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Discovering biomarkers and druggable targets in uveal melanoma

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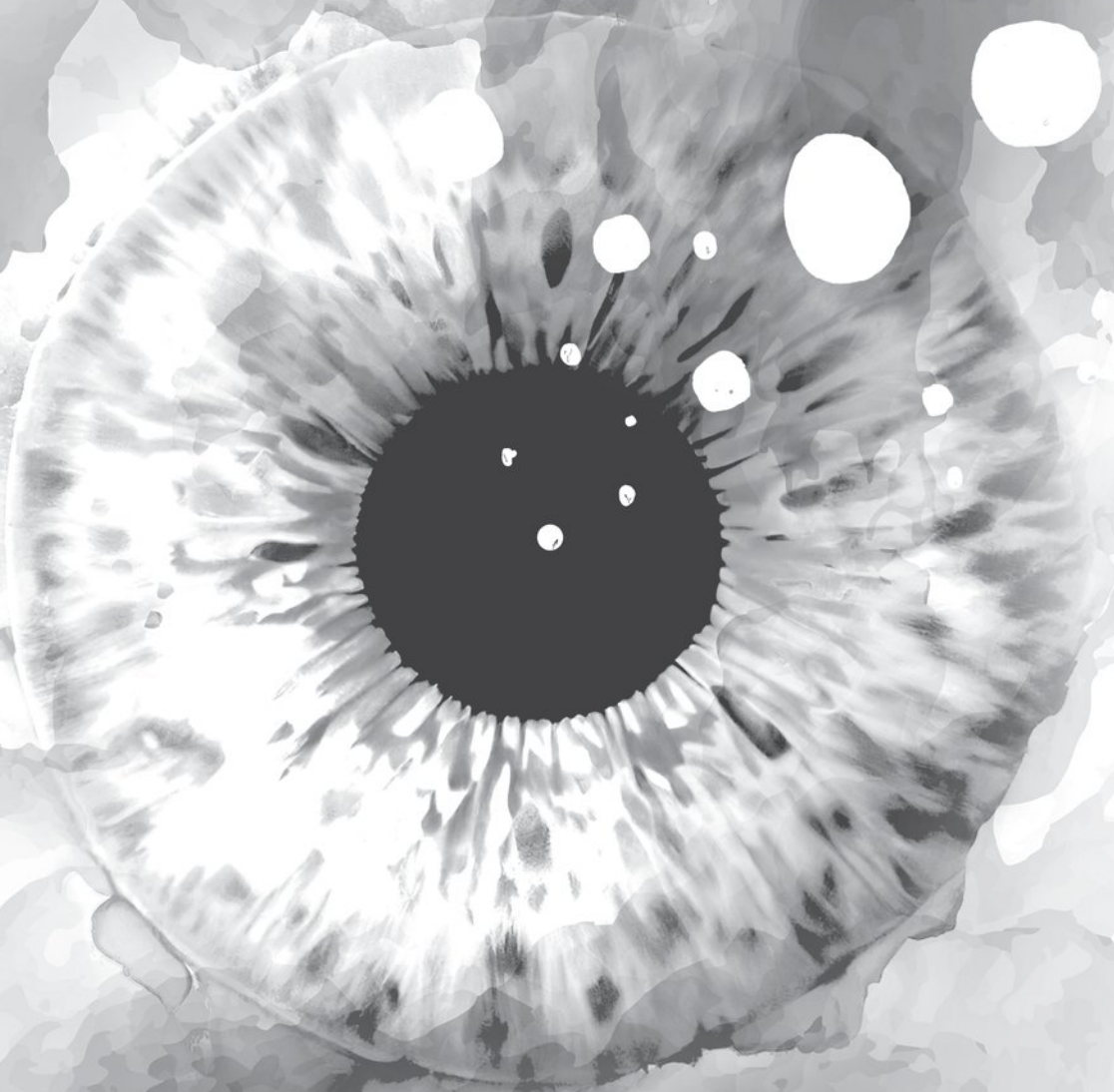
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Chapter 10





Summary and Discussion

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The papers described in this thesis are based on research projects that led the way into new areas. The thesis is part of UM CURE, European Union's Horizon 2020 research and innovation programme. When it comes to rare diseases, international collaborative efforts are key in facilitating scientific progress. The collaborative efforts and discussions stimulated research at the time that treatment with immune checkpoint inhibitors was initiated, and when studying intra-ocular fluids became possible thanks to new techniques [1, 2].

We first investigated whether survival of patients who underwent an enucleation for Uveal Melanoma (UM) has improved over the years. The Leiden University Medical Centre (LUMC) has been the major centre for the diagnosis and treatment of UM since it started with P32 analysis and subsequently with plaque irradiation [3]. We analysed the characteristics of patients with UM and their tumors between 1973 and 2019, using data from medical records and pathology reports from UM patients treated with an enucleation. We noticed that changes over time included an increase in the mean age of the patient at the time of enucleation, a more frequent involvement of the ciliary body and tumors were more frequently classified in a high TNM/AJCC class. Despite years of scientific research, no improvement in survival over time was found. These data indicate how important it is to find new targets for the treatment of metastases. This is precisely what this thesis focused on.

The use of immune checkpoint inhibitors has proven successful in the treatment of different tumors, prolonging life and curing patients. We analysed the potential role of checkpoint inhibitors in the treatment of conjunctival and UM. Immune checkpoints act as the “brakes” of immune responsiveness. They are co-stimulatory molecules on T-cells; when they bind to their receptors, they stop T-cell responses. However, when the receptors are blocked by the immune checkpoint inhibitors, the T cell is no longer inhibited, allowing the T cell to attack its target. While this treatment helps to release life-saving immune responses in patients with metastases of cutaneous melanoma, there is a lack of success in UM patients. A select few cases where a clinical response to the checkpoint inhibitor PD-1 was described were patients in which hypermutated phenotypes of MBD4 germline mutation had been identified [4]. Interestingly, successful treatment of metastatic disease has been described for patients with a conjunctival melanoma (CM) [2, 5].

With these clinical successes in cutaneous and conjunctival melanoma in mind, we wanted to focus on whether these targets for immune checkpoint inhibitors are expressed in patients with UM. Because of the lower clinical complexity to perform

a liquid biopsy rather than a tumor biopsy, we tested the anterior chamber fluid of 84 UM patients on the presence of PD-L1. We found a higher expression levels of PD-L1 in the fluid of the anterior chamber when tumors were larger in prominence and diameter (higher AJCC stage) and involved the ciliary body. When compared to genetic data, the eyes with a high PD-L1 expression more often had a gain of 8q and tended to have monosomy 3. The group of patients with a high PD-L1 expression had a significantly worse survival. Even though clinical responses did not show success, these data showed differential expression of the treatment target between cases. It may be that the lack of effect of immune checkpoint inhibitors is not due to lack of expression of the checkpoint molecules, but lack of targetable antigens that can be attacked by T cells. This is however contradicted by the use of another type of immunotherapy (with tebentafusp), which uses a bispecific antibody that stimulates T cells to attack pigmented cells carrying gp100. Nathan et al. described a phase 3-trial, screening patients from March 2017 through June 2020, in which HLA-A*02:01-positive patients with metastatic UM were included. They describe that metastatic UM patients treated with tebentafusp showed overall longer survival in comparison to control therapy [6]. More recently, it was shown in animal experiments and in a first study in uveal melanoma patients, that combining immune checkpoint inhibitors with tumor-destroying therapy may be more effective than immune checkpoint inhibitor therapy alone [7, 8, 9].

In order to identify new potential targets or indicators of prognosis in UM by liquid biopsy, we studied the presence of 84 preset biomarkers related to oncology or immunology in the anterior chamber fluid of UM eyes. The presence of the biomarkers in the aqueous humor was compared to clinical data, pathology reports as well as cytogenetic data and a cluster analysis was performed. Three distinct clusters were defined based on overall protein expression, in which the aqueous humor either lacked proteins or showed a high expression of almost all inflammation-related biomarkers. A third group showed an intermediate expression pattern. Significant differences were found between these three clusters and the cluster with the overall highest protein expression had the worst prognostic outcome. These data indicate that protein analysis of the aqueous humor can be used for prognostication and for identifying potential targets. These studies demonstrated the identification of biomarkers available in the aqueous humor and stimulated investigations on the potential clinical use for liquid biopsies to identify high risk patients. Peng et al. analyzed protein expression of 1472 targets and found that 45 proteins that were differentially expressed could differentiate tumors into either GEP class 1 or class 2 at diagnosis [10]. However, when analysing their data, one may also conclude that tumor size influenced protein expression levels. The newest approaches are able to identify even up to 20.000 proteins, which also result in identifying clusters of UM cases (presentation ISOO conference 2026 by Dr Prithvi Mruthyunjaya).

We subsequently focussed on two different sets of proteins, soluble HLA, which may play a role in immune responses, and angiopoietins. We know that prognostically bad tumors have a so-called inflammatory phenotype, where loss of chromosome 3/BAP1 expression is associated with a high HLA expression and an increased number of lymphocytes and macrophages in UM [11, 13]. Metastases in general follow the genetics and inflammatory phenotype of the original tumor, and it may well be that especially tumors with an inflammatory phenotype are susceptible to immune therapy (antigens are presented to T cells on HLA molecules). We set out to investigate the possibility to detect soluble HLA (sHLA) in the aqueous humor. In 19 out of 108 patient samples we observed measurable levels of sHLA. We further compared the sHLA to clinical data, pathology reports and cytogenetic data. The tumors of the sHLA positive samples were significantly larger, more often involved the ciliary body and belonged to the higher AJCC stages. The tumors of the sHLA positive samples differed significantly in their cytogenetic data. These tumors more often had a monosomy 3 status, a gain of 8q and loss of BAP1 expression in immunohistochemical staining. This fits with our original findings in which tumors with a high HLA Class I expression on the tumor cells were found to have monosomy 3 and carry a worse prognosis than tumors with a low HLA Class I expression.

We then looked at two potential druggable targets, angiopoietin-1 (ANG-1) and angiopoietin-2 (ANG-2), combining information from protein expression levels of ANG-1 and ANG-2 in the anterior chamber fluid with mRNA gene expression data obtained from tumor tissue of the same 64 enucleated tumors. Samples were collected from patients undergoing primary enucleation at the LUMC between 1999 and 2017 [11]. We found that gene expression of ANG-2, but not of ANG-1, was associated with a worse prognostic outcome. The tumors were larger and more often involved the ciliary body. High ANG-2 expression correlated significantly to UM related death. We found that the protein expression in the aqueous humor correlated to the ANG-2 gene expression data. We thus conclude that ANG-2 can be a potential targetable cytokine in uveal melanoma. As there now is a drug that targets ANG-2 together with VEGF (faricimab), one may not only look into the role of ANG-2 in tumor development, but also whether it is involved in radiation retinopathy.

While we studied protein expression, others focused on genomic biomarkers. Im et al. described the use of liquid biopsies by obtaining anterior chamber fluid to collect circulating tumor DNA (ctDNA) [14]. They described the possibility and identification of mutations, and found high enough levels of ctDNA to detect mutations in eyes in which the uveal melanoma involved the ciliary body. Higher ctDNA levels were observed in post radiation eyes. Nell et al. reported on using a droplet PCR technique on vitreous

fluid obtained from eyes enucleated for uveal melanoma. While they were often able to pinpoint which mutations were present, quite a large number of samples did not contain enough DNA for proper evaluation [15].

While Nell et al. studied DNA, other studies focus on proteins in vitreous biopsies. E.g. Velez et al. focused on vitreous proteins and their potential to become a biomarker instead of needing tumor material through a tumor biopsy [16]. They found elevated expression levels of growth factors HGFR, HGF, and SCFR and a lower expression of KLK7. They further investigated which FDA-approved drugs are available that potentially influence these elevated proteins. SCFR is inhibited by imatinib, and the authors thus suggest imatinib as a drug candidate for adjuvant therapy. With these results in mind, the authors conclude that vitreous biopsies could not only help to identify potential mechanisms for tumor proliferation but also aid in defining adjuvant therapies, designing future clinical trials but can also be used in metastatic risk surveillance.

Aqueous humor taps are often performed in the clinic for different diagnostic purposes. To perform an aqueous humor tap is a simple clinical procedure with minor risks. This could potentially benefit patients in consultation for metastatic risk. A vitreous fluid biopsy is a more elaborate procedure but also easier than taking a tumor biopsy. Even though the tumor itself is not altered, limitations of liquid biopsies include heterogeneity of the ocular fluids as well as the fact that proteomic changes are dynamic [16].

When identifying targets for treatment of a tumor, one focusses on specific characteristics of a tumor. A differentiating characteristic of UM from other tumors is pigmentation. UM arises from a uveal melanocyte and can have different clinical phenotypes, from amelanotic to moderate and even highly pigmented, as well as a combination of these. When looking at the literature, pigmentation is associated with a higher risk of developing metastases. One of the factors that influences pigmentation levels is the type of tumor mutation, with loss of chromosome 3/a mutation in *BAP1* being related to higher pigmentation [17]. We wondered whether iris pigmentation influenced tumor pigmentation. Having a light eye color is a risk factor for developing UM. After reviewing the literature, we studied the relation between eye color and clinical, histopathologic and genetic characteristics of UM. While all expected relationships were confirmed in light-eyed patients, the differential effect of monosomy 3 and extra copies of 8q was not so obvious in patients with a dark iris color. This leads to the question whether UM in patients from populations with mainly dark eyes (most populations outside Northern Europe and their descendants) also differ from what are now considered standard risk factors.

As one can identify patients with UM who are at high risk of developing metastases, prevention of outgrowth of metastases should be attempted. One can already provide systemic treatment before or at the time of treatment of the primary tumor, which is known as neo-adjuvant treatment. Otherwise, one can start treatment after enucleation or irradiation. These treatments can be immunological or target essential molecules that play a role in tumor proliferation [18]. We analysed the presence of a wide range of kinase and phosphatases in a series of primary and metastatic UM. We found that protein tyrosine peptide (PTK) phosphorylation was differentially expressed between M3 and D3 tumors. Upon further comparison with expression of infiltrate marker of the corresponding tumor we observed that members of the protein tyrosine kinase family were especially expressed on tumor-infiltrating leukocytes. Treatment with kinase inhibitors would thereby not only attack the tumor cells but also the immune cells. Whether that is a good or a bad thing for the patient needs further investigation.

Conclusion

People with a light eye color carry a higher risk to develop UM than people with a dark eye color. Most work involving UM has been performed in countries with many patients, and thus especially in Northern Europe and in countries where people live who are descendants of Northern Europeans. In this thesis, we show “standard” prognostic factors may be influenced by the genetic background of patients, i.e. by having blue or brown eyes. This indicates that populations with mainly brown eyes may not follow the algorithms for UM prognostication that have mainly been developed in countries with many light-eyed people.

Apart from using parameters derived from tumor tissues, one may also use liquid biopsies: blood, aqueous humor, or vitreous fluid. We analysed aqueous humor obtained when eyes with UM were being enucleated, and showed that an analysis can provide excellent information on prognostic factors and druggable targets. Further research will show whether aqueous or vitreous is more suitable, and which target should be analysed (Protein, RNA, DNA). Finding the right druggable target will need elaborate collaborations between basic and translational researchers and clinicians who treat metastases. With the first successful neo-adjuvant treatments that combine reduction in tumor size prior to e.g. irradiation with treatment of metastases while not yet detectable, this is a very exciting time, which may finally provide better survival chances for uveal melanoma patients. This thesis looked for new biomarkers that can be used to determine the nature of a uveal melanoma and be a potential druggable target or a biomarker of value for prognostication.

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