Pigmentation and prognosis in uveal melanoma

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Chapter 5

SUMMARY AND DISCUSSION

This thesis aims to explore the role of tumour pigmentation and eye colour in the prognosis of uveal melanoma. It also aims to present the complexity of uveal melanoma (UM) prognostication and to contribute to the field of therapeutic target discovery. We tackled these issues from multiple angles (literature review, clinical data analysis, genetic analysis and cell line work) and we are now integrating our findings and observations in one single dissertation.

This thesis starts by explaining the many methods and systems used for prognostication in **chapter 2.1**, which also presents evidence suggesting that combining different systems together is beneficial, especially in tumours of intermediate groups (**chapter 2.2**). Next, we focused on eye colour and pigmentation in **chapter 3**. **Chapters 3.1 and 3.2** raise the possibility that genetic factors related to eye colour may influence the tumour's prognostic factors. **Chapter 3.3** sheds more light on the role of tumour pigmentation and pose the question whether tumour pigmentation actively contributes to UM prognosis or if it is an innocent bystander. Lastly, we touch on the topic of discovering new targets, discussing MITF as a potential pigment-related target (**chapters 4.1-4.2**), spotlighting PRAME as a potential target for therapy (**chapter 4.3**) and issuing caution in the choice of cell lines to use for *in vitro* testing (**chapter 4.4**).

Prognosis of uveal melanoma: which factors should we consider?

Uveal melanoma is a rare disease, but carries a high risk of metastases. Extensive efforts have been directed towards predicting the risk of metastasis in patients with UM, with two main aims: tailoring the follow up plan and giving patients reliable information on their prospects. An additional motivation to focus on prognostication is to identify patients who may benefit from future therapies to prevent or treat metastases, even if we have no such therapy at the moment. Finally, understanding the pathophysiology of prognostic factors may help to obtain targets for therapy.

At the moment, many methods and systems are used for prognostication and there is no worldwide gold standard**.** As in many other types of cancer, one of the most widespread systems is the American Joint Committee on Cancer (AJCC) staging system[1, 2], which is revised periodically and combines clinical knowledge about metastases with tumour size (diameter and thickness) and parameters of local invasiveness (ciliary body involvement and extraocular extension). More recently, genetic factors have become more important for prognostication, in terms of chromosome aberrations, genetic mutations and gene expression. Monosomy of chromosome 3 and gain of the long arm of chromosome 8 are strongly associated with a high risk of developing metastases[3-5] and are the basis of The Cancer Genome Atlas (TCGA) genetic prognostication 4-group system: A (disomy 3, normal 8q), B (disomy 3, any 8q gain), C (monosomy 3, one extra copy of 8q), and D (monosomy 3, multiple extra 8q copies).[6, 7]

Our work showed that tumours in TCGA group C (monosomy 3, gain of one copy of 8q) can be further stratified by their AJCC stage and that UM with AJCC classes II and III have a different prognosis based on the number of extra copies of chromosome 8q (TCGA group D vs C). This finding is important for two reasons. First, it supports the thesis that combining multiple prognostication systems together allows to predict the risk of metastasis more accurately.[8, 9] This approach is behind tools like the Liverpool Uveal Melanoma Prognosticator Online (LUMPO)[10, 11] and follows the trend towards precision medicine. However, one should carefully select which parameters to consider at the same time, as many are interrelated. The second reason is that it highlights the fact that, within the context of 8q gain, prognosis is worse in tumours with a higher number of extra 8q copies. It is not yet known which gene on 8q is responsible for the worsening of prognosis, but literature evidence points towards

8q24 as one of the most relevant segments.[12, 13] This region contains the proto/oncogene *C-MYC* (MYC Proto-Oncogene, BHLH Transcription Factor) on 8q24.1[14-19], *DDEF1* (ArfGAP with SH3 domain, ankyrin repeat and PH domain 1) on 8q24.21[20], *PTK2* (Protein tyrosine kinase 2) on 8q24.3[21] and *PTP4A3* (Protein tyrosine phosphatase 4A3) on 8q.24.3[22]. Interestingly, we found PTP4A3 as one of the most upregulated genes in *PRAME*-positive UM compared to *PRAME*-negative UM.

PRAME is interesting both as a prognostic marker and a potential therapeutic target in many cancers, among which UM. The expression of *PRAME* in UM has been studied by different groups in the past decade, and it is now considered an independent negative prognostic factor.[23-26] Indeed, *PRAME* expression will be included in the Collaborative Ocular Oncology Group 2 (COOG2) study, along with GEP class and *BAP1*, *SF3B1*, and *EIF1AX* mutations.[27]

Is pigmentation an active player or a bystander?

Tumour pigmentation is a prominent tumour feature that has been studied by many authors over the years, but its role in tumour progression and prognosis is debated. On one hand, several groups have repeatedly shown that darker tumour pigmentation is related to a shorter patient survival.[28-32] We have confirmed this association in a large cohort of 1058 patients with UM who underwent an enucleation at the LUMC and who had macroscopic tumour pigmentation recorded in our database. One may wonder how melanin could influence tumour behaviour. One potential mechanism that has been tested in zebrafish models involves ferroptosis[33]: pigmented melanoma cells have a greater metastatic colonisation potential compared to non-pigmented melanoma cells, as the presence of melanin protects cells from death by ferroptosis.

However, one could argue that pigmentation does not play an active role in tumour behaviour but may just be a bystander. This hypothesis stems from the fact that dark tumours, in addition to having a worse prognosis, often have bad prognostic features, i.e. a large diameter, a high number of epithelioid cells and macrophages.[28-31] We show that molecular factors such as monosomy of chromosome 3 and gain of chromosome 8q occur more frequently in dark tumours. We also show that, once chromosome 3 and 8q are included in the regression model, tumour pigmentation is not an independent prognostic factor. This may mean that the presence and amount of melanin in the tumour is just a bystander. However, statistical significance and biology do not always match. The presence and amount of melanin in tumours may be increased thanks to chromosome aberrations, which decreases its statistical significance in a multifactorial model, but it may still be involved in malignancy. A further element in favour of tumour pigmentation having an active role in prognosis is the fact that, in a cohort of patients enucleated for a UM in Leiden, monosomy 3 has a larger impact on the survival of patients with light tumours than in patients with dark tumours.

Eye colour: more than a predisposing factor?

A light iris colour is known to be associated with an increased risk of developing UM. Indeed, UM is more common in populations (mainly Northern and Western Europe and Oceania) with a high incidence of fair skin and light eyes.[34-36] Indeed, UM has a higher incidence in people with a blue or green iris than in people with dark eyes.[37, 38] We decided to explore the role of eye colour more deeply and we studied if it may have a role not only in tumour development but in tumour behaviour as well. We first analysed clinical or self-reported iris colour in a cohort of 412 patients from the LUMC and confirmed our results in a second cohort of 934 patients treated at the Wills Eye Hospital. We showed that clinical eye colour is not predictive of survival when considered in isolation but that in patients with light eyes monosomy 3 and 8q gain had a large impact on prognosis, while these factors had a much smaller impact in patients with brown eyes.

Since eye colour is largely genetically-determined[39-41], we looked deeper into the subject and sequenced the single nucleotide polymorphisms (SNPs) related to eye, skin and hair colour in a cohort of 394 patients with UM. We used the SNP array from the HIrisplex-S system[42-44], which includes SNPs that have been linked to eye, skin and hair colour in previous genome-wide association study (GWAS) analyses. This system can be used to predict the pigmentation phenotype, starting from the SNPs. Our data show that the SNP that is most predictive of eye colour, rs12913832 (*HERC2*) is associated with prognosis: patients with the G/G (light iris) genotype had a shorter survival than patients with the A/G or A/A (dark iris) genotype. Moreover, monosomy 3 was more frequent in the G/G genotype than in the A/G + A/A genotype group.

These results suggest that the genetic makeup of a patient may influence some of the tumour features or the susceptibility of tumours to develop certain characteristics. One may hypothesise that populations with different pigmentation features may have different susceptibility to the well-established prognostic factors, that were discovered in studies performed in populations with a high risk of developing UM (i.e. populations with many people with fair skin and light eyes).

New therapeutic targets

The treatment of primary UM is highly successful in managing the tumour in the eye. The main challenges in this disease are the high rate of metastases and the lack of effective preventive strategies and treatment options for metastatic disease. Hence, the community is constantly in search of potential new targets. A therapeutic option that recently showed promise is Tebentafusp, [45-49] which is a bispecific molecule consisting of an anti-CD3 single chain antibody fragment and a monoclonal high-affinity T cell receptor (TCR) targeting the tumour antigen gp100. Tebentafusp is classified as an Imm-TAC (immune-mobilizing monoclonal T-cell receptors against cancer) and it can be used in patients with HLA-A*2:01.We explored two potential targets: Microphthalmia-associated transcription factor (MITF) and PRAME.

Because dark pigmentation is associated with a bad prognosis, we were interested in the regulation of melanin synthesis. Since MITF is the master regulator of melanin synthesis and is important for melanocyte development, one would expect MITF expression to be higher in darker tumours compared to lighter ones. However, we were surprised to find that the opposite was true when we looked at a Leiden cohort of 64 cases. We noticed that MITF may not only be involved in melanin synthesis and pigmentation, but it may have other functions related to cell survival and behaviour. To further complicate the picture, evidence on the role of MITF in cutaneous and uveal melanoma is not unanimous. On one hand, MITF promotes tumour cell survival and proliferation, [50-57], which are needed for local tumour growth. On the other hand, it inhibits invasion, epithelial-to-mesenchymal transition[58-60] and de-differentiation[50, 61-64], which are needed for metastatic spread. This balance would make targeting MITF in UM treatment particularly challenging.

In addition, we focused on a more promising target: PRAME. The fact that PRAME is highly expressed in tumours and in the testes but has no/very low expression in other healthy tissues makes it an attractive therapeutic target.[65] Immune therapies that target PRAME, alone or in combination with other antigens, are being developed.[66-70] T cell therapy is a promising option, and Gezgin et al. showed that T cells are able to recognize PRAME-expressing UM cells.[24] Two different approaches exist: expansion of pre-existing PRAME-specific T cells[71-77] and engineered TCR-transduced T cells[78, 79] targeting PRAME. The negative selection of high-avidity T cells against self-antigens in the thymus may make the latter option more feasible. At the moment, PRAME-specific TCR-transduced T cells are being tested in patients with recurrent or relapsing advanced or metastatic solid tumours, among which is UM (NCT03686124 and NCT04262466).

The search of new therapeutic targets usually starts with *in vitro* studies, using cell lines to study disease mechanisms and to test potential agents. It is, therefore, extremely important to use cell lines that are truly representative of the disease under investigation. Two of the UM cell lines that are frequently used in UM research lack the typical UM mutations in *GNAQ* and *GNA11*: Mel285 and Mel290.[80, 81] We decided to study these two cell lines in more depth and confirmed that they do not harbour other UM-related mutations either (*CYSLTR2 or PLCB4*) and that they express extremely low levels of melanocyte and melanoma markers, which calls into question their identity of UM cell lines. While we could not identify an alternative origin or any specific source of contamination, we can state that they are not typical UM cell lines and may not be representative models to test potential therapeutic targets for UM. The main proteomic and gene expression differences between 12 other cell lines were due to mutations in the BAP1 gene, which is one of the most important determinants of prognosis.

Conclusions and future perspectives

Uveal melanoma is still a focus of study for many groups around the world, because there are still several questions open and there is much room for improvement in the treatment of metastases.

Over time, we have gained better understanding of the disease and we have improved our diagnostic and prognostic accuracy. There are multiple valid prognostication tools available and, even though there is no gold standard, it is becoming increasingly clear that combining histo-pathological, patient-related and molecular prognostic markers allows more accurate prediction of metastatic risk. However, most of the studies are performed in populations with high risk of UM, which also have a high incidence of fair skin and blue eyes. Based on our data, we can hypothesise that different populations may show a different sensitivity to develop uveal melanoma and uveal melanoma metastases and may need different prognostication algorithms.

As for the treatment, local tumour control can be achieved effectively but it is not yet possible to prevent or treat metastases with the current therapeutic options. Nonetheless, the efforts to find new therapeutic targets continue and progress is being made, especially in the field of targeted therapy. We believe that we should keep looking for therapeutic targets and that T cell therapy is a promising strategy and that PRAME should be considered among the targets to study.

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