.149	.24

mir-429	.388	**.002**	-.047	.71	.171	.18	.272	**.03**	-.151	.23
mir-448	-.075	.56	-.234	.06	-.042	.74	-.036	.78	-.190	.13
mir-450-A1	-.119	.35	-.033	.80	-.122	.34	-.108	.40	-.222	.08
mir-486	-.314	**.01**	-.176	.16	-.259	.04	-.333	**.01**	-.001	.99
mir-489	.048	.70	-.029	.82	.104	.41	.093	.47	.096	.45
mir-496	-.041	.75	-.132	.30	-.215	.09	-.226	.07	-.189	.14
mir-504	.086	.50	-.023	.86	.030	.82	.103	.42	.091	.47
mir-505	-.029	.82	-.057	.65	-.044	.73	-.119	.35	-.052	.68
mir-518-E	.102	.42	.090	.48	.133	.29	.177	.16	.121	.34
mir-525	.011	.93	-.080	.53	.098	.44	.171	.18	.041	.75
mir-526-A2	-.219	.08	-.160	.21	-.011	.93	-.051	.69	-.142	.26
mir-539	-.207	.10	-.267	**.03**	-.128	.31	-.158	.21	-.190	.13
mir-541	.242	.05	.016	.90	.262	**.04**	-.004	.97	-.035	.78
mir-551-A	.001	.99	-.024	.85	-.112	.38	.022	.87	.069	.59
mir-557	-.189	.13	-.208	.10	-.143	.26	-.376	**.002**	-.191	.13
mir-558	-.103	.42	-.089	.49	-.054	.67	.043	.74	-.276	**.03**
mir-559	.200	.11	.038	.77	.091	.47	.215	.09	.052	.68
mir-568	.016	.89	-.165	.19	-.108	.39	-.212	.09	-.313	**.01**
mir-574	.074	.56	.158	.21	.063	.62	.067	.60	.180	.15
mir-576	-.183	.15	-.066	.60	.024	.85	-.263	**.04**	.082	.52
mir-577	.183	.15	-.059	.65	.084	.51	-.102	.42	.098	.44
mir-590	.044	.73	-.203	.11	.032	.80	.100	.43	.022	.86
mir-593	-.175	.17	-.143	.26	-.217	.08	-.157	.22	-.134	.29
mir-604	.199	.11	.017	.90	.155	.22	.018	.90	.283	**.02**
mir-630	.083	.51	.178	.16	.104	.41	.229	.07	.221	.08
mir-631	.229	.07	.035	.78	.008	.95	.060	.64	-.018	.89
mir-637	-.054	.67	-.253	**.04**	-.076	.55	-.069	.59	-.254	**.04**
mir-638	.032	.80	-.193	.13	-.032	.80	.088	.49	-.235	.06
mir-639	-.019	.88	.117	.36	.103	.42	.047	.71	-.018	.89
miR-640	-.009	.95	-.046	.72	.061	.63	-.156	.22	.029	.82
mir-642-A	.085	.50	.001	.99	.014	.91	.066	.60	.157	.22
mir-645	-.170	.18	.054	.67	-.012	.92	-.017	.89	-.035	.78
mir-657	.077	.54	-.066	.61	-.041	.75	.008	.95	-.144	.26
mir-708	-.060	.63	-.092	.47	.051	.69	-.116	.36	.011	.93
mir-759	-.066	.61	-.134	.29	.031	.81	-.042	.74	-.125	.32
mir-760	.141	.27	.290	**.02**	.125	.32	.169	.18	.359	**.004**
miR-877	-.025	.85	.043	.74	.153	.23	.259	**.04**	.093	.46
mir-933	.023	.85	-.106	.40	-.133	.30	-.071	.58	-.159	.21
mir-939	.205	.10	.106	.41	.223	.08	.104	.41	.109	.39
mir-940	.144	.25	.011	.93	.040	.75	.198	.12	.044	.73
mir-943	-.006	.96	-.053	.67	-.045	.72	-.069	.59	.074	.56
miR-1208	.052	.68	-.185	.14	.001	.99	-.336	**.01**	.052	.68
miR-1224	.157	.22	.161	.20	.262	**.04**	.085	.51	.227	.07
miR-1237	.145	.25	.138	.28	.156	.22	.245	**.05**	.242	**.05**
miR-1246	-.002	.99	-.135	.29	-.072	.57	-.008	.95	-.084	.51
miR-1247	-.149	.24	-.178	.16	-.105	.41	-.160	.21	-.230	.07
miR-1253	.059	.64	.140	.27	.032	.80	.227	.07	.144	.26
mir-1267	.107	.40	-.042	.74	.173	.17	-.013	.92	-.178	.16
mir-1269-A	-.011	.93	-.078	.54	.114	.37	.011	.93	.088	.49

mir-1271	-.204	.11	-.339	**.006**	-.217	.08	-.201	.11	-.169	.18
mir-1277	-.117	.36	.089	.48	.010	.94	-.088	.49	.201	.11
mir-1281	-.240	.06	-.117	.36	-.091	.47	-.277	**.03**	.159	.21
mir-1282	-.081	.52	.011	.93	.059	.64	.026	.84	-.034	.79
mir-1323	-.019	.88	-.104	.41	-.096	.45	-.003	.98	-.174	.17
mir-1537	.033	.79	.033	.79	.022	.86	.113	.37	.019	.88
mir-1909	.121	.34	.101	.43	.027	.83	.015	.91	.102	.42
mir-1914	-.065	.61	-.053	.68	-.044	.73	-.082	.52	-.066	.60
mir-1915	-.082	.52	-.116	.36	-.180	.15	.071	.58	-.132	.30
mir-2115	.043	.73	.053	.68	-.031	.81	-.110	.39	-.117	.36
mir-2116	.042	.74	.020	.88	-.024	.85	.240	.06	-.243	**.05**
mir-2278	.002	.99	-.127	.32	-.237	.06	-.175	.17	.020	.87

Table S4. Correlation between miRNA gene expression and HLA Class I and infiltrate markers in a cohort of 26 UM tumours from Rotterdam; Spearman correlation, $p \leq 0.05$ considered significant and indicated as bold.

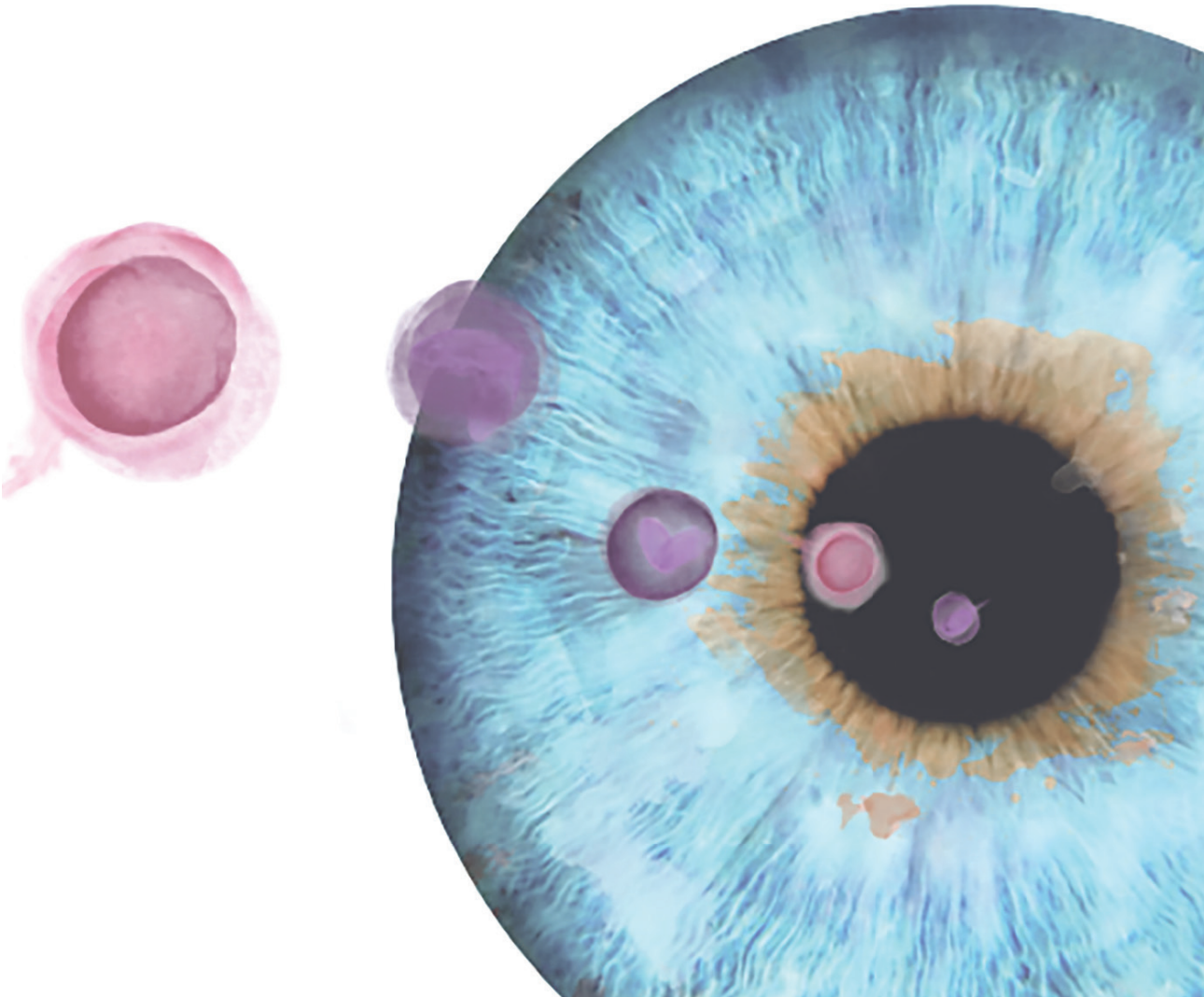
	Descriptive		**HLA-A**		**HLA-B**		**TAP1**		**CD4**		**CD8**		**CD163**	
	Mean	**SD**	**R**	***p***	**R**	***p***	**R**	***p***	**R**	***p***	**R**	***p***	**R**	***p***
mir-125B1_3p	9	12	-.248	.29	-.116	.63	-.392	.09	-.247	.29	-.373	.10	-.420	.06
mir-125b2_3p	36	24	-.585	**.007**	-.456	**.05**	-.617	**.004**	-.473	**.03**	-.317	.17	-.441	**.05**
mir-125b_5p	1088	2358	-.525	**.02**	-.359	.12	-.620	**.004**	-.411	.072	-.360	.12	-.469	**.04**
mir-155_5p	94	100	.397	.08	.415	.07	.502	**.02**	.526	**.02**	.308	.19	.355	.12
mir-18B_5p	45	38	-.403	.08	-.156	.51	-.268	.25	-.144	.543	-.237	.31	-.271	.25
mir-211_3p	273	141	-.420	.06	-.316	.17	-.580	**.007**	-.524	**.02**	-.489	**.03**	-.397	.08
mir-211_5p	26040	14088	-.442	**.05**	-.364	.11	-.605	**.005**	-.594	**.006**	-.449	**.05**	-.403	.08
mir-22_5p	268	113	.317	.17	.275	.24	.080	.74	.066	.78	.105	.66	-.027	.91
mir-31_3p	9	12	-.180	.45	-.206	.38	-.080	.74	.160	.502	.159	.50	-.033	.89
mir-31_5p	75	76	-.077	.75	-.009	.97	.117	.62	.287	.22	.271	.25	-.036	.88
mir-361_3p	539	214	.395	.08	.389	.09	.499	**.02**	.187	.43	.513	**.02**	.149	.53
mir-361_5p	1351	515	-.314	.17	-.346	.13	-.392	.09	-.548	**.01**	-.283	.23	-.302	.19
mir-454_3p	202	43	.006	.98	.042	.86	.111	.64	-.157	.51	-.002	.99	-.152	.52
mir-507	65	117	-.200	.40	-.111	.64	-.210	.37	-.136	.57	-.012	.96	-.267	.25
mir-7_1_3p	55	27	-.414	.07	-.227	.34	-.411	.07	-.276	.24	-.191	.42	-.205	.39
mir-98_3p	23	7	-.239	.31	-.223	.35	-.432	.06	-.351	.13	-.136	.57	-.319	.17
mir-98_5p	270	64	.388	.09	.389	.090	.120	.61	.011	.96	.196	.41	-.023	.92

Chapter 7

Expression of LAG3 and its ligands is increased in high risk Uveal Melanoma

Zahra Souri, Annemijn P.A. Wierenga, Wilma G.M. Kroes, Pieter A. van der Velden, Robert M. Verdijk, M. Eikmans ,Gregorius P.M. Luyten, Martine J. Jager

Under review



Simple Summary: Uveal melanoma (UM) is a rare type of intraocular malignancy, which often gives rise to metastases. While treatment with immune checkpoint inhibitors is often effective in the treatment of cutaneous melanoma metastases, it is hardly effective in case of UM metastases. Lymphocyte Activation Gene 3 (LAG3) is a recently recognized immune checkpoint; we determined the distribution of LAG3 expression and its ligands in three sets of primary UM. High risk UM (epithelioid cell type, loss of chromosome 3/BAP1 staining) had a higher expression of LAG3 and its ligands, which correlated with the presence of infiltrating immune cells. We conclude that LAG3 is especially expressed in high risk UM and may be a target for adjuvant immunotherapy in UM.

Abstract

Uveal melanoma (UM) is a rare ocular malignancy which originates in the uveal tract, and often gives rise to metastases. A potential target for immune checkpoint inhibition is Lymphocyte-Activation Gene 3 (LAG3). We set out to analyse the distribution of this immune checkpoint and its ligands in UM. Expression of mRNA was determined using an Illumina array in 64 primary UM from Leiden. The T lymphocyte fraction was determined by digital droplet PCR. In a second cohort, mRNA expression was studied by Fluidigm qPCR, while a third cohort consisted of 80 UM from TCGA. In the Leiden cohort, LAG3 expression was associated with the presence of epithelioid cells ($p = 0.002$), Monosomy of chromosome 3 ($p = 0.004$), and loss of BAP1 staining ($p = 0.001$). In Leiden and the TCGA, LAG3 expression correlated positively with expression of its ligands: LSECtin, Galectin-3, and the HLA Class II molecules HLA-DR, HLA-DQ, and HLA-DP (all $p < 0.001$).

Furthermore, the LAG3 ligands Galectin-3 and HLA Class II were higher in monosomy 3 tumours and expression of LAG3 correlated with the presence of an inflammatory phenotype (T cell fraction, macrophages, HLA-A and HLA-B expression: all $p < 0.001$). High expression levels of LAG3 ($p = 0.01$), Galectin-3 ($p = 0.001$), HLA-DRA1 ($p = 0.002$), HLA-DQA1 ($p = 0.04$), HLA-DQB2 ($p = 0.03$), and HLA-DPA1 ($p = 0.007$) were associated with a worse survival.

We conclude that LAG3 shows a higher expression in high risk Monosomy 3 UM than in low risk Disomy 3 tumours, and that this expression is strongly correlated with expression of its ligands

Galectin-3 and HLA Class II. The distribution suggests a potential benefit of anti-LAG3 monoclonal antibodies as adjuvant treatment in patients with high risk UM.

Keywords: Uveal Melanoma, Inflammation, Metastasis, Immunotherapy, LAG3

Introduction

Uveal Melanoma (UM) is a rare ocular malignancy which is especially seen in people with a fair skin and light eyes [1, 2]. Up to 50% of patients develop metastases and no improvement in survival has occurred over the last 50 years [3, 4]. The presence of an inflammatory phenotype is associated with a bad prognosis and involves the presence of a mixed leukocytic infiltrate, which is made up of tumour-infiltrated lymphocytes (TIL), tumour-associated macrophages (TAM), and an increased HLA Class I and II expression [5-9]. In spite of the presence of large numbers of lymphocytes and macrophages, local immune responses are not effective against intraocular tumours and do not inhibit their growth and metastases formation; instead, the presence of infiltrating leukocytes seems to stimulate growth [10-12]. In order to get an effective immune response, it is important to find the reason behind the unresponsiveness of the immune cell population. T cell responses are modulated through binding of stimulatory and inhibitory ligands to cell surface receptors. Some of these ligands are known as immune checkpoints, which can prevent immune overstimulation and auto-immune responses. Well-known immune checkpoints are Cytotoxic T-Lymphocyte-Associated Protein 4 (CTLA-4) and Programmed Cell Death Protein-1 (PD-1) [13, 14]. Another immune checkpoint is Lymphocyte Activation Gene-3 (LAG3), which is present on the surface of T cells, NK cells, and plasmacytoid dendritic cells [15]. The LAG3 protein forms a stable connection with HLA Class II via its 30-amino acid loop structure, and selectively binds to peptide-containing Major Histocompatibility Class II (MHC II) molecules [16, 17]. Under normal circumstances, LAG3 may help to prevent auto-immune responses, or excessive responses against virus infections [18]. However, tumour cells may use immune checkpoints to avoid immune recognition and exhaust cytotoxic T cells. LAG3 is highly associated and synergistic with PD-1 as it is co-expressed with this immune checkpoint on CD4 and CD8 T cells: one study found that more macrophages, CD3, CD4, and CD8 T cells were present in murine tumours when LAG3 and PD-1 were both knocked out, which suggests that the

combined deletion of these two factors alters regulator T cell homeostasis while enhancing tumour immunity [19].

Recently, Durante et al. called attention to the potential role of LAG3 in UM, indicating that LAG3 may be the dominant "exhaustion" marker in this malignancy [20]. Single cell RNA sequencing showed that most CD8 cytotoxic T cells in UM expressed LAG3 at a high level. The authors suggested that this was the reason that anti PD-1 and anti-CTLA-4 therapies had not been effective [21, 22]. Several studies have addressed the beneficial usage of anti-LAG3 therapy in different malignancies: one monoclonal anti-LAG3 antibody, IMP321, was able to activate Antigen-Presenting Cells (APCs) and T cells in breast cancer [23]. Another anti-LAG3 therapeutic approach proved efficacious with a high safety profile when it was combined with anti-PD-1 blockade in cutaneous melanoma patients [24, 25]. Many different trials, especially combining antibodies against different immune checkpoints, are ongoing. While LAG3 is expressed on regulator T cells, it may interact with multiple cell surface receptors, such as LSECtin, Galectin-3, and HLA Class II. In general, the effectiveness of targeted therapies is influenced by the expression of the target and this would also apply to the use of monoclonal antibodies against immune checkpoints; with this assumption, we analysed the expression of LAG3 and its ligands in primary UM to determine which tumours can be a target for adjuvant therapy.

2. Materials and Methods

2.1. Study Population

UM tissue was obtained from two groups of patients from the Leiden University Medical Center (LUMC) in Leiden, The Netherlands: the first group consisted of 64 patients who underwent an enucleation for UM between 1999 and 2008, of which 51% were male and 49% female.

The mean age at the time of enucleation was 61 years. The mean follow-up time (defined as the time period between enucleation and death) was 83 months (range 2 to 229 months). Follow-up was updated in 2020. At the end of follow up, 17 (27%) patients were alive, 37 (58%) patients had died because of metastasis, four (6%) had died because of other causes and six (9 %) had died but the cause of death was unknown; the second group was made up of 15 patients who underwent an

enucleation for UM at the LUMC between 2016 and 2017: 60% of these patients were male while 40% were female. The mean age at the time of enucleation was 59 years; the mean follow-up time was 26 months (range 9 to 34 months). At the end of follow up, 13 (87%) patients were alive, while 2 (13%) patients had died because of metastases. We also looked at mRNA levels of tumours included in the TCGA database (n=80) [9].

2.2. Chromosome Status

DNA was isolated by using the QIAmp DNA Mini kit (Qiagen, Venlo, The Netherlands). Chromosome 3 aberrations were detected by SNP analysis using the Affymetrix 250K_NSP and Affymetrix SNP 6.0 array [26, 27].

2.3. Illumina array

Gene expression was determined with the Illumina HT12v4 array (Illumina, Inc., San Diego, CA, US) for LAG3, Galectin-3, LSECtin, HLA Class II (DRalpha, DQalpha1, DQbeta2, DPalpha1), and immune cell markers (CD3, CD4, CD8, CD68, CD163), as described previously [28].

2.4. T lymphocyte fraction

The T cell fraction was obtained as previously described, by using a Digital Droplet PCR (ddPCR) assay directed at a specific locus of the TCR-β gene [12, 29].

2.5. Fluidigm qPCR

Fluidigm qPCR (Fluidigm Corporation, South San Francisco, CA, USA) was performed on 15 UM tumours, as previously described [30]. Briefly, total RNA (50–200 ng) was used for cDNA synthesis. Complementary DNA was diluted ten times (1.25 µL) and amplified with 2.5 µL of Taqman Preamp master mix (Applied Biosystems, Foster City, CA, USA) and 1.25 µL pooled primer mix for 14 cycles. QPCR reactions were performed using Eva-green dye and final results were collected using the BioMark HD system (Fluidigm). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), β-actin, Hypoxanthine Phosphoribosyltransferase 1 (*HPRT-1*), Ribosomal Protein L13a (*RPL13a*) and Hydroxymethylbilane Synthase (*HMBS*) were used as reference genes. The geometric mean of these references genes was used to standardize the gene expression signals of interest. The normalized data were log-transformed using Z-scores.

2.6. Statistical Analysis

Data were analyzed by SPSS version 22.0 (SPSS, nc., Chicago, Il, USA). Graphs were obtained by GraphPad Prism version 5.0 for windows (GraphPad Software, La Jolla, CA, USA). Spearman correlation was performed for correlations between non-parametric data. The Mann-Whitney U test was used to compare non-normal groups. A log-rank test was used for the significance analysis of survival graphs.

3. Results

3.1. Association between LAG3 and high risk characteristics of UM

We compared the expression level of LAG3 with the distribution of clinico-pathological characteristics in a cohort of 64 cases of UM from Leiden, The Netherlands. For this, we first sorted LAG3 expression from lowest to highest and observed two potential inflection points (Figure 1). We subsequently separated the cohort into two groups, with expression below and above the inflection point of 6.87. Forty-six tumours fell into the low expression group, and 18 into the high expression group. LAG3 expression was increased in cases with age >60 years (p = 0.04), in the presence of epithelioid cells (p = 0.002), loss of BAP1 staining (p = 0.001) (Table 1), and chromosome 3 loss (Monosomy 3, p = 0.004) (Figure 3).

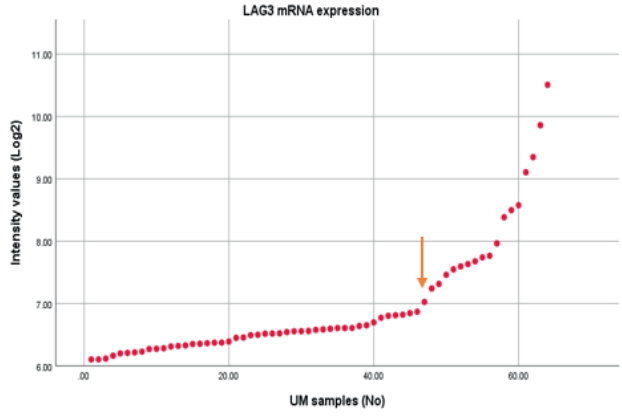


Figure 1. LAG3 expression in UM. The LAG3 expression level of 64 UM as seen in an Illumina array was sorted from low to high; the slope showed two inflection points, and we selected the inflection point of 6.87 to divide the cases into a low and high expression group.

Table 1. LAG3 expression compared to clinico-pathological and genetic characteristics of a set of 64 UM. Groups were separated according to the inflection point of 6.87. L = low expression, H = high expression. Using Pearson's Chi Square test, $p \leq 0.05$ is considered significant. Numbers in brackets represent percentages.

	Number of Patients (%)		
Clinical and Histopathologic Characteristics	**L**	**H**	***P***
Gender			
Male	23 (36%)	10 (16%)	
Female	23 (36%)	8 (12%)	0.69
Age (Years) at Enucleation (SD)			
≤60	26 (41%)	5 (8%)	
>60	20 (31%)	13 (20%)	**0.04**
Cell Type			
Spindle	21 (33%)	1 (1%)	
Mixed/epithelioid	25 (37%)	17 (26%)	**0.002**
Largest Tumour Diameter (LBD) in mm			
< 13.0 (median)	20 (31%)	7 (11%)	
>= 13.0 (median)	26 (41%)	11 (17%)	0.74
Tumour Prominence in mm			
< 8.0 (median)	24 (37%)	5 (8%)	
>= 8.0 (median)	22 (34%)	13 (20%)	0.08
Ciliary Body Involvement			
Not involved	31 (48%)	9 (14%)	
Involved	15 (23%)	9 (14%)	0.20
cTNM Stage (n = 62)			
Stage I-IIB	27 (43%)	10 (16%)	
Stage IIIA-IIIB	17 (27%)	8 (13%)	0.67
Metastasis			
No	22 (34%)	4 (6%)	
Yes	24 (37%)	14 (22%)	0.06
BAP1 status (n = 55)			
BAP1 staining positive	24 (44%)	1 (2%)	
BAP1 staining negative	15 (27%)	15 (27%)	**0.001**

3.2 Association between LAG3 and cell surface ligands

As LAG3 on T lymphocytes binds to ligands on APCs or tumour cells, we subsequently analysed which tumours express these ligands. LAG3 is known to form stable complexes with different cell surface receptors such as LSECtin, Galectin-3, and HLA Class II isoforms. In our cohort of 64 UM, the expression of LSECtin and Galectin-3 was positively correlated with LAG3 (both p <0.001). Messenger RNA expression levels of the HLA Class II genes (HLA-DRalpha, HLA-DQalpha, and HLA-DQbeta2 and HLA-DPalpha1) were positively associated with LAG3 expression (all $p \leq 0.001$) (Figure 2; supplemental Table 1A).

In order to validate our findings, we made the same analysis using the TCGA database of 80 UM samples [9] and found almost identical results: LAG3 expression showed a positive correlation with LSECtin, Galectin-3, HLA-DRalpha, HLA-DQalpha, HLA-DQbeta2, and HLA-DPalpha (all $p \leq 0.001$) (Supplemental Table 1B).

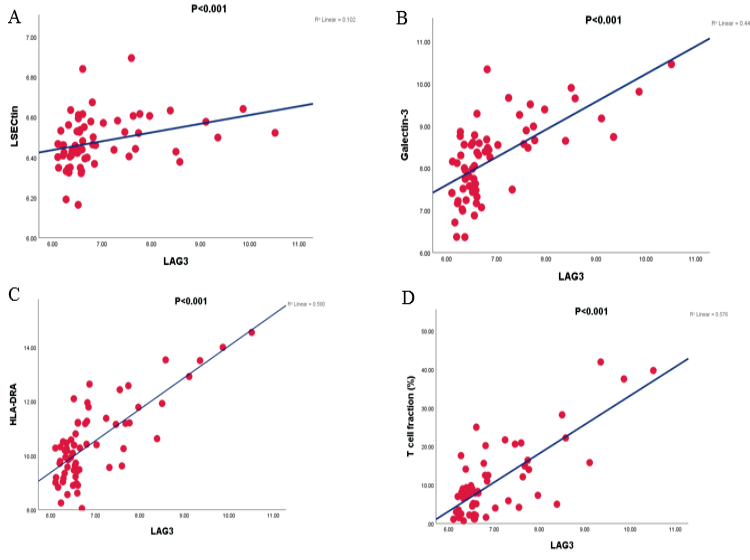


Figure 2. Correlation between LAG3, LSECtin, Galectin-3, and HLA-DRalpha in 64 UM from Leiden cohort (A-C), and between LAG3 and lymphocyte fraction as determined by ddPCR (D); a Spearman correlation was applied.

3.3. LAG3 and inflammatory phenotype

As chromosome status is related to the presence of an inflammatory phenotype, we checked the associations of LAG3 with immune cell markers: LAG3 showed a positive association with the presence of TILs and TAMs (Spearman correlation coefficient, CD3E R = 0.727, CD4 R = 0.596, CD8A R= 0.832, CD68 R = 0.542, CD163 R = 0.485, all p <0.001), as well as with HLA-Class I expression (HLA-A probe 1 R= 0.712, HLA-A probe 2 R= 0.731, HLA-B R= 0.791, all p <0.001) (Supplemental Table 1A); a similar pattern was observed in the TCGA cohort (Supplemental Table 1B). As mRNA and RNAseq data show expression levels but not cell numbers, we additionally looked at the T cell fraction as determined using a specifically-developed ddPCR that quantifies the rearranged T cell genes [12, 29]. A good correlation was present between LAG3 levels and the tumour's T lympocyte fraction (R= 0.553, p < 0.001, Figure 2D).

3.4. UM with Monosomy 3 express higher levels of LAG3 ligands than with Disomy 3

As we had found a positive association between LAG3 expression and its ligands, and an association between LAG3 and loss of BAP1 expression/the presence of Monosomy 3, we wondered whether the ligands would similarly show associations with high risk tumour characteristics. When looking at the Leiden 64-case cohort, expression of Galectin-3 (p = 0.002), of one of the probes for HLA-DRalpha (p = 0.02), of HLA-DQbeta2 (p = 0.02) and of HLA-DPalpha (p = 0.01) was significantly higher in high risk M3 tumours compared to low risk D3 tumours (Figure 3).

In the TCGA cohort, we observed similar correlations: M3 tumours showed a higher expression than D3 tumours for LAG3, LSECtin, Galectin-3, HLA-DRalpha, HLA-DQalpha, and HLA-DPalpha (all p = 0.001, Mann-Whitney U test).

As a control for having used mRNA/RNAseq expression levels, we analysed an additional 15 UM from Leiden by Fluidigm qPCR, and compared the expression of LAG3, HLA-DR, and Galectin-3 between D3 vs M3 tumours (Table 2). Similar to prior results, the mean value of LAG3 (p = 0.004) and HLA-DR (p = 0.004) was higher in M3 than in D3 tumours while this was not the case for Galectin-3 (p = 0.61).

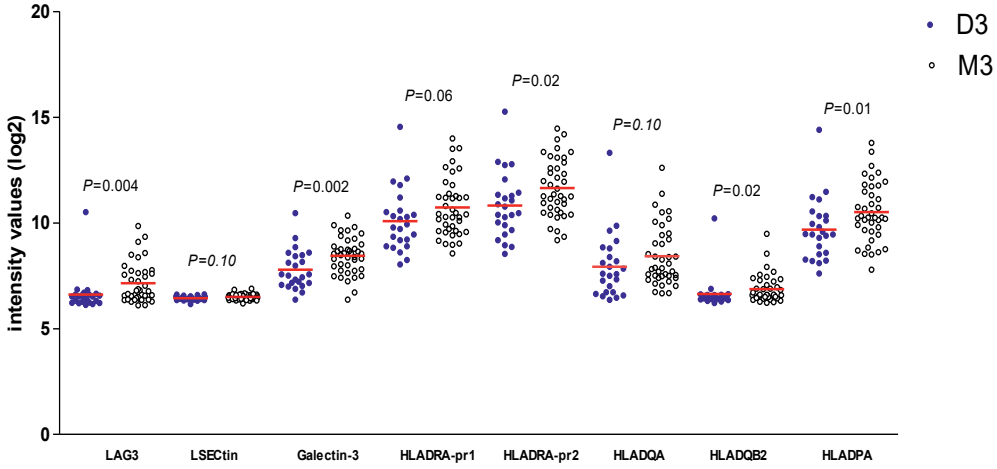


Figure 3. Comparison of gene expression of LAG3, LSECtin, Galectin-3, and several HLA Class II molecules in 64 UM from Leiden between D3 (n=24) and M3 (n=40) UM; a Mann-Whitney U test was applied. Horizontal bars indicate mean gene expression.

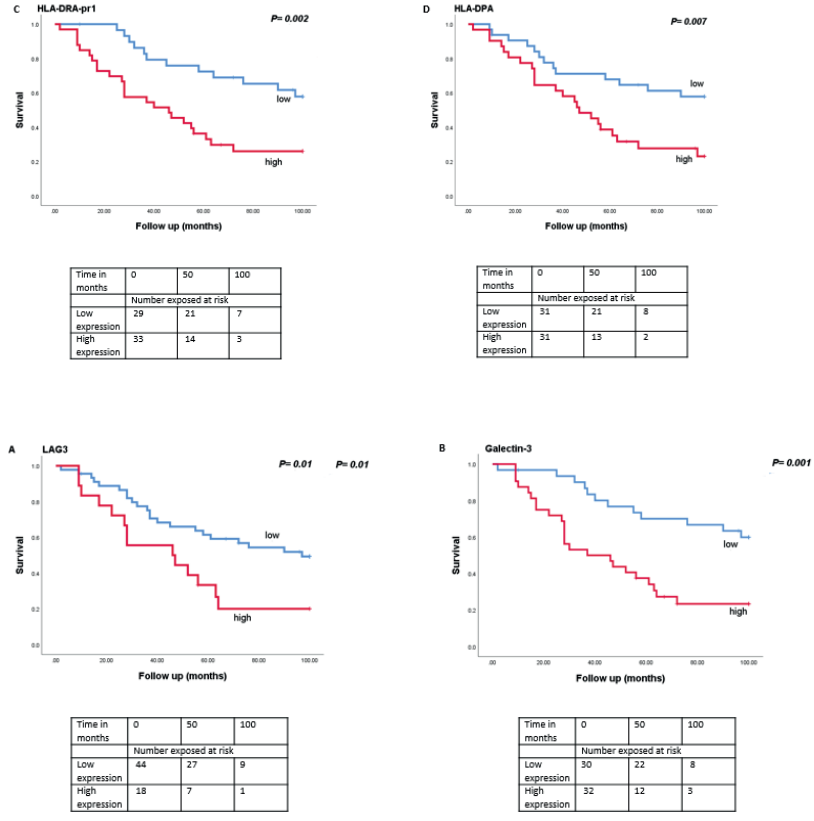
Table 2. Mean mRNA expression levels of different immune-modulators defined by qPCR in UM tumours (n=15). A Mann-Whitney U test was performed. $p \leq 0.05$ is considered significant (indicated in bold).

	D3 (n=9) Mean ± SD	M3 (n=6) Mean ± SD	*p*
LAG3	4 ± 3	98. ± 80	**0.004**
Galectin-3	1179 ± 1489	581± 345	0.61
HLA-DR	721 ± 606	4953 ± 4751	**0.004**

3.5. Association between LAG3 and its ligands with survival

As monosomy 3 is associated with a worse prognosis, we then analysed the relation between expression levels of LAG3, LSECtin, Galectin-3, and HLA Class II molecules with survival in the Leiden set of 64 UM (Figure 4). Tumours with a high expression of LAG3 (split at the inflection

point into high and low, $p = 0.01$), Galectin-3 (split at the median, $p = 0.001$), HLA-DRalpha (split at the median, $p = 0.002$), HLA-DQalpha (split at the median, $p = 0.04$), HLA-DQbeta2 (split at the median, $p = 0.02$) and HLA-DPalpha (split at the median, $p = 0.007$) were associated with a significantly worse metastasis-related survival, while mRNA expression levels of LSECtin (not shown) did not show an association with survival ($p = 0.39$).



Time in months	0	50	100
	Number exposed at risk		
Low expression	29	21	7
High expression	33	14	3

Time in months	0	50	100
	Number exposed at risk		
Low expression	31	21	8
High expression	31	13	2

Time in months	0	50	100
	Number exposed at risk		
Low expression	44	27	9
High expression	18	7	1

Time in months	0	50	100
	Number exposed at risk		
Low expression	30	22	8
High expression	32	12	3

Figure 4. Kaplan-Meier survival curves based on mRNA expression of LAG3, Galectin-3, and HLA Class II molecules. A log-rank test was used for the significance analysis.

3.6. LAG3 and immunemodulators

Combining several monoclonal antibodies directed against checkpoint inhibitors as treatment might boost the overall response to therapy in patients [24, 25]. We therefore decided to look at the correlation between expression of LAG3 and other immune-modulators: LAG3 showed a positive correlation with PD-1 (two-tailed Spearman correlation coefficient R = 0.655, $p < 0.001$), CTLA-4 (R = 0.298, $p = 0.02$), IDO-1 (R = 0.759, $p < 0.001$) and one of the probes of TIGIT (R = 0.413, $p = 0.001$), while it did not show a significance association with TIGIT second probe (R = -0.207, $p = 0.10$) (Supplemental Table 1A). Even higher associations were seen in the TCGA cohort (Supplemental Table 1B).

4. Discussion

High risk primary UM is associated with inflammation and its metastases are non-responsive to most current immunotherapy approaches. A treatment for metastases is eagerly awaited: many different studies have tested anti-PD-1 and anti-CTLA-4 therapies in UM patients but results have been unsatisfactory, especially in comparison with metastasized cutaneous melanoma, with very few complete or partial responses [31-33]. As tumours with a high risk of developing metastases can be recognized because they carry specific chromosome aberrations and mutations, developing an early adjuvant therapy for preventing metastases would be most welcome. Such an adjuvant treatment has proved useful in treating cutaneous melanoma patients [34]. Chromosome 3/BAP1 loss is a leading event in the development of the highest risk UM, which often give rise to metastases. A strong association has been observed between Chromosome 3/BAP1 loss and an inflammatory phenotype, consisting of a mixed arrangement of leukocytes and enhanced inflammation [7, 8, and 35].

The immune checkpoint that we investigated here is LAG3, which is expressed on a wide range of lymphocytes. We found that high levels of LAG3 expression in UM were positively associated with high risk tumour parameters, such as epithelioid/mixed cell type and chromosome 3/BAP1 loss, and the presence of inflammatory cells. Although we and others have previously indicated that there are more T cells and macrophages in M3 than D3 tumours, this association between LAG3 and Monosomy 3 has as far as we know not yet been described [7, 9, 27]. A high expression of LAG3 and of its ligands was found to be associated with bad survival.

Recently, the group of Harbour commented that LAG3 was the most dominant immune checkpoint molecule in UM [20]. They studied a small series of primary UM and metastases by single cell RNAseq analysis. It might be that the CD8 cytotoxic T cell population in UM is functionally exhausted by the LAG3-signaling cascade. Such a situation was described in follicular lymphoma, with T cells forming a heterogenic population: some of the T cells which expressed PD-1 were also LAG3-positive. The PD-1+LAG-3+ CD8 population was poor in cytokine production compared to the group which was LAG3 negative and hence was immunologically non-functional; moreover, in accordance to what we found in UM, the high level of LAG3 correlated with worse survival in this disease [36]. Hoefsmit recently described that liver metastases from UM patients often contain LAG3-expressing lymphocytes [37].

One of the ligands for LAG3 is LSECtin which is a member of the Selectin family, and is highly expressed in liver tissue [38]. In one study, LSECtin was expressed on murine B16 melanoma cells: interaction between LSECtin and LAG3 led to inhibition of Interferon γ (IFNγ) secretion by CD8 T cells and reduced their cytotoxic activity, promoting tumour growth [39]. We found positive associations of this protein with the expression of LAG3 (p<0.001), but no significant difference between D3 and M3 tumours. Galectin-3 is a type of Lectin with immune checkpoint inhibitory activity through its capacity to bind to LAG3 on CD8+ effector T cells. It can serve as a chemoattractant for macrophages and is reported to be highly expressed during inflammation [40]. One biological process in which this protein might be involved is the suppression of T cell-mediated lysis: Galectin-3 was shown to interact with LAG3 present on CD8 cells in pancreatic ductal adenocarcinoma. Depletion of Galectin-3 improved CD8 cytotoxicity [41]. In cutaneous melanocytic lesions, melanoma and metastases more often displayed nuclear and cytoplasmic Galectin-3 than naevi [42]. In accordance to what we observed, Galectin-3 has been identified as a marker for a low overall survival of different cancers such as colorectal, ovarian, and non-small cell lung cancer, serving as an anti-apoptotic, proangiogenic and invasive agent [43]. It has been reported that Galectin-3 promotes ocular inflammation and when knocked down decreases angiogenesis signaling pathways in human endothelial cells [44]. From these studies and the association of elevated expression of Galectin-3 and LAG3, we can assume that the interaction

between Galectin-3/LAG3 may take place in UM and may be a cause for the blunted activity of CD8 cells.

A third set of LAG3 ligands is made up of the HLA Class II antigens, which are expressed on tumour cells, antigen-presenting cells, and a subset of T cells. These heterodimer cell surface molecules consist of alpha and beta chains. The HLA antigens can present antigens to CD4 T cells through binding to their T cell receptors. HLA Class II molecules consist of three isotypes: DR, DQ, and DP which differ slightly in structure [45, 46]. Cutaneous melanoma cells express high levels of HLA Class II, which have been shown to inhibit T cell anti-tumoural activity through their LAG3 receptor [47]. In UM, HLA Class II is expressed in primary UM and carries prognostic significance [6]. In 1988, Jager et al. reported that HLA Class II is expressed in UM although less than HLA Class I. Our prior study showed that HLA-DQ was associated with increased numbers of infiltrating TILs [48]. When we compared expression levels between D3 and M3 tumours in the 64-case Leiden cohort, most Class II molecules were higher in high risk M3 tumours. We confirmed that HLA Class II expression was enhanced in M3 tumours by analysing the TCGA study and by testing a second Dutch cohort of fifteen UM using Fluidigm PCR. It has been reported that HLA-DR is the dominant isoform for antigen-restricted T cell stimulation with HLA-DQ contributing less in different diseases [49-51].

IFNγ is a strong inducer for HLA Class II molecules in UM [52]; we suggest a positive feedback loop between the tumour cells HLA Class II expression, and the infiltrate: genetically bad tumours with loss of one chromosome 3 and a mutation in the BAP1 gene upregulate surface LAG3 ligands (LSECtin, Galectin-3, and HLA Class II) to attract T cells which express LAG3. High risk M3 tumours not only contain more infiltrating lymphocytes and macrophages, but also express higher levels of immune check-point inhibitors: we show that LAG3 is co-expressed with other immune checkpoints (PD-1, CTLA-4, and IDO-1), suggesting that the combination of anti-checkpoint therapies might be more appropriate in UM than single anti-checkpoint antibodies, as others also find this appropriate in other diseases such as cutaneous melanoma [24].

5. Conclusions

Taken together, our data demonstrate elevated levels of LAG3 checkpoint expression in high risk M3 tumours and associations between LAG3 expression and bad prognosis. Moreover, we indicate that a positive association is found between expression of LAG3, its ligands, and other immune checkpoints. As increased levels of CD8 cells are present in the same tumours, suggesting that LAG3 could be considered as a potential silencer for CD8 T cell cytotoxicity in UM.

Author Contributions: Conceptualisation, Z.S., M.J.J.; methodology, Z.S., W.G.M.K., R.M.V., M.E; formal analysis, Z.S.; data curation, A.P.A.W.; writing—original draft preparation, Z.S. and M.J.J.; writing—review and editing, A.P.A.W., W.G.M.K., P.A.v.d.V., R.M.V., M.E, G.P.M.L. and M.J.J.; supervision, M.J.J.; funding acquisition, P.A.v.d.V., and M.J.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by a grant to Z.S. from The Iranian Ministry of Science Research and Technology, Grant UL 2011-4991 from the Dutch Cancer Society (KWF), Stichting Leids Oogheelkundig Onder-steunings Fonds LOOF and a Horizon 2020 grant from the European Community, CURE UM, nr. 667787.

Institutional Review Board Statement:

This project was approved by the METC of the LUMC (B14.003/SH/sh Approval Biobank OOG-2 “Oogtumoren (of een verdenking hierop)”, protocol Uveamelanoom-lab B20.026, approval June 2020). The research adhered to Dutch law and the tenets of the Declaration of Helsinki (World Medical Association of Declaration 2013; ethical principles for medical research involving human subjects).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Metzelaar-Blok, J.A.; ter Huurne, J.A.; Hurks, H.M.; et al. Characterization of melanocortin-1 receptor gene variants in uveal melanoma patients. Invest. Ophthalmol. Vis. Sci 2001, 42(9), 1951-1954.
2. Houtzagers, L.E.; Wierenga, A.P.A.; Ruys, A.A.M.; et al. Iris Colour and the Risk of Developing Uveal Melanoma. Int. J. Mol. Sci. 2020, 21(19), 7172-7189. DOI: 10.3390/ijms21197172.
3. Kujala, E.; Mäkitie, T., Kivelä, T. Very long-term prognosis of patients with malignant uveal melanoma. Invest. Ophthalmol. Vis. Sci. 2003, 44(11), 4651-4660. DOI: 10.1167/iovs.03-0538.
4. Roelofsen, C.D.M.; Wierenga, A.P.A.; van Duinen, S.; et al. Five decades of enucleations for uveal melanoma in one center: more tumours with high risk factors, no improvement in survival over time. Ocul. Oncol. Pathol. 2020, 7(2), 133-141. DOI: 10.1159/000509918
5. Blom, D.J.; Luyten, G.P.; Mooy, C.; et al. Human leukocyte antigen class I expression. Marker of poor prognosis in uveal melanoma. Invest. Ophthalmol. Vis. Sci. 1997, 38(9), 1865-1872.
6. Ericsson, C.; Seregard, S.; Bartolazzi, A.; et al. Association of HLA class I and class II antigen expression and mortality in uveal melanoma. Invest. Ophthalmol. Vis. Sci. 2001, 42(10), 2153-2156.
7. Maat, W.; Ly, L.V.; Jordanova, E.S.; et al. Monosomy of chromosome 3 and an inflammatory phenotype occur together in uveal melanoma. Invest. Ophthalmol. Vis. Sci. 2008, 49(2), 505-510. DOI: 10.1167/iovs.07-0786.
8. Bronkhorst, I.H.; Vu, T.K.; Jordanova, E.S.; et al. Different subsets of tumour-infiltrating lymphocytes correlate with macrophage influx and monosomy 3 in uveal melanoma. Invest. Ophthalmol. Vis. Sci. 2012, 53(9), 5370-5378. DOI: 10.1167/iovs.11-9280.
9. Robertson, A.G.; Shih, J.; Yau, C.; et al. Integrative analysis identifies four molecular and clinical subsets in Uveal Melanoma. Cancer Cell 2017, 32(2), 204-220. DOI: 10.1016/j.ccell.2017.07.003.
10. Niederkorn, J.Y. Immune escape mechanisms of intraocular tumours. Progr. Ret. Eye Res. 2009, 28(5), 329-347. DOI: 10.1016/j.preteyeres.2009.06.002.
11. Ly, L.V.; Baghat, A.; Versluis, M.; et al. In aged mice, outgrowth of intraocular melanoma depends on proangiogenic M2-type macrophages. J. Immunol. 2010, 185(6), 3481-3488. DOI: 10.4049/jimmunol.0903479
12. De Lange, M.J.; Nell, R.J.; Lalai, R.N.; et al. Digital PCR-Based T-cell Quantification-Assisted deconvolution of the microenvironment reveals that activated macrophages drive tumour inflammation in Uveal Melanoma. Mol. Cancer Res. 2018, 16(12), 1902-1911. DOI: 10.1158/1541-7786.MCR-18-0114.

13. Ishida, Y.; Agata, Y.; Shibahara, K.; et al. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. EMBO journal 1992, (11), 3887-3895.
14. Wierenga, A.P.; Cao, J.; Luyten, G.P.; et al. Immune checkpoint inhibitors in uveal and conjunctival melanoma. Int. Ophthalmol. Clin. 2019, 59(2), 53-63. DOI: 10.1097/IIO.0000000000000263.
15. Triebel, F.; Jitsukawa, S.; Baixeras, E.; et al. LAG-3, a novel lymphocyte activation gene closely related to CD4. J. Exp. Med. 1990, 171(5), 1393-1405.
16. Huard, B.; Mastrangeli, R.; Prigent, P.; et al. Characterization of the major histocompatibility complex class II binding site on LAG-3 protein. Proc. Natl. Acad. Sci. U S A 1997, 94(11), 5744-5749. DOI: 10.1084/jem.171.5.1393.
17. Maruhashi, T.; Okazaki, I.M.; Sugiura, D.; et al. LAG-3 inhibits the activation of CD4+ T cells that recognize stable pMHCII through its conformation-dependent recognition of pMHCII. Nat. Immunol. 2018, 19(12), 1415-1426. DOI: 10.1038/s41590-018-0217-9.
18. Andrews, L.P.; Marciscano, A.E.; Drake, C.G.; et al. LAG3 (CD223) as a cancer immunotherapy target. Immunol. Rev. 2017, 276(1), 80-96. DOI: 10.1111/imr.12519.
19. Woo, S.R.; Turnis, M.E.; Goldberg, M.V.; et al. Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoural immune escape. Cancer Res. 2012, 72(4), 917-927. DOI: 10.1158/0008-5472.CAN-11-1620.
20. Durante, M.A.; Rodriguez, D.A.; Kurtenbach, S.; et al. Single-cell analysis reveals new evolutionary complexity in uveal melanoma. Nat. Commun. 2020, 11(1), 1-10. DOI: 10.1038/s41467-019-14256-1.
21. Piulats, R.J.; De La Cruz, M.L.; Espinosa, E.; et al. Phase II multicenter, single arm, open label study of nivolumab in combination with ipilimumab in untreated patients with metastatic uveal melanoma. Ann. Oncology 2018, 29, viii443. doi:10.1093/annonc/mdy289.
22. Rozeman, E.A.; Prevoo, W.; Meier, M.A.; et al. Phase Ib/II trial testing combined radiofrequency ablation and ipilimumab in uveal melanoma (SECIRA-UM). Melanoma Res. 2020, 30(3), 252-260. DOI: 10.1097/CMR.0000000000000653.
23. Duhoux, F. P.; Dirix, L.Y.; Huizing, M.T. Combination of paclitaxel and a LAG-3 fusion protein (eftilagimod alpha), as a first-line chemoimmunotherapy in patients with metastatic breast carcinoma (MBC): final results from the run-in phase of a placebo-controlled randomized phase II. J. Clin. Oncol. 2018, 36 (15), 1050-1050. DOI: 10.1200/JCO.2018.36.15.
24. Ascierto, P.A.; Bhatia, S.; Bono, P.; et al. Initial efficacy of anti-lymphocyte activation gene-3 (anti–LAG-3; BMS-986016) in combination with nivolumab (nivo) in PTS with melanoma (MEL) previously

treated with anti–PD-1/PD-L1 therapy. J. Clin. Oncol. 2017, 35 (15), 9520-9520. DOI: 10.1200/JCO.2017.35.15.

25. Khan, S. and Carvajal, R.D. Dual Immunological Checkpoint Blockade for Uveal Melanoma. J. Clin. Oncol. 2021, 39 (6), 586-599. DOI: 10.1200/JCO.20.03274.
26. Versluis, M.; De Lange, M.J.; Van Pelt, S.I.; et al. Digital PCR Validates 8q Dosage as Prognostic Tool in Uveal Melanoma. PLoS ONE 2015, 10, e0116371. DOI: 10.1371/journal.pone.0116371.
27. Gezgin, G.; Dogrusöz, M., van Essen, T.H.; et al. Genetic evolution of uveal melanoma guides the development of an inflammatory microenvironment. Cancer Immunol. Immunother. 2017, 66(7), 903-912. DOI: 10.1007/s00262-017-1991-1.
28. Van Essen, T.; Van Pelt, S.I.; Bronkhorst, I.H.G.; et al. Upregulation of HLA Expression in Primary Uveal Melanoma by Infiltrating Leukocytes. PLoS ONE 2016, 11, e0164292. DOI: 10.1371/journal.pone.0164292.
29. Zoutman, W.H.; Nell, R.J.; Versluis, M.; et al. Accurate quantification of T cells by measuring loss of germline T-cell receptor loci with generic single duplex droplet digital PCR assays. J. Mol. Diagn. 2017, 19(2), 236-243. DOI: 10.1016/j.jmoldx.2016.10.006.
30. Wierenga, A.; Gezgin, G.; van Beelen, E.; et al. Soluble HLA in the aqueous humour of uveal melanoma is associated with unfavourable tumour characteristics. Cancers 2019, 11(8), 1202- 1213. DOI: 10.3390/cancers11081202.
31. Jochems, A.; van der Kooij, M.K.; Fiocco, M.; et al. Metastatic uveal melanoma: Treatment strategies and survival—results from the Dutch melanoma treatment registry. Cancers 2019, 11(7), 1007-1020. DOI: 10.3390/cancers11071007.
32. Jager, M.J.; Shields, C.L.; Cebulla. et al. Uveal melanoma. Nat. Rev. Disease Primers 2020, 6(1), 1-25. DOI: 10.1038/s41572-020-0158-0.
33. Rodrigues, M.; de Koning, L.; Coupland, S.E.; et al. So close, yet so far: discrepancies between uveal and other melanomas. A position paper from UM Cure 2020. Cancers 2019 11(7), 1032-1040. DOI: 10.3390/cancers11071032.
34. Eggermont, A.M.M.; Chiarion-Sileni, V.; Grob, J.J.; et al. Prolonged survival in Stage III melanoma with ipilimumab adjuvant therapy. N. Engl. J. Med. 2016, 375(19), 1845-1855. DOI: 10.1056/NEJMoa1611299.
35. Souri, Z.; Wierenga, A.; van Weeghel.; et al. Loss of BAP1 is associated with upregulation of the NFKB pathway and increased HLA class I expression in uveal melanoma. Cancers 2019, 11(8), 1102-1118. DOI: 10.3390/cancers11081102.

36. Yang, Z.Z.; Kim, H.J.; Villasboas, J.C.; et al. Expression of LAG-3 defines exhaustion of intratumoural PD-1+ T cells and correlates with poor outcome in follicular lymphoma. Oncotarget 2017, 8(37), 61425-61439. DOI: 10.18632/oncotarget.18251.

37. Hoefsmit, E.P.; Rozeman, E.A.; Van, T.M.; et al. Comprehensive analysis of cutaneous and uveal melanoma liver metastases. J. Immunother. Cancer 2020, 8(2), PMC7713183. DOI: 10.1136/jitc-2020-001501.

38. Liu, W.; Tang, L.; Zhang, G., et al. Characterization of a novel C-type lectin-like gene, LSECtin: demonstration of carbohydrate binding and expression in sinusoidal endothelial cells of liver and lymph node. J. Biol. Chem. 2004, 279(18), 18748–18758. DOI: 10.1074/jbc.M311227200.

39. Xu, F.; Liu, J.; Liu, D. et al. LSECtin expressed on melanoma cells promotes tumour progression by inhibiting antitumour T-cell responses. Cancer Res. 2014, 74(13), 3418-3428. DOI: 10.1158/0008-5472.CAN-13-2690.

40. Flotte, T. J., T. A. Springer, and G. J. Thorbecke. Dendritic cell and macrophage staining by monoclonal antibodies in tissue sections and epidermal sheets. Am. J. Pathol. 1983, 111(1), 111-112.

41. Kouo, T.; Huang, L.; Pucsek, A.B; etal. Galectin-3 shapes antitumour immune responses by suppressing CD8+ T cells via LAG-3 and inhibiting expansion of plasmacytoid dendritic cells. Cancer Immune Res. 2015, 3(4), 412-423. DOI: 10.1158/2326-6066.CIR-14-0150.

42. Prieto, V.G.; Mourad-Zeidan, A.A.; Melnikova, V.; et al. Galectin-3 expression is associated with tumour progression and pattern of sun exposure in melanoma. Clin. Cancer Res. 2006, 12(22), 6709-6715. DOI: 10.1158/1078-0432.CCR-06-0758.

43. Wang, Y.; Liu, S.; Tian, Y.; et al. Prognostic role of galectin-3 expression in patients with solid tumours: a meta-analysis of 36 eligible studies. Cancer Cell Internat. 2018, 18(1), 1-5. DOI: 10.1186/s12935-018-0668-y.

44. Markowska, A.I.; Jefferies, K.C.; Panjwani, N. Galectin-3 protein modulates cell surface expression and activation of vascular endothelial growth factor receptor 2 in human endothelial cells. J. Biol. Chem. 2011, (34), 29913-29921. DOI: 10.1074/jbc.M111.226423.

45. Korman, A.J.; Boss, J.M.; Spies, T.; et al. Genetic complexity and expression of human class II histocompatibility antigens. Immunol. Rev. 1985, 85, 45-86. DOI: 10.1111/j.1600-065x.1985.tb01130.x.

46. Trowsdale, J.; Young, J.A.; Kelly, A.P.; et al. Structure, sequence and polymorphism in the HLA-D region. Immunol. Rev. 1985, 85, 5-43. DOI: 10.1111/j.1600-065x.1985.tb01129.x.

47. Hemon, P.; Jean-Louis, F.; Ramgolam, K.; et al. MHC Class II Engagement by Its Ligand LAG-3 (CD223) Contributes to Melanoma Resistance to Apoptosis. J. Immunol. 2011, 186(9), 5173-5183. DOI: 10.4049/jimmunol.1002050.
48. Jager, M.J.; van der Pol, J.P.; de Wolff-Rouendaal, D.; et al. Decreased expression of HLA class II antigens on human uveal melanoma cells after in vivo X-ray irradiation. Am. J. Ophthalmol. 1988, 105(1), 78-86. DOI: 10.1016/0002-9394(88)90125-0.
49. Karp, D.R.; Teletski, C.L.; Jaraquemada, D.; et al. Structural requirements for pairing of alpha and beta chains in HLA-DR and HLA-DP molecules. J. Exp. Med 1990, 171(3), 615-628. DOI: 10.1084/jem.171.3.615.
50. Ulvestad, E.; Williams, K.; Bø, L.; et al. HLA class II molecules (HLA-DR,-DP,-DQ) on cells in the human CNS studied in situ and in vitro. Immunology 1994, 82(4), 535-541.
51. Grifoni, A.; Moore, E.; Voic, H.; et al. Characterization of magnitude and antigen specificity of HLA-DP, DQ, and DRB3/4/5 restricted DENV-specific CD4+ T cell responses. Front. Immunology 2019, 10, 1568-1578. DOI: 10.3389/fimmu.2019.01568.
52. De Waard-Siebinga, I.; Creyghton, W.M.; Kool, J.; et al. Effects of interferon alfa and gamma on human uveal melanoma cells in vitro. Br. J. Ophthalmol. 1995, 79(9), 847-855. DOI: 10.1136/bjo.79.9.84.

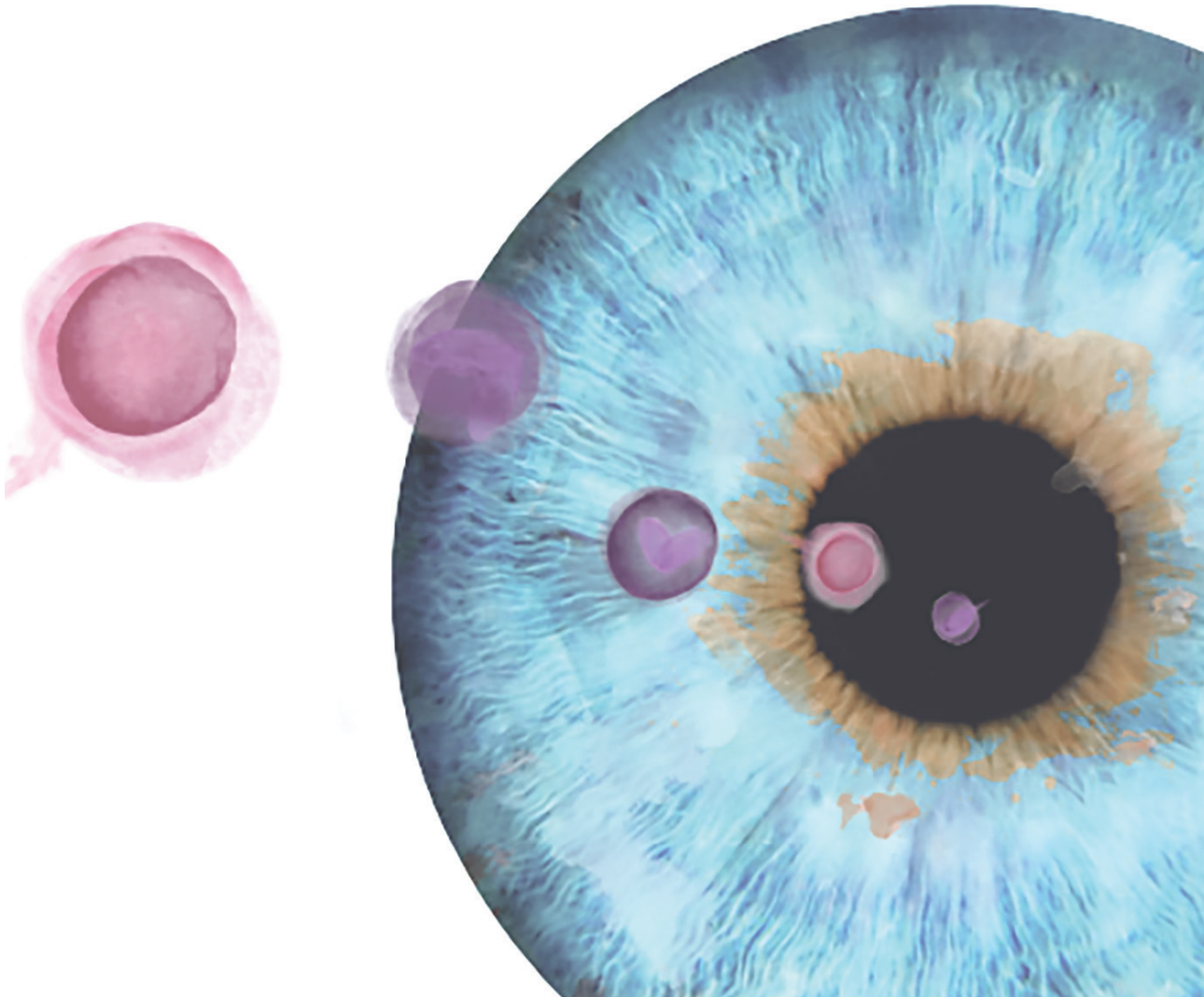
Supplementary Table 1. Correlation between mRNA expression levels (determined by Illumina array) of LSECtin, Galectin-3, HLA Class II genes and infiltrate markers versus expression of LAG3 in: A. the Leiden cohort (n=64), and B. the TCGA cohort (n = 80). R = two-tailed Spearman correlation coefficient.

A. Leiden cohort	LAG3	
	R	***p***
LSECtin	.428	<0.001
Galectin-3	.672	<0.001
HLA-DRalpha-pr1	.633	<0.001
HLA-DRalpha-pr2	.669	<0.001
HLA-DQalpha1	.607	<0.001
HLA-DQbeta2	.735	<0.001
HLA-DPalpha1	.619	<0.001
CD3	.727	<0.001
CD4	.596	<0.001
CD8	.832	<0.001
CD68	.542	<0.001
CD163	.485	<0.001
HLA-A-pr1	.712	<0.001
HLA-A-pr2	.731	<0.001
HLA-B	.791	<0.001
PD-1	.655	<0.001
CTLA-4	.298	0.02
IDO-1	.759	<0.001
TIGIT-pr1	.413	.001
TIGIT-pr2	-.207	.10

B. TCGA cohort	LAG3	
	R	***p***
LSECtin	.579	<0.001
Galectin-3	.666	<0.001
HLA-DR-alpha	.780	<0.001
HLA-DQalpha1	.787	<0.001
HLA-DQbeta2	.665	<0.001
HLA-DPalpha1	.805	<0.001
CD3	.886	<0.001
CD4	.609	<0.001
CD8	.846	<0.001
CD68	.301	.007
CD163	.638	<0.001
HLA-A	.773	<0.001
HLA-B	.796	<0.001
PD-1	.878	<0.001
CTLA-4	.690	<0.001
IDO-1	.838	<0.001
TIGIT	.831	<0.001

Chapter 8

Summary and discussion



Summary and discussion

While Uveal Melanoma (UM) is quite rare, it is often a deadly disease, with 50% of UM patients developing metastases (Augsburger 2008). The increased risk of metastases is associated with loss of one chromosome 3 and a mutation in the BAP1 gene in the tumour cells (Harbour 2010), while other genetic factors such as additional copies of chromosome 8q or mutations in the SF3B1 gene also play a role (Versluis 2015, Yavuzyigitoglu 2016). Loss of one chromosome 3/BAP1 expression in UM is associated with the presence of an inflammatory phenotype, although the mechanism that leads to inflammation has not yet been properly identified (Robertson 2017, Gezgin 2017). The survival rate of UM has not improved over the last five decades (Roelofsen 2021), and even the introduction of the new immune checkpoint inhibitors has not led to improved survival of UM patients (Rodrigues 2019).

The purpose of this thesis is to increase insight into the regulation of inflammation in UM. We study the main features of the inflammatory phenotype in UM (the presence of tumour-infiltrating lymphocytes and macrophages and the expression of HLA antigens), analysing the relation of these factors with the NFkB system, epigenetic HDAC regulators and miRNAs, and finally the relation with the LAG3 immune checkpoint molecule.

A comprehensive overview regarding the HLA system in UM showed that the level of HLA expression is determined by tumour genetics (**chapter 2**). HLA molecules are cell surface glycoproteins that present antigens to the immune system. This molecular mechanism can be hijacked by tumour cells and expression of HLA molecules downregulated to avoid T cell recognition. While specific HLA antigens may be downregulated, the overall expression of HLA antigens in UM may be upregulated: a high expression of HLA Class I and II is associated with high-risk tumour characteristics (de Waard-Siebinga 1996, Blom 1997, Ericsson 2001).

As already shown by Maat in 2008, upregulation of backbone HLA Class I is seen in tumours with loss of chromosome 3 (Maat 2008). However, UM cells might alter the expression of specific alleles or loci, which helps to avoid T cell immunity. This could take place at different levels such as genetic haplotype loss, individual allele loss and complete HLA-A or HLA-B loss (Hurks 2000, Anastassiou 2003). When investigating the expression of individual HLA-A and HLA-B alleles in

UM cell lines, we found defects in HLA-B locus which could not be revived by IFNɤ, although a genetic analysis showed the presence of the specific genes. This confirmed earlier results (de Waard-Siebinga 1995, Hurks 2000).

Following our review of the published literature about HLA expression as an inducer of metastasis in UM, we next investigated the possible role of the NFkB pathway in the inflammation of high-risk UM (**chapter 3**). NFkB is a transcription factor activated by inflammatory cytokines which, upon activation, is known to enhance the expression of proliferative, anti-apoptotic and pro-inflammatory downstream genes, affecting cancer cells at an early stage (as reviewed by Taniguchi 2018).

Using data from an Illumina mRNA array and long-term follow-up, we analyzed the mRNA expression of inflammatory markers in 64 primary UM samples. We found that components of the NFkB pathway are positively associated with the expression of HLA-A, HLA-B, major HLA Class I regulators including IRF1 and IRF8, and components of the antigen-processing machinery TAP1 and TAP2. Moreover, we highlighted the fact that the NFkB pathway is associated with infiltrating inflammatory cells, and with the tumour's chromosome/mutation status: high levels of HLA-A, HLA-B, NFkB1 and NFkB2 expression were associated with loss of one chromosome 3 and loss of expression of BAP1, which characterizes high-risk tumours. It may be that upon chromosome 3 loss combined with a BAP1 mutation, the inflammatory pathway loses its normal regulation. It is important to consider the possible role of PPAR-ɤ in NFkB regulation: this gene is located on chromosome 3 and may therefore be influenced by chromosome 3 loss. The reason for the high expression of NFkB pathway components in M3 tumours might be explained by insufficient negative regulation provided by PPAR-ɤ.

Inflammatory cytokines such as TNF-α, IL-1 and IL-17 can upregulate NFkB in colon cancer (Terzic 2010); here in UM, similarly, the inflammatory cytokines might contribute to the upregulation of this signalling pathway in the tumour cell which in turn can lead to the upregulation of HLA Class I and secretion of chemoattractants, thereby further enhancing local inflammation.

In addition to genetic aberrations, epigenetic changes may also alter gene expression. Identifying epigenetic alterations in high-risk UM is very important as the effects could be reversed by certain

drugs, and specific changes have been associated with the risk of metastases development (Robertson 2017). Different types of epigenetic regulation have been observed in the development of different cancers such as cutaneous melanoma, with DNA methyltransferases and Histone deacetylases playing a major role (Sigalotti 2010).

In **chapter 4**, we analysed the distribution of a wide range of histone deacetylase (HDAC) enzymes and a histone methyltransferase, enhancer of zeste homolog 2 (EZH2), and compared the level of their expression between low and high-risk UM tumours (disomy 3 = D3 vs monosomy 3 = M3 tumours). Some HDACs (HDAC1, HDAC3, HDAC4, and HDAC8) were higher while HDAC11 was lower in M3 compared to D3 tumours. The high expression of HDACs in high-risk UM tumours could be a possible mechanism for the enhancement of inflammation since HDAC enzymes have been shown to play a stimulating role in NFkB and IFNɣ signalling (Shakespear 2011). The association between a low expression of HDAC11 and M3 can be explained by the location of the HDAC11 gene on chromosome 3.

Because we found that HDACs are expressed in UM and are co-expressed with HLA Class I, we focused on the potential use of a combination of immunotherapy and anti-HDAC chemotherapy. To take an initial step towards this idea, we performed in-vitro studies using the HDAC inhibitor Quisinostat, which reduced cell growth in all three tested UM cell lines (Heijkants 2017). Although Quisinostat reduced UM cell growth, it also modified the expression of HLA Class I gene expression, increasing it both at the protein and mRNA level. The upregulation of HLA Class I is possibly due to the drugs ability to open the chromatin structure by inhibiting de-acetylation and increasing gene expression. This could have consequences: if the metastasis has already developed and reached the liver, the use of this drug could enhance HLA Class I expression and thereby T cell-mediated lysis; if only the primary tumour is present, this drug might contribute to the progression of the disease by further upregulation of HLA Class I, which may block NK cell-mediated lysis in the blood.

As we observed that especially high-risk UM showed a high expression of different epigenetic HDAC enzymes, together with upregulation of HLA Class I, we wondered whether HDAC expression is also associated with the presence of infiltrating leukocytes. Indeed, the expression of some of the HDACs (1, 3 and 8) was related to the presence of lymphocytes (**chapter 5**).

These HDACs were subsequently identified as being susceptible to upregulation by IFNɣ. This also explains the positive correlation observed between several HDACs and the loss of one copy of chromosome 3: it is likely that not only HLA expression but also expression of these HDACs is stimulated by the secretion of inflammatory cytokines.

Not only HDACs are involved in epigenetic regulation, but there are many other regulators as well. In **chapter 6** we focused on a set of non-translational RNAs named miRNAs as epigenetic regulators. MiRNAs have been shown to be involved in the progression of UM (reviewed by Smit 2019). We looked at the distribution of 125 miRNAs for which we had Illumina array expression information from 64 UM and investigated their association with different factors related to the inflammatory characteristics in UM. Since we know from previous studies that elevated HLA Class I expression is related to a bad prognosis and chromosome 3 loss, we wondered whether we could find any associations between miRNA expression and HLA-A and HLA-B expression levels. We found that among the 125 miRNAs, 24 showed significant associations: four miRNAs (22, 155, 635, and 1276) were positively correlated with HLA-A and HLA-B expression, while the other 21 miRNAs showed a negative correlation. Moreover, miRNAs with a positive association with HLA expression were often upregulated in M3 UM, while the opposite pattern existed for many of the downregulated miRNAs.

Loss of chromosome 3 together with a mutation in the BAP1 gene probably affects the normal miRNA regulatory network: the upregulated miRNAs might be involved in silencing tumour suppressors and act as positive regulators of inflammatory pathways (Onco-miRs) while the miRNAs which are lower in M3 UM may have an opposite role (a tumour-suppressive role).

It has been reported that inflammation can modify miRNA expression: the level of mir-192 expression decreased in the inflammatory disease ulcerative colitis; the identified target for this miRNA was the inflammatory chemokine, macrophage inhibitory peptide (MIP)-2α (Wu 2008).

Despite the presence of immune cells in UM, the local immune responses seem to be ineffective. In order to mount an anti-tumour action, it is very important to increase the knowledge on how immune cells and especially CD8 T cells are regulated. The immune checkpoint Lymphocyte activation gene-3 (LAG3) has recently been shown to be highly expressed in UM (Durante 2020).

Immune checkpoints are cell surface inhibitory receptors that normally control autoimmune reactions in the body.

In **chapter 7**, we studied LAG3 expression in 64 primary UM samples from the Leiden cohort and verified our results using an independent cohort of another set of 15 tumours from Leiden and 80 samples from the TCGA cohort. With the knowledge that loss of chromosome 3 contributes to inflammation, we hypothesized that this cell surface inhibitory receptor would be especially expressed in M3 tumours and therefore could be used as a target for anti-immune checkpoint therapy. We found that elevated levels of LAG3 are associated with the presence of prognostically-bad markers such as an epithelioid cell type, loss of chromosome 3 and loss of BAP1 expression. Positive associations were found between LAG3 and the presence of tumour-infiltrating immune cells.

LSECtin, HLA Class II and Galectin-3 are ligands of LAG3. We found that HLA Class II molecules are highly correlated with LAG3. In addition to HLA Class I, HLA Class II expression is also linked to higher numbers of infiltrating T cells (Jager 1988) and metastasis formation (Ericsson 2001, Krishnakumar 2003). In addition to HLA Class II molecules, we also found Galectin-3 to have an increased expression in M3 tumours. This lectin has been shown to decrease the cytotoxic activity of CD8 cells (Kouo 2015).

It has been proposed that one of the mechanisms behind the exhaustion of CD8 cells is the upregulation of LAG3 ligands on the tumour, attracting LAG3-positive infiltrating lymphocytes into the tumour microenvironment and transmitting inhibitory signals to them (Durante 2020, Figueiredo 2020).

As especially high-risk UM carry high numbers of tumour-infiltrating lymphocytes (TILs) and tumour-associated macrophages (TAM), the high LAG3 distribution in these tumours suggests a potential benefit of anti-LAG3 monoclonal antibodies as adjuvant treatment in patients with high-risk UM.

Conclusions and future perspective

In this thesis, we focus on the role of different factors that may enhance the inflammatory phenotype in UM and we investigate how an anti-tumour immune attack might be disabled in this disease. We aim to increase the knowledge of the pathogenesis of inflammation in order to define possible new therapeutic targets.

Inflammation is a protective strategy of the body which normally shuts down after healing a wound. One-fourth of all cancers are caused by chronic inflammation (as reviewed by Coussens 2002); chronic inflammation fails to heal the tumour (Dvorak 1986). Inflammation is associated with the development of metastasis in UM. In recent years, different studies have identified several genetic prognostic markers highly associated with inflammation, including chromosome aberrations such as loss of chromosome 3, extra copies of chromosome 8q gain, as well as mutations, causing loss of BAP1 expression. This information classifies tumours into different risk subtypes and could help to identify patients who might be candidates for neoadjuvant trials and frequent screening. It is important to increase knowledge concerning different molecular factors related to these genetic abnormalities to understand why inflammation occurs in this disease.

Our results show that many different mediators are collectively dysregulated in inflamed tumours; upregulation of HLA-Class I was associated with upregulation of NFkB pathway components, with upregulation of a specific group of HDACs and with several specific miRNAs. All of these are upregulated in high-risk M3/BAP1-negative tumours. An exception is HDAC11, which is located on chromosome 3, and is downregulated in tumours with loss of chromosome 3.

The deregulation observed in different epigenetic modulators and the NFkB pathway might be connected: for example, the inhibition of HDAC enzymes in non-small cell lung cancer reduced NFkB transcriptional activity (Imre 2006); moreover, mir-155 which we found to be increased in M3 tumours with positive correlation with HLA Class I, is shown to be up-regulated by NFkB in lung cancer (Chiu 2016).

Based on the results of this, we hypothesise that loss of chromosome 3/BAP1 expression induces HLA expression through upregulation of the NFkB pathway and epigenetic reprogramming. The latter involves HDACs and miRNA to recruit infiltrating macrophages and lymphocytes to the

microenvironment, exhausting their cytotoxic activity via LAG3/HLA Class II or Galectin-3 interaction and at the same time using immune cells as a source of growth factors. This leads to more inflammation in the microenvironment and increases angiogenesis and subsequently metastatic spreading.

One central regulator may be the presence of ischemia. Jehs (Jehs 2014) showed that stimulation of UM cell lines led to the secretion of monocyte-attracting cytokines, while Bronkhorst proposed that it was the presence of ischemia which led to the production of pro-inflammatory cytokines (Bronkhorst 2014). Brouwer (Brower 2019) recently showed that ischemia is seen more often in UM with M3/loss of BAP1. It is interesting to see that drugs that interfere with a very important ischemia-induced cytokine, namely HIF-1 alpha, are being developed and tested for UM (Dong 2019).

Many questions remain, such as the question of whether chromosome 3/BAP1 loss and subsequent increases in HLA Class I expression on the tumour cells lead to the attraction of immune cells. Or is it the inflammatory microenvironment that upregulates HLA Class I expression and enhances genomic instability in UM? Understanding the main reason for the presence of immune cells in the eye is critical to understand the pathogenesis of this disease to be able to design proper anti-inflammatory strategies.

We hope our attempts have been useful towards the understanding of the regulation of inflammation in uveal melanoma and that it will benefit survival of UM patients.

References

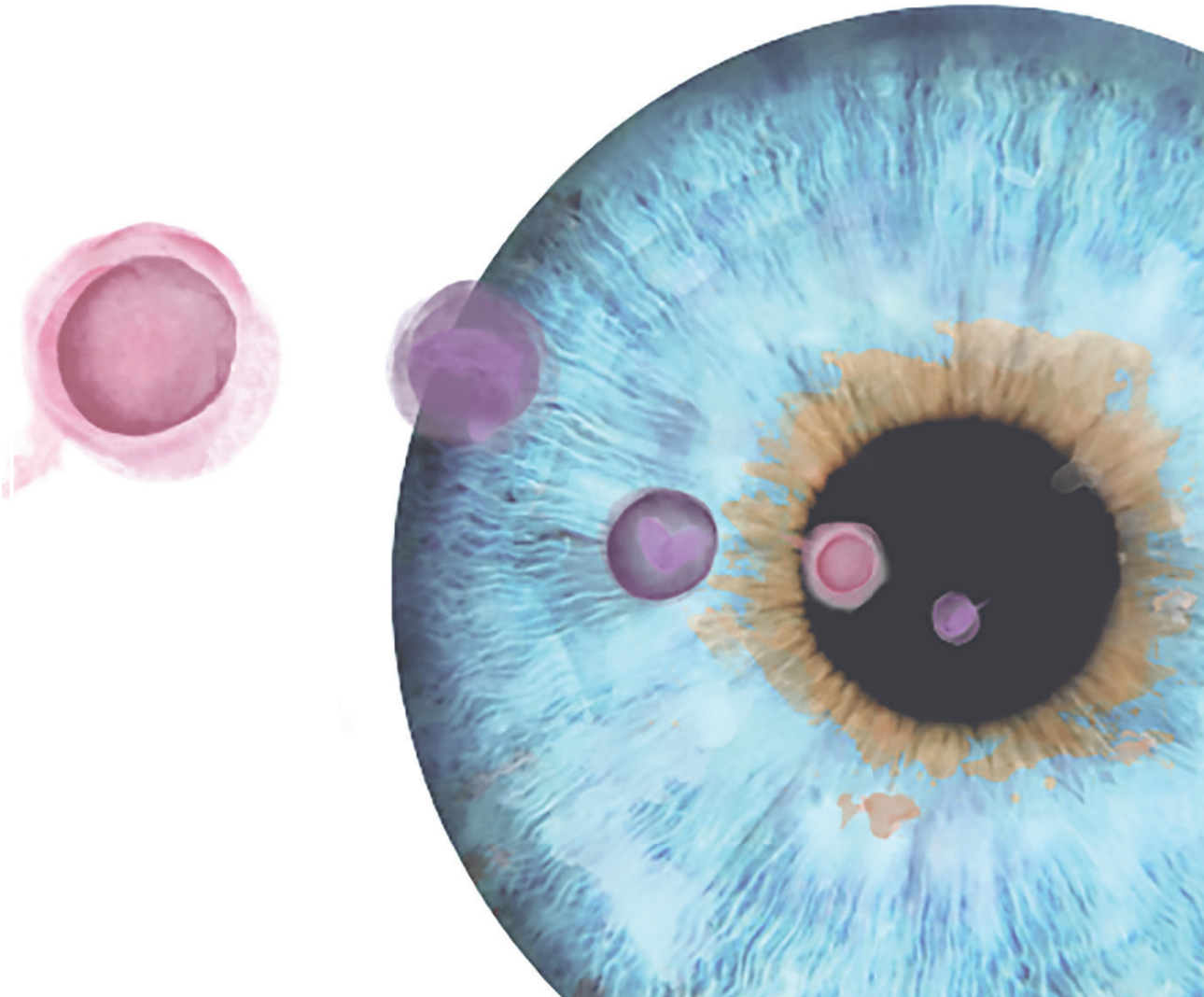
1. Augsburger, J.J.; Corrêa, Z.M.; Shaikh, A.H. Quality of evidence about effectiveness of treatments for metastatic uveal melanoma. Trans. Am. Ophthalmol. Soc. 2008, 106, 128-137.
2. Harbour, J.W.; Onken, M.D.; Roberson, E.D.; et al. Frequent mutation in BAP1 in metastasizing uveal melanomas. Science 2010, 330 (6009) 1410-1413.
3. Versluis, M.; de Lange, M.J.; van Pelt, S.I.; et al. Digital PCR validates 8q dosage as prognostic tool in uveal melanoma. PLoS One 2015, 10(3), e0116371.
4. Yavuzyigitoglu, S.; Koopmans, A.E.; Verdijk, R.M.; et al; Rotterdam Ocular Melanoma Study Group. Uveal Melanomas with SF3B1 Mutations: A distinct subclass associated with late-onset metastases. Ophthalmology 2016, 123(5), 1118-1128.
5. Robertson, A.G.; Shih, J.; Yau, C.; et al. Integrative analysis identifies four molecular and clinical subsets in uveal melanoma. Cancer Cell 2017, 32, 204-220
6. Gezgin, G.; Dogrusöz, M.; van Essen, T.H.; et al. Genetic evolution of uveal melanoma guides the development of an inflammatory microenvironment. Cancer Immunol. Immunother. 2017, 66, 903-912.
7. Roelofsen, C.D.M.; Wierenga, A.P.A.; van Duinen, S.; et al. Five Decades of Enucleations for Uveal Melanoma in One Center: More Tumors with High Risk Factors, No Improvement in Survival over Time. Ocul. Oncol. Pathol. 2021, 7(2), 133-141.
8. Rodrigues, M.; Koning, L.; Coupland, S.E.; et al. UM Cure 2020 Consortium. So close, yet so far: Discrepancies between Uveal and other Melanomas. A position paper from UM Cure 2020. Cancers (Basel). 2019, 11(7), 1032.
9. De Waard-Siebinga, I.; Houbiers, J.G.; Hilders, C.G.; et al. Differential expression of HLA-A and B alleles on uveal melanoma as determined by immuno-histology. Ocul. Immunol. Inflamm. 1996 4, 1-14.
10. Blom, D.J.; Luyten, G.P.; Mooy, C.; et al. Human Leukocyte Antigen class I expression. Marker of poor prognosis in uveal melanoma. Investig. Ophthalmol. Vis. Sci. 1997, 38, 1865-1872.
11. Ericsson, C.; Seregard, S.; Bartolazzi, A.; et al. Association of HLA class I and class II antigen expression and mortality in uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2001 42, 2153-2156.
12. Maat, W.; Ly, L.V.; Jordanova, E.S.; et al. Monosomy of chromosome 3 and an inflammatory phenotype occur together in uveal melanoma. Invest. Ophthalmol. Vis. Sci. 2008, 49(2), 505-510.

13. Hurks, H.M.H.; Metzelaar-Blok, J.A.W..; Mulder, A.; et al. High frequency of allele-specific down-regulation of HLA Class I expression in uveal melanoma cell lines. Int. J. Cancer 2000, 85, 697-702.
14. Anastassiou, G.; Rebmann, V.; Wagner, S.; et al. Expression of classic and non-classic HLA class I antigens in uveal melanoma. Investig. Ophthalmol. Vis. Sci 2003, 44, 2016-2019.
15. De Waard-Siebinga, I.; Creyghton, W.M.; Kool, J.; Jager, M.J. Effects of interferon alfa and gamma on human uveal melanoma cells in vitro. Br. J. Ophthalmol. 1995, 79, 847-855.
16. Taniguchi, K.; Karin, M. NF-κB, inflammation, immunity and cancer: coming of age. Nature Rev. Immunol. 2018, 18(5), 309-324.
17. Terzic, J.; Grivennikov, S.; Karin, E.; et al. Inflammation and colon cancer. Gastroenterology. 2010, 138, 2101-2114.
18. Sigalotti, L.; Covre, A.; Fratta, E.; et al. Epigenetics of human cutaneous melanoma: setting the stage for new therapeutic strategies. J Translat. Med. 2010, 8(1), 1-22.
19. Shakespear, M.R.; Halili, M.A.; Irvine, K.M.; et al. Histone deacetylases as regulators of inflammation and immunity. Trends Immunol. 2011, 32, 335-343.
20. Heijkants, R.; Willekens, K.; Schoonderwoerd, M.; et al. Combined inhibition of CDK and HDAC as a promising therapeutic strategy for both cutaneous and uveal metastatic melanoma. Oncotarget 2017, 9(5), 6174-6187.
21. Smit, K.N.; Chang, J.; Derks, K.; et al. Aberrant MicroRNA expression and its implications for uveal melanoma metastasis. Cancers 2019, 11(6), 815-829.
22. Wu, F.; Zikusoka, M.; Trindade, A.; et al. MicroRNAs are differentially expressed in ulcerative colitis and alter expression of macrophage inflammatory peptide-2α. Gastroenterology. 2008, 135(5), 1624-1635.
23. Durante, M.A.; Rodriguez, D.A.; Kurtenbach, S.; et al. Single-cell analysis reveals new evolutionary complexity in uveal melanoma. Nature Commun. 2020, 11(1), 1-10.
24. Jager, M.J.; van der Pol, J.P.; de Wolff-Rouendaal, D.; et al. Decreased expression of HLA class II antigens on human uveal melanoma cells after in vivo X-ray irradiation. American Journal Ophthalmology 1988, 105(1), 78-86.
25. Krishnakumar, S.; Abhyankar, D.; Lakshmi, S.A.; et al. HLA class II antigen expression in uveal melanoma: Correlation with clinicopathological features. Exp. Eye Res. 2003, 77,175-180.
26. Kouo, T.; Huang, L.; Pucsek, A.B.; et al. Galectin-3 shapes antitumor immune responses by suppressing CD8+ T cells via LAG-3 and inhibiting expansion of plasmacytoid dendritic cells. Cancer Immunol. Res. 2015, 3, 412-423.

27. Figueiredo, C.R.; Kalirai, H.; Sacco, J.J.; et al. Loss of BAP1 expression is associated with an immunosuppressive microenvironment in uveal melanoma, with implications for immunotherapy development. J. Pathol. 2020, 250(4), 420-439.
28. Coussens, L.M.; Werb, Z. Inflammation and cancer. Nature 2002,420 (6917), 860-867.
29. Dvorak, H.F. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N. Engl. J. Med. 1986, 315 (26), 1650-1659.
30. Imre, G.; Gekeler, V.; Leja, A.; et al. Histone deacetylase inhibitors suppress the inducibility of nuclear factor-κB by tumor necrosis factor-α receptor-1 down-regulation. Cancer Res. 2006, 66(10), 5409-5418.
31. Chiu, C.F.; Chang, Y.W.; Kuo, K.T.; et al. NF-κB-driven suppression of FOXO3a contributes to EGFR mutation-independent gefitinib resistance. Proc. Natl. Acad. Sci. USA 2016, 13(18), 2526-2535.
32. Jehs, T.; Faber, C.; Juel, H.B.; et al. Inflammation-induced chemokine expression in uveal melanoma cell lines stimulates monocyte chemotaxis. Invest. Ophthalmol. Vis. Sci. 2014, 55(8), 5169-5175.
33. Bronkhorst, I.H.G.; Jehs, T.M.L.; Dijkgraaf, E.M.; et al. Effect of hypoxic stress on migration and characteristics of monocytes in uveal melanoma. JAMA Ophthalmol. 2014, 132 (5), 614-621.
34. Brouwer, N.J.; Wierenga, A.P.A.; Gezgin, G.; et al. et al. Ischemia is related to tumour genetics in uveal melanoma. Cancers. 2019, 11, 1004.
35. Dong, L.; You, S.; Zhang, Q.; et al. Arylsulfonamide 64B inhibits hypoxia/HIF-induced expression of c-Met and CXCR4 and reduces primary tumor growth and metastasis of uveal melanoma. Clin. Canc. Res. 2019, 25(7), 2206-2218.

Chapter 8

Samenvatting en discussie (Nederlandse)



Samenvatting en discussie (Nederlandse)

Hoewel het oogmelanoom (uveaal melanoom, afgekort UM) vrij zeldzaam is, is het vaak een dodelijke ziekte, waarbij 50% van de UMpatiënten metastasen zal ontwikkelen (Augsburger 2008). Het verhoogde risico op uitzaaiingen gaat vaak samen met verlies van één kopie van chromosoom 3 met tegelijkertijd een mutatie in het *BAP1* gen in de tumorcellen (Harbour 2010). Daarnaast spelen andere genetische factoren, zoals extra kopieën van chromosoom 8q of mutaties in bv. het SF3B1-gen, ook een rol (Versluis 2015, Yavuzyigitoglu 2016). Verschillende onderzoeken hebben aangetoond dat verlies van één kopie van chromosoom 3/BAP1 expressie in UM geassocieerd is met ontsteking in de tumor, hoewel het mechanisme dat leidt tot een inflammatoir fenotype nog niet is geïdentificeerd (Robertson 2017, Gezgin 2017). De overlevingskans van de UM patiënt is de afgelopen vijf decennia niet verbeterd (Roelofsen 2021) en zelfs de introductie van de nieuwe immuuncheckpoint remmers heeft niet geleid tot een betere overleving van UM patiënten (Rodrigues 2019).

Het doel van dit proefschrift is om meer inzicht te krijgen in de regulatie van ontstekingen in UM. We bestudeerden de belangrijkste kenmerken van het inflammatoire fenotype in UM (de tumor-infiltrerende lymfocyten en macrofagen en de expressie van HLA antigenen) en onderzochten de relatie met oa. het NFkB systeem, epigenetische regulatoren (HDACs) en miRNA's, en tenslotte met checkpoint inhibitor LAG3.

In een uitgebreid overzicht van het HLA systeem in UM laten wij zien dat het niveau van HLA expressie wordt bepaald door tumorgenetica **(hoofdstuk 2)**. HLA moleculen zijn glycoproteïnen op het celoppervlak die antigenen aan het immuunsysteem presenteren. Dit moleculaire mechanisme kan door tumorcellen worden gekaapt om herkenning door T cellen te voorkomen. Hierdoor kan de expressie van specifieke HLA antigenen ontbreken. Bij een ontstekingsfenotype van het UM is echter de algehele expressie van HLA antigenen vaak verhoogd: een hoge expressie van HLA-klasse I en II wordt geassocieerd met tumorkenmerken met een hoog risico op uitzaaiing (de Waard-Siebinga 1996, Blom 1997, Ericsson 2001).

Zoals Maat al in 2008 liet zien, wordt opregulatie van HLA Klasse I gezien bij tumoren met verlies van één kopie van chromosoom 3 (Maat 2008). Er kan echter verlies van specifieke allelen of loci

optreden. Dit kan op verschillende niveaus plaatsvinden: verlies van het gehele haplotype, individueel allelverlies of volledig HLA-A of HLA-B verlies op gen niveau (Hurks 2000, Anastassiou 2003). Bij het onderzoeken van de expressie van afzonderlijke HLA-A- en HLA-B allelen in UM cellijnen vonden we defecten in het HLA-B locus die niet door toevoeging van Interferonγ ongedaan konden worden gemaakt, hoewel een genetische analyse de aanwezigheid van de specifieke genen wel aantoonde. Dit bevestigde eerder door onze groep gevonden resultaten (de Waard-Siebinga 1995, Hurks 2000), en verdient meer onderzoek.

Na het bestuderen van de gepubliceerde literatuur over HLA expressie in relatie tot metastasering bij UM, onderzochten we de mogelijke rol van de NFkB route in de ontsteking van UM **(hoofdstuk 3)**. NFkB is een transcriptiefactor welke wordt geactiveerd door inflammatoire cytokinen waarvan bekend is dat ze, na activering, de expressie van proliferatieve, anti-apoptotische en pro-inflammatoire genen verbetert, waardoor kankercellen in een vroeg stadium worden gestimuleerd (zoals beschreven door Taniguchi 2018).

Met behulp van data van een Illumina mRNA array, in combinatie met lange follow-up, analyseerden we de mRNA expressie van inflammatoire markers in stukjes weefsel van 64 primaire UM. We ontdekten dat activatie van de NFkB route samengaat met expressie van HLA-A, HLA-B, belangrijke HLA Klasse I regulatoren, waaronder IRF1 en IRF8, en componenten van de antigeenverwerkingsmachines TAP1 en TAP2. Bovendien benadrukten we het feit dat de NFkB-route geassocieerd is met de aanwezigheid van infiltrerende ontstekingscellen en met de chromosomale en mutatiestatus van de tumor: hoge niveaus van HLA-A, HLA-B, NFkB1 en NFkB2 expressie waren geassocieerd met verlies van één kopie van chromosoom 3 en verlies van BAP1 expressie, d.w.z. in de categorie van tumoren met een hoog risico. Bij het verlies van één kopie van chromosoom 3 in combinatie met een BAP1 mutatie in het resterende chromosoom 3 kan deze ontstekingsroute zijn normale regulatie verliezen. Het is nutig om de mogelijke rol van PPAR-γ in NFkB regulatie te overwegen: dit gen bevindt zich op chromosoom 3 en kan daarom worden beïnvloed door chromosoom 3 verlies. De reden voor de hoge expressie van NFkB pathway componenten in UM met monosomie 3 zou kunnen worden verklaard door de onvoldoende negatieve regulatie door PPAR-γ.

Inflammatoire cytokinen zoals TNF-α , IL-1 en IL-17 kunnen NFkB bij darmkanker opreguleren (Terzic 2010); in UM kunnen de inflammatoire cytokinen op vergelijkbare wijze bijdragen aan de opregulatie van deze signaalroute in de tumorcel, wat vervolgens kan leiden tot opregulatie van HLA-klasse I en de afscheiding van andere cytokines, waardoor een lokale ontsteking verder wordt versterkt.

Naast genetische afwijkingen kunnen ook epigenetische veranderingen de genexpressie veranderen. Het identificeren van epigenetische veranderingen in UM met een hoog risico is erg belangrijk omdat deze effecten door bepaalde medicijnen teruggedraaid zouden kunnen worden. Specifieke epigenetische patronen zijn in verband gebracht met het risico op de ontwikkeling van metastasen (Robertson 2017).

Er zijn verschillende soorten epigenetische regulatie waargenomen bij de ontwikkeling van verschillende kankers zoals huidmelanoom, waarbij DNA-methyl-transferasen en histon-deacetylasen een belangrijke rol spelen (Sigalotti 2010). **In hoofdstuk 4** analyseerden wij de distributie van een breed scala aan histon deacetylase (HDAC) enzymen en een histon-methyl-transferase (EZH2, enhancer of zeste homolog 2), en vergeleken wij het niveau van hun expressie tussen laag en hoog risico UM (disomie 3 = D3 vs monosomie 3 = M3 tumoren). Sommige HDAC's (HDAC1, HDAC3, HDAC4 en HDAC8) waren hoger, terwijl HDAC11 lager was in M(monosomie)3 tumoren in vergelijking met D(Disomie)3 tumoren. De hoge expressie van HDAC's in UM met een hoog risico zou een mogelijk mechanisme kunnen zijn voor het versterken van ontsteking, aangezien is aangetoond dat HDAC enzymen een stimulerende rol spelen in NFkB en IFNɣ signalering (Shakespear 2011). De associatie tussen een lage expressie van HDAC11 en M3 kan worden verklaard door de locatie van het HDAC11 gen op chromosoom 3.

Omdat we ontdekten dat HDAC's tot expressie worden gebracht in UM, samen met HLA Klasse I, hebben we ons gericht op het mogelijke gebruik van een combinatie van immunotherapie en anti-HDAC chemotherapie. We hebben *in vitro* studies uitgevoerd met de HDAC-remmer Quisinostat, die de celgroei verminderde in alle drie de geteste UM-cellijnen (Heijkants 2017). Hoewel Quisinostat de groei van UM cellen verminderde, wijzigde het ook de expressie van HLA Klasse I expressie, zowel op eiwit als mRNA niveau. De opregulatie van HLA klasse I is mogelijk te wijten aan het vermogen van geneesmiddelen om de chromatinestructuur te openen door

deacetylering te remmen en daarmee genexpressie te verhogen. Dit kan gevolgen hebben: als de metastase zich al heeft ontwikkeld en de lever heeft bereikt, kan het gebruik van dit medicijn de HLA-klasse I expressie en daarmee de T cel-gemedieerde lysis verhogen; als alleen de primaire tumor aanwezig is, kan dit medicijn bijdragen aan de progressie van de ziekte door verdere opregulatie van HLA-klasse I, die door NK cellen gemedieerde lysis in het bloed kan blokkeren.

Omdat we zagen dat vooral UM met een hoog risico een hoge expressie van verschillende epigenetische HDAC enzymen vertoonden, samen met opregulatie van HLA Klasse I, vroegen we ons af of HDAC expressie ook geassocieerd is met de aanwezigheid van infiltrerende leukocyten. De expressie van sommige HDAC's (1, 3 en 8) was inderdaad gerelateerd aan de aanwezigheid van lymfocyten **(hoofdstuk 5)**. Deze HDAC's werden vervolgens geïdentificeerd als gevoelig voor opregulatie door IFNɣ. Dit verklaart ook de positieve correlatie die is waargenomen tussen verschillende HDAC's en het verlies van één kopie van chromosoom 3: het is waarschijnlijk dat niet alleen HLA expressie, maar ook expressie van deze HDAC's wordt gestimuleerd door de secretie van inflammatoire cytokines.

Niet alleen HDAC's zijn betrokken bij epigenetische regulatie, maar er zijn ook veel andere regulatoren. **In hoofdstuk 6** hebben wij ons gericht op een reeks niet-translationele RNA's die miRNA's worden genoemd. Het is aangetoond dat miRNA's betrokken zijn bij de progressie van UM (beoordeeld door Smit 2019). We keken naar de verdeling van 125 miRNA's waarvan wij van 64 UM expressieinformatie hadden en onderzochten hun associatie met verschillende factoren die verband houden met de inflammatoire kenmerken in UM. Omdat we uit eerdere onderzoeken weten dat verhoogde HLA Klasse I expressie verband houdt met een slechte prognose en verlies van chromosoom 3, vroegen we ons af of we associaties konden vinden tussen miRNA expressie en HLA-A en HLA-B expressie. We ontdekten dat van de 125 gemeten miRNA's, er 24 significante associaties vertoonden: vier miRNA's (22, 155, 635 en 1276) waren positief gecorreleerd met HLA-A en HLA-B expressie, terwijl de andere 21 miRNA's een negatieve correlatie vertoonden. Bovendien waren miRNA's met een positieve associatie met HLA expressie vaak opgereguleerd in M3 UM, terwijl het tegenovergestelde patroon bestond voor veel van de gedownreguleerde miRNA's.

Verlies van chromosoom 3, in combinatie met een mutatie in het BAP1 gen, beïnvloedt waarschijnlijk het normale miRNA regulatienetwerk: de opgereguleerde miRNA's kunnen betrokken zijn bij het tot zwijgen brengen van tumorsuppressors en fungeren als positieve regulatoren van ontstekingsroutes (Onco-miR's), terwijl de miRNA's die lager zijn in M3 UM een tegengestelde rol hebben (een tumoronderdrukkende rol).

Het kan zijn dat een ontsteking de miRNA expressie zou kunnen wijzigen: het niveau van mir-192-expressie nam af bij de ontstekingsziekte colitis ulcerosa; het geïdentificeerde doelwit voor dit miRNA is het inflammatoire chemokine, Macrofage Inhibiting Peptide (MIP)-2α (Wu 2008).

Ondanks de aanwezigheid van immuuncellen in UM, lijken de lokale immuunresponsen niet effectief te zijn. Om een anti-tumorwerking te verkrijgen is het erg belangrijk om de kennis te vergroten over hoe immuuncellen, en vooral CD8 T cellen, worden gereguleerd. Het immuuncheckpoint Lymfocyt-Activatingsgene-3 (LAG3) is recentelijk in de belangstelling komen te staan (Durante 2020).

Immuuncheckpoints zijn receptoren op het celoppervlak die normaal gesproken auto-immuunreacties in het lichaam afremmen. **In hoofdstuk 7** hebben we de LAG3-expressie bestudeerd in weefsel van 64 primaire UM van het Leidse cohort en onze resultaten geverifieerd met behulp van een onafhankelijk cohort van een andere set van 15 tumoren uit Leiden en 80 tumoren van de TCGA studie. Met de kennis dat verlies van één kopie van chromosoom 3 bijdraagt aan ontsteking, veronderstelden we dat deze checkpoint inhibitor vooral tot expressie zou worden gebracht in M3 tumoren en daarom zou kunnen worden gebruikt als een doelwit voor behandeling met checkpoint remmers. We ontdekten dat verhoogde niveaus van LAG3 geassocieerd zijn met de aanwezigheid van prognostisch slechte markers, zoals een epithelioïd celtype, verlies van chromosoom 3 en verlies van BAP1 expressie. Er werden positieve associaties gevonden tussen LAG3 en de aanwezigheid van tumor-infiltrerende immuuncellen.

LSECtin, HLA Klasse II en Galectin-3 zijn liganden van LAG3. We ontdekten dat expressie van HLA klasse II-moleculen sterk correleert met LAG3. Naast HLA Klasse I wordt HLA Klasse II expressie ook in verband gebracht met hogere aantallen infiltrerende T cellen (Jager 1988) en metastasevorming (Ericsson 2001, Krishnakumar 2003). Naast HLA Klasse II-moleculen vonden

we ook dat Galectin-3 een verhoogde expressie heeft in M3 tumoren. Van dit lectine is aangetoond dat het de cytotoxische activiteit van CD8 cellen verlaagt (Kouo 2015).

Er is voorgesteld dat één van de mechanismen achter de uitputting van CD8 cellen de opregulatie van LAG3 liganden op de tumor is, waardoor LAG3-positieve T cellen worden aangetrokken en remmingssignalen naar hen worden overgebracht (Durante 2020, Figueiredo 2020).

Aangezien UM met een bijzonder hoog risico grote aantallen tumor-infiltrerende lymfocyten (TIL's) en tumor-geassocieerde macrofagen (TAM) bevat, suggereert de hoge LAG3 distributie in deze tumoren een mogelijk voordeel van anti-LAG3-monoklonale antilichamen als adjuvante behandeling bij patiënten met UM met hoog risico.

Conclusie en toekomstperspectief

In dit proefschrift hebben wij ons gericht op de rol van verschillende factoren bij de versterking van het inflammatoire fenotype in UM en onderzochten wij hoe anti-tumor immuunaanvallen bij deze ziekte worden uitgeschakeld. We willen de kennis van de pathogenese van ontsteking vergroten om mogelijke nieuwe therapeutische doelen te definiëren.

Ontsteking is een beschermende strategie van het lichaam die normaal gesproken stopt na het genezen van een wond. Een kwart van alle kankers wordt veroorzaakt door chronische ontstekingen (zoals beschreven door Coussens 2002); bij een chronische ontsteking geneest de tumor niet en stopt daarom niet bij kanker (Dvorak 1986). Ontsteking wordt geassocieerd met de ontwikkeling van metastasen in UM. In de afgelopen jaren hebben verschillende onderzoeken verschillende genetisch voorspellende markers geïdentificeerd die sterk geassocieerd zijn met ontsteking, waaronder chromosoomafwijkingen zoals verlies van één kopie van chromosoom 3 en extra kopieën van de lange arm van chromosoom 8, evenals mutaties, die verlies van BAP1-expressie veroorzaken. Deze informatie classificeert tumoren in verschillende risicosubtypes en kan helpen bij het identificeren van patiënten die mogelijk in aanmerking komen voor neoadjuvante therapie en frequente screening. Het is belangrijk om de kennis over verschillende moleculaire factoren die door deze genetische afwijkingen worden beïnvloed te vergroten om te begrijpen waarom er bij deze ziekte ontstekingen optreden.

Onze resultaten laten zien dat veel verschillende mediatoren collectief ontregeld zijn in ontstoken tumoren: opregulatie van HLA-klasse I was geassocieerd met opregulatie van de NFkB pathway, met opregulatie van een specifieke groep HDAC's en met verschillende specifieke miRNA's. Deze zijn allemaal opgereguleerd in M3/BAP1-negatieve tumoren met een hoog risico. Een uitzondering is HDAC11, dat zich op chromosoom 3 bevindt en dat lager tot expressie komt in tumoren met verlies van chromosoom 3.

De deregulering die wordt waargenomen in verschillende epigenetische modulatoren en de NFkB route kunnen met elkaar in verband staan: de remming van HDAC enzymen bij niet-kleincellig longkanker verminderde bijvoorbeeld de transcriptie van NFkB (Imre 2006); bovendien is aangetoond dat mir-155, waarvan wij vonden dat het verhoogd was in M3 tumoren met een positieve correlatie met HLA Klasse I, bij longkanker wordt opgereguleerd door NFkB (Chiu 2016).

Op basis van de resultaten van dit proefschrift veronderstellen wij dat verlies van één kopie van chromosoom 3/BAP1-expressie leidt tot een hogere HLA expressie door opregulatie van de NFkB-route en epigenetische herprogrammering. Dit kan er dan toe leiden dat HDACs en miRNA leukocyten aantrekken, waar de lokale microenvironment vervolgens hun cytotoxische activiteit uitput via een interactie met LAG3/HLA klasse II of Galectin-3 en tegelijkertijd de uitgeputte immuuncellen gebruikt als bron voor voedings- en groeifactoren om de angiogenese aan te zetten. Dit verhoogt de kans op metastasen.

Een centrale regulator kan de aanwezigheid van ischemie zijn. Jehs (Jehs 2014) toonde aan dat stimulatie van UM cellijnen leidde tot de afscheiding van cytokines die monocyten aantrekken, terwijl Bronkhorst met *in vitro* proeven liet zien dat het de aanwezigheid van ischemie was die leidde tot de productie van pro-inflammatoire cytokines (Bronkhorst 2014). Brouwer (Brouwer 2019) toonde onlangs aan dat ischemie vaker voorkomt in UM met M3/verlies van BAP1. Het is interessant om te zien dat geneesmiddelen die interfereren met een zeer belangrijk ischemie-geïnduceerd cytokine, HIF-1 alfa, nu worden getest voor toepassing bij UM (Dong 2019).

Het begrijpen van de belangrijkste reden voor de aanwezigheid van immuuncellen in UM is van cruciaal belang om de pathogenese van deze ziekte te begrijpen en om de juiste ontstekingsremmende strategieën te kunnen ontwerpen.

Wij hopen dat onze pogingen nuttig zijn geweest voor het begrip van de regulatie van ontsteking bij oogmelanoom en mogen leiden tot de toename en verbetering van de overleving bij UM patiënten.

References

1. Augsburger, J.J.; Corrêa, Z.M.; Shaikh, A.H. Quality of evidence about effectiveness of treatments for metastatic uveal melanoma. Trans. Am. Ophthalmol. Soc. 2008, 106, 128-137.
2. Harbour, J.W.; Onken, M.D.; Roberson, E.D.; et al. Frequent mutation in BAP1 in metastasizing uveal melanomas. Science 2010, 330 (6009) 1410-1413.
3. Versluis, M.; de Lange, M.J.; van Pelt, S.I.; et al. Digital PCR validates 8q dosage as prognostic tool in uveal melanoma. PLoS One 2015, 10(3), e0116371.
4. Yavuzyigitoglu, S.; Koopmans, A.E.; Verdijk, R.M.; et al; Rotterdam Ocular Melanoma Study Group. Uveal Melanomas with SF3B1 Mutations: A distinct subclass associated with late-onset metastases. Ophthalmology 2016, 123(5), 1118-1128.
5. Robertson, A.G.; Shih, J.; Yau, C.; et al. Integrative analysis identifies four molecular and clinical subsets in uveal melanoma. Cancer Cell 2017, 32, 204-220
6. Gezgin, G.; Dogrusöz, M.; van Essen, T.H.; et al. Genetic evolution of uveal melanoma guides the development of an inflammatory microenvironment. Cancer Immunol. Immunother. 2017, 66, 903-912.
7. Roelofsen, C.D.M.; Wierenga, A.P.A.; van Duinen, S.; et al. Five Decades of Enucleations for Uveal Melanoma in One Center: More Tumors with High Risk Factors, No Improvement in Survival over Time. Ocul. Oncol. Pathol. 2021, 7(2), 133-141.
8. Rodrigues, M.; Koning, L.; Coupland, S.E.; et al. UM Cure 2020 Consortium. So close, yet so far: Discrepancies between Uveal and other Melanomas. A position paper from UM Cure 2020. Cancers (Basel). 2019, 11(7), 1032.
9. De Waard-Siebinga, I.; Houbiers, J.G.; Hilders, C.G.; et al. Differential expression of HLA-A and B alleles on uveal melanoma as determined by immuno-histology. Ocul. Immunol. Inflamm. 1996 4, 1-14.
10. Blom, D.J.; Luyten, G.P.; Mooy, C.; et al. Human Leukocyte Antigen class I expression. Marker of poor prognosis in uveal melanoma. Investig. Ophthalmol. Vis. Sci. 1997, 38, 1865-1872.
11. Ericsson, C.; Seregard, S.; Bartolazzi, A.; et al. Association of HLA class I and class II antigen expression and mortality in uveal melanoma. Investig. Ophthalmol. Vis. Sci. 2001 42, 2153-2156.
12. Maat, W.; Ly, L.V.; Jordanova, E.S.; et al. Monosomy of chromosome 3 and an inflammatory phenotype occur together in uveal melanoma. Invest. Ophthalmol. Vis. Sci. 2008, 49(2), 505-510.

13. Hurks, H.M.H.; Metzelaar-Blok, J.A.W..; Mulder, A.; et al. High frequency of allele-specific down-regulation of HLA Class I expression in uveal melanoma cell lines. Int. J. Cancer 2000, 85, 697-702.
14. Anastassiou, G.; Rebmann, V.; Wagner, S.; et al. Expression of classic and non-classic HLA class I antigens in uveal melanoma. Investig. Ophthalmol. Vis. Sci 2003, 44, 2016-2019.
15. De Waard-Siebinga, I.; Creyghton, W.M.; Kool, J.; Jager, M.J. Effects of interferon alfa and gamma on human uveal melanoma cells in vitro. Br. J. Ophthalmol. 1995, 79, 847-855.
16. Taniguchi, K.; Karin, M. NF-κB, inflammation, immunity and cancer: coming of age. Nature Rev. Immunol. 2018, 18(5), 309-324.
17. Terzic, J.; Grivennikov, S.; Karin, E.; et al. Inflammation and colon cancer. Gastroenterology. 2010, 138, 2101-2114.
18. Sigalotti, L.; Covre, A.; Fratta, E.; et al. Epigenetics of human cutaneous melanoma: setting the stage for new therapeutic strategies. J Translat. Med. 2010, 8(1), 1-22.
19. Shakespear, M.R.; Halili, M.A.; Irvine, K.M.; et al. Histone deacetylases as regulators of inflammation and immunity. Trends Immunol. 2011, 32, 335-343.
20. Heijkants, R.; Willekens, K.; Schoonderwoerd, M.; et al. Combined inhibition of CDK and HDAC as a promising therapeutic strategy for both cutaneous and uveal metastatic melanoma. Oncotarget 2017, 9(5), 6174-6187.
21. Smit, K.N.; Chang, J.; Derks, K.; et al. Aberrant MicroRNA expression and its implications for uveal melanoma metastasis. Cancers 2019, 11(6), 815-829.
22. Wu, F.; Zikusoka, M.; Trindade, A.; et al. MicroRNAs are differentially expressed in ulcerative colitis and alter expression of macrophage inflammatory peptide-2α. Gastroenterology. 2008, 135(5), 1624-1635.
23. Durante, M.A.; Rodriguez, D.A.; Kurtenbach, S.; et al. Single-cell analysis reveals new evolutionary complexity in uveal melanoma. Nature Commun. 2020, 11(1), 1-10.
24. Jager, M.J.; van der Pol, J.P.; de Wolff-Rouendaal, D.; et al. Decreased expression of HLA class II antigens on human uveal melanoma cells after in vivo X-ray irradiation. American Journal Ophthalmology 1988, 105(1), 78-86.
25. Krishnakumar, S.; Abhyankar, D.; Lakshmi, S.A.; et al. HLA class II antigen expression in uveal melanoma: Correlation with clinicopathological features. Exp. Eye Res. 2003, 77,175-180.
26. Kouo, T.; Huang, L.; Pucsek, A.B.; et al. Galectin-3 shapes antitumor immune responses by suppressing CD8+ T cells via LAG-3 and inhibiting expansion of plasmacytoid dendritic cells. Cancer Immunol. Res. 2015, 3, 412-423.

27. Figueiredo, C.R.; Kalirai, H.; Sacco, J.J.; et al. Loss of BAP1 expression is associated with an immunosuppressive microenvironment in uveal melanoma, with implications for immunotherapy development. J. Pathol. 2020, 250(4), 420-439.
28. Coussens, L.M.; Werb, Z. Inflammation and cancer. Nature 2002,420 (6917), 860-867.
29. Dvorak, H.F. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N. Engl. J. Med. 1986, 315 (26), 1650-1659.
30. Imre, G.; Gekeler, V.; Leja, A.; et al. Histone deacetylase inhibitors suppress the inducibility of nuclear factor-κB by tumor necrosis factor-α receptor-1 down-regulation. Cancer Res. 2006, 66(10), 5409-5418.
31. Chiu, C.F.; Chang, Y.W.; Kuo, K.T.; et al. NF-κB-driven suppression of FOXO3a contributes to EGFR mutation-independent gefitinib resistance. Proc. Natl. Acad. Sci. USA 2016, 13(18), 2526-2535.
32. Jehs, T.; Faber, C.; Juel, H.B.; et al. Inflammation-induced chemokine expression in uveal melanoma cell lines stimulates monocyte chemotaxis. Invest. Ophthalmol. Vis. Sci. 2014, 55(8), 5169-5175.
33. Bronkhorst, I.H.G.; Jehs, T.M.L.; Dijkgraaf, E.M.; et al. Effect of hypoxic stress on migration and characteristics of monocytes in uveal melanoma. JAMA Ophthalmol. 2014, 132 (5), 614-621.
34. Brouwer, N.J.; Wierenga, A.P.A.; Gezgin, G.; et al. et al. Ischemia is related to tumour genetics in uveal melanoma. Cancers. 2019, 11, 1004.
35. Dong, L.; You, S.; Zhang, Q.; et al. Arylsulfonamide 64B inhibits hypoxia/HIF-induced expression of c-Met and CXCR4 and reduces primary tumor growth and metastasis of uveal melanoma. Clin. Canc. Res. 2019, 25(7), 2206-2218.

List of publications

1. **HLA expression in Uveal Melanoma: an indicator of malignancy and a modifiable immunological target**
   Z. Souri, A.P.A. Wierenga, A. Mulder, A.G. Jochemsen, M. J. Jager.
   Cancers 2019, 11, 1132-1150. Doi: 10.3390/cancers11081132.

2. **Loss of BAP1 is associated with upregulation of the NFkB pathway and increased HLA Class I expression in Uveal Melanoma**
   Z. Souri, A.P.A. Wierenga, C. van Weeghel, P.A. van der Velden, W.G.M. Kroes, G.P.M. Luyten, S.H. van der Burg, A.G. Jochemsen, M.J. Jager.
   Cancers 2019, 11, 1102-1118. Doi: 10.3390/cancers11081102.

3. **HDAC inhibition increases HLA Class I expression in Uveal Melanoma**
   Z. Souri, A.G. Jochemsen, M. Versluis, A.P.A. Wierenga, F. Nemati, P. A. van der Velden, W. G.M. Kroes, R.M. Verdijk, G.P.M. Luyten, M.J. Jager.
   Cancers 2020, 12, 3690-3704. Doi: 10.3390/cancers12123690.

4. **Expression of HDACs 1, 3, and 8 is upregulated in the presence of infiltrating lymphocytes in Uveal Melanoma.**
   Z. Souri, A.G. Jochemsen, A.P. A. Wierenga, W.G. M. Kroes, R.M. Verdijk , P.A. van der Velden, G.P. M. Luyten and M.J. Jager.
   Cancers 2021

5. **MiRNAs correlate with HLA expression in Uveal Melanoma: both up- and downregulation are related to Monosomy 3**
   Z. Souri, A.P. A. Wierenga, E. Kiliç, E. Brosens, S. Böhringer, W.G.M. Kroes, R.M. Verdijk, P.A. van der Velden, G.P. M. Luyten and M.J. Jager.
   Cancers 2021

6. **LAG3 and its Ligands show increased Expression in High Risk Uveal Melanoma**
   Z. Souri, A.P.A. Wierenga, W.G.M. Kroes, P.A. van der Velden, R.M. Verdijk, M. Eikmans, G.P.M. Luyten and M.J. Jager.
   Under review

List of presentations

1. **Regulation of PD-L1 in uveal melanoma**
   Z. Souri, A.P.A. Wierenga, M.J Spruyt- Gerritse, M. Eikmans, M.J. Jager.
   The Association for Research in Vision and Ophthalmology (ARVO 2019)

2. **HDAC inhibitor Quisinostat upregulates HLA Class I expression in Uveal Melanoma cell lines**
   Z. Souri, M. Vesluis, A.P.A. Wierenga, P.A.van der Velden, G.P.M. Luyten, A.G. Jochemsen, M.J. Jager.
   Dutch Ophthalmology PhD student congress (DOPS 2020)

3. **HDAC expression in uveal melanoma**
   Z. Souri, R.M. Verdijk, G.P.M. Luyten, M.J. Jager.
   Universal Scientific Education and Research Network (USERN2020)

4. **Inflammation-related MiRNAs are associated with Monosomy 3 in Uveal Melanoma.**
   Z. Souri, P.A. van der Velden, G.P.M Luyten, M.J. Jager.
   "Gold Medal"
   European Association for Vision and Eye Research (EVER 2020)

5. **HDAC and HLA Class I expression in uveal melanoma.**
   Z. Souri, A.G. Jochemsen, R.M. Verdijk, P.A. van der Velden, G.P.M Luyten, M.J. Jager.
   European Association for Vision and Eye Research (EVER2020)

6. **HDAC inhibitors in UM**

   Z. Souri, A.G. Jochemsen, M.J. Jager.

   The 10th annual meeting of the Iranian Research Association for Vision and Ophthalmology (Iravo 2021)

7. **Expression of Immune checkpoint LAG3 is associated with high risk uveal melanoma**

   Z. Souri, A.P.A. Wierenga, G.P.M Luyten, M.J. Jager.

   The Association for Research in Vision and Ophthalmology (ARVO 2021).

8. **Relation between Histone deacetylase expression in uveal melanoma and the presence of infiltrating leukocytes**

   Z. Souri, A.G. Jochemsen, R.M. Verdijk, M.J. Jager

   European Association for Vision and Eye Research (EVER2021).

Acknowledgements (Dankwoord)

It all started with sending an email to Professor Dr. Martine J. Jager. After several months I saw myself waving goodbye to my family and landing in a flat green country with rain in the summer; it was then that my exciting journey began...

When I look back I am very happy that I decided to study in The Netherlands; besides all beauty of the country, I met a wonderful, friendly group of experts in my life who I would like to give my deepest appreciation to.

Dear Professor Jager, I am truly grateful for having the chance to be one of your students. Your immense knowledge always encouraged me to do more during my PhD. You were my biggest supporter in the Netherlands. You taught me a lot, both in personal and scientific aspects; you inspired me to present myself as a strong lady, showed me how to be precise and creative and helped me to reach my goal. Thank you for always going above and beyond to ensure the success of our projects. I will miss you so much.

I would like to give a special thanks to Dr Aart G. Jochemsen; dear AG, you showed me so many useful skills while working with the cells; without your great advice during my experiments, my results would not have been produced so smoothly.

I wish to show my gratitude to Professor Dr. Gre Luyten. It was an honor for me to be a member of your department. You were always very kind to me; you taught me how one could have a superb position and be friendly with the staff at the same time!

Dr Pieter A. van der Velden and Dr Robert M. Verdijk thank you very much for all your great points of view, making my papers better and better each time by teaching me how to think deeper and consider different aspects during a study.

Special thanks to Dr. Annemijn P.A. Wierenga and Dr. Maria Chiara Gelmi for accepting to be my paranymphs! Dear friends, we had a lot of fun together and I am so happy that I met you. I will miss you so much and hope to see you again!

I would like to show my great appreciation to Dr. Mieke Versluis. Dear Mieke thank you so much for taking the time to show me the necessary techniques during my experiments.

And all other friends and colleagues in LUMC, I thank you very much for making this such a wonderful place to work every day.

Getting through my PhD required more than academic support, therefore, I would like to express my appreciation to the Iranian Ministry of Science Research and Technology.

I would like to express my gratitude to Professor Dr. Abolghasem Esmaeili my scientific advisor and all staff members of the Cell and Molecular Biology Department of University of Isfahan.

Last but not least, my family members. My dear father, Professor Dr. Manouchehr Souri and my mother Katrin, my amazing brothers Dr. Amir Mohammad Souri together with his family and Mohammad Amin Souri, words won't be enough to express how thankful I am; thank you for giving me the power to decide; for being available for me in every single second; I always felt you by my side. I would never have been able to reach my goal without having you in my life.

I was thousands of kilometers away from home, but I made it and it is all because I had all you precious and wonderful people in my life, which I am very grateful for.

Now I exactly understand what it means when it is said that time flies!

I guess it's time for me to begin my next chapter of life...

Sincerely,

Zahra Souri

Leiden 2021

Curriculum vitae

The author of this thesis was born on March 26th, 1989 in Kermanshah, Iran. In 2008, after finishing her high school education in the field of Science at the Razi University high school in Kermanshah, she decided to study Animal Biology at the Department of Biology, Razi University. The author obtained her BSc degree in 2012, and was subsequently admitted to the Master's program in the field of Cell and Molecular Biology from the Iranian Ministry of Science, Research and Technology. After completion of her Master's degree in 2014, she decided to participate in the Iranian PhD national exam implemented by the Ministry of Science, Research and Technology and became 2nd place in approximately 400 participants in the field of Cell and Molecular Biology; with this score, she was admitted to a PhD position at the Department of Cell and Molecular Biology of Isfahan University in 2015; moreover she was awarded an international PhD scholarship. In August 2017, she eventually decided to come to The Netherlands and started her new journey at the Ophthalmology Department of Leiden University Medical Center under Professor Martine Jager's supervision.

During her PhD, the author had the opportunity to introduce and present her work at congresses held in Europe (Ever 2020, Ever 2021), Asia (USERN 2020, IRAVO 2021) and the United States (ARVO 2019, 2021) and received a gold medal from the European Association for Eye and Vision Research (EVER 2020).

Upon completion of her PhD program, the author will go back to Iran where she will be enrolled as an assistant professor at the University of Isfahan and continue her scientific research.