New approaches to imaging and treatment of ocular melanoma

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GENERAL DISCUSSION AND APPENDICES

Chapter 7: Summary and General Discussion

Chapter 8: Appendices

7

Summary and General Discussion

IMAGING AND TREATMENT OF OCULAR MELANOMA

The aims of this thesis were to better diagnose ocular melanoma lesions using new imaging techniques, and to identify new targets for ocular melanoma therapy. Both conjunctival melanoma (CoM) and uveal melanoma (UM) have been studied: malignancies that share a need for better understanding and therapy, yet each with its distinct genetic background and clinical presentation. A common link in several projects of this thesis is 'angiogenesis'. This was assessed to better understand tumour growth, for diagnostic use, and as a target for therapy. We believe that the inclusion of UM as well as CoM in this thesis, and the inclusion of basic projects as well as clinical projects, resulted in a comprehensive overview with a better understanding of both malignancies.

PART I – CONJUNCTIVAL MELANOMA

Summary and discussion

CoM is a rare ocular tumour with an incidence of 0.3-0.8 per million in Caucasians.[1-4] It has a high recurrence rate (of approximately 40% in 5 years)[1,5] and high metastatic potential (of approximately 20% in 10 years)[6,7]. There is a need to diagnose patients early, and to develop better therapies, especially for advanced and metastatic disease.

This thesis starts by analyzing current CoM patients and their clinical outcome (**chapters 2.1, 2.2, 2.3**). The findings support the call for better therapies and provide recommendations regarding clinical follow up. Next, we summarize the current knowledge on the genetic and immunologic background of CoM (**chapters 3.1, 3.2**). This provides a basis for diagnostic and prognostic purposes, and indicates targets for new therapies based on genetic and immunologic principles.

CoM has high recurrence and metastasis rates

Better therapies in CoM are urgently needed because of the substantial rates of recurrences and metastases.[4,5] While several studies have reported on this topic, most study groups are small and only assess a limited follow-up time. This may be not surprising due to the rarity of CoM and fragmented healthcare systems in many countries, however it compromises conclusions on prognostic features. In the Netherlands, national referral centers for ocular oncology have been appointed and a national oncology registry exists (i.e. OncDoc / RANK), which allowed us to obtain a large cohort of 70 patients with good-quality follow-up data (**chapter 2.1**). We identified the importance of early referral to a center with expertise, as patients who had a first excision elsewhere had a significantly higher recurrence rate. This may be due to incomplete excision or suboptimal surgical approach with a risk for tumour dissemination. For localized CoM, we found that surgical excision alone is not an appropriate therapy, and that adjuvant strategies are required. There is currently no data favouring a particular strategy.[8,9] Our approach includes brachytherapy (currently with Ru-106

plaques) for bulbar lesions, and addition of mitomycin c drops when a component of primary acquired melanosis (PAM) is present. Results of this approach in development of recurrences and patient survival are favourable compared to the literature and could be advised for other centers as well.[10]

Tumour pigmentation is an important clinical feature of CoM

While assessing our cohort of CoM patients, we were struck by the variety of clinical presentations. CoM may range from amelanotic and pink to black, reflecting different types of tumour pigmentation. Melanin has a role in melanoma formation and behaviour – as is known from work on skin melanoma and UM[11]- and this posed the question whether pigment characteristics are related to CoM behaviour. We studied pigment in a combined set of 444 CoM patients from Leiden and Philadelphia (USA), notably one of the largest reported cohorts on CoM. In **chapter 2.2** we describe that lightly-pigmented CoM have a worse clinical outcome compared to darker lesions. This may result from characteristics of different types of melanin,[12] but also from treatment-related factors such as early identification and visualization of tumour margins. In **chapter 2.3** we compared the original CoM lesions with their recurrences. We show that recurrences are more often lightly-pigmented than their parent lesions, but any pigmentation status can occur. This finding may be due to a loss of pigment-producing ability in more malignant melanocytes, or because primary amelanotic lesions are more easily overlooked. As clinical outcome did not relate to pigmentation of recurrences (as it did to pigmentation of the primary lesion), this may imply that metastases have an early origin more related to the primary lesion than to the recurrence, or that recurrences have been treated more heavily.

CoM requires a thorough and lengthy follow-up

Regarding the clinical management of CoM, we emphasize the importance of proper follow-up and identification of conjunctival lesions. Recurrences of CoM may not only show a variety of pigmentation (**chapter 2.3**), but also occur even after several years, as we illustrate by a patient who developed two late recurrent lesions; one recurrence developed 21 years after excision and cryotherapy, the other developed 4 years after orbital exenteration (**chapter 6.3)**. This implies that CoM is prone to 'tumour dormancy'[13] with cells that spread prior to surgical therapy. Proper identification of conjunctival lesions during follow-up is therefore important to provide appropriate care. Importantly, when assessing conjunctival lesions, clinicians should always be wary of secondary causes of melanoma, as the conjunctiva is prone to harbour metastases of distantly-located melanoma types.[14] We present a patient with a conjunctival lesion that proved to be a metastasis of a cutaneous melanoma (**chapter 6.1**). This patients was treated successfully with new targeted/immunotherapy, stating the relevance of these new therapies. Illustrating that not every pigmented conjunctival

lesion is malignant, however, was our observation in a patient who received brachytherapy for UM and later developed two pigmented spots on the sclera, presumably consisting of pigment-loaded macrophages requiring no further treatment (**chapter 6.4**).

The genetic background of CoM is that of an extraocular melanoma

Recent work shows that CoM harbours mutations in genes such as *BRAF*, *NRAS*, *NF1* and *TERT*, and that rare mutations can occur in *KIT* and other genes.[15-19] This profile resembles cutaneous melanoma[20,21] and illustrates the position of CoM as an *extraocular* tumour different from UM (e.g. with mutations in *GNAQ/11* and *BAP1*).[22-24] Assessment of genetic mutations in CoM confirms that ultraviolet (UV) radiation is a contributing factor for tumour development, with many C>T alterations and a high mutational burden;[25-27] however, CoM can develop both at sun-exposed as well as non-exposed sites, implying that UV is not a necessity for its development.

Precursor lesions of CoM, such as conjunctival nevi and PAM,[28] harbour similar mutations as found in CoM and while frequencies in reported genes differ, no truly exclusive mutations are known.[17,29-32] This limits the use of genetics to differentiate benign from malignant lesions and illustrates that key moments in tumorigenesis of CoM are yet to be identified. Mutational status can be used to differentiate melanocytic lesions with a conjunctival origin from a uveal origin however, relevant in specific cases of UM tumour outgrowth or in cases with an unknown primary lesion. Very recent reports show that (anterior) uveal melanoma may harbour *BRAF* mutations,[33] and CoM may sporadically harbour BAP1 mutations however,[34] which though unlikely, limits this approach.

The prognostic relevance of mutations in primary CoM is currently limited since studies are not consistent regarding their clinical outcome, and hampered by small sample sizes. Recent work shows that *TERT* mutations may relate to metastasis, and that these mutations are very rare in benign disease, so this may become an important new factor in CoM staging.[35] A promising approach regarding the genetic traits of CoM is that of micro RNA (miRNA) analysis, which - although in an early phase - may be informative by analysing many genes at once to differentiate and prognosticate lesions.[36-38]

Presence of immune infiltrate in CoM suppresses tumour growth but needs further identification

In addition to tumour genetics, inflammation is one of the hallmarks of cancer and has been recognized as an important factor for tumour development and behaviour.[39] Tumour infiltrate in CoM consists of several cell types, including lymphocytes and macrophages with different effector functions. The presence of inflammatory cells is known to be favourable in CoM,[40-42] suggesting benefit from tumour surveillance. This observation shows that – also in this matter – CoM resembles cutaneous melanoma while this is in contrast with UM where inflammation is a sign of malignancy and worse clinical outcome.[43]

The role of macrophages in CoM is poorly understood, but as these cells can promote angiogenesis (especially the predominantly identified M2 subtype),[44] it is likely that they exert an unfavourable effect on CoM growth as is known from cutaneous melanoma and also from UM.[45]

One of the important immunological mechanisms (checkpoints) of host-tumour interactions is the PD-1/PD-L1 pathway.[46] In this, expression of molecules causes downregulation of the immune system and thus allows unrestricted tumour growth. We showed that PD-L1 is expressed in CoM and this expression relates to worse survival as can be hypothesized by the mechanism of action (**chapter 3.2**). This is similar to observations from cutaneous melanoma.

New therapies for CoM follow genetic and immunologic findings

A consequence of the findings on tumour genetics and immunologic behaviour of CoM are the theoretical benefit of 'targeted' and 'checkpoint inhibitor' therapies, as were recently introduced for cutaneous melanoma. New therapies like these are urgently awaited for CoM cases where conventional therapy is not sufficient. To our knowledge, no clinical trials or large series on this topic exist, but small reports on CoM patients illustrate the benefit for locally advanced as well as metastatic disease.

Targeted therapy includes *BRAF* and MEK inhibitors, and several reports have been presented on successful tumour control in CoM (reviewed in **chapter 3.1**). In addition, a plethora of drugs is being evaluated in preclinical studies (targeting eg *KIT*, *TERT*, or EZH2).

Checkpoint inhibitors act by host-tumour interaction, as by the earlier mentioned PD-1/PD-L1 axis. Looking at tumour sections and in vitro models, we showed a rationale for usage of anti PD-1/PD-L1 drugs in CoM (**chapter 3.2**). Cases of patients who were treated with these drugs have been reported with successful outcome (reviewed in **chapter 3.1**).

Drawbacks to new therapies for CoM

Two unfortunate drawbacks of the currently-available new therapies are to be mentioned: *treatment resistance* and the *development of side effects*.[47-50] To overcome the first issue, a combination of therapies may be required, targeting several pathways simultaneously. Importantly, genetic screening and typing of CoM allows for a personalized approach to best fit patients and drugs. Side effects of the new therapies should be monitored to adapt the therapy, or to allow for side effect treatment. Since immune-related side effects are a relatively new phenomenon in medicine, this calls for clinical attention. Notably, immune-related side effects can be ocular – while admission of new drugs is systemic – and ophthalmologists should therefore be wary of these in any oncology patients treated with immunologic drugs for non-ocular malignancies.[51] We show a case of development of ocular rosacea following ipilimumab and nivolumab use, that was effectively treated with topical steroids (**chapter 6.2**).

Future perspectives

Current studies on genetics and immunology in CoM demonstrate that much is still to be learned about tumour development and behaviour. Similarly though, it shows that by this knowledge new promising therapies are visible around the corner. A better characterization of CoM (based on genetics, precursor lesions, and external stimuli such as melanin and UV-radiation) will allow for better prognostication and individualized therapies. In addition to drugs targeting *BRAF* and MEK, and immunotherapy against PD-1 and CTLA4, new drugs targeting *Kit, NF1, TERT*, or *EZH2* are awaited. New drugs will mostly benefit metastatic patients, but may also be beneficial to patients with advanced local disease as an alternative to extensive surgery. A secondary effect of these new therapeutic options is the relevance of better tumour staging. Apart from staging based on tumour material, this includes the use of lymph node staging by the sentinel lymph node biopsy[52] and imaging.

A promising development in ocular oncology is the recognition of CoM as distinct disease entity within ocular melanoma, and the awareness of clinicians worldwide about this. Early referral to tertiary centers should become regular practice, as should be the use of appropriate adjuvant therapy. Besides a direct benefit for current patients to receive best treatment, this facilitates research on larger numbers of patients, benefiting future patients as well.

PART II – UVEAL MELANOMA

Summary and discussion

UM is the most common type of ocular melanoma with an incidence of 5.1-8.6 per million in Caucasians.[53,54] It comprises melanoma of the choroid, ciliary body and iris. Up to 50% of patients die from metastatic disease,[55] with unchanged numbers over the last five decades.[56] Many concepts and therapies that apply to other forms of melanoma are not effective for UM due to its distinct genetic background and immune-privileged position in the eye.[57] Differentiating benign from malignant uveal lesions can be challenging, while the first are harmless and there is an urgent need for development of better therapies for the latter.

In this part of the thesis, we first address the genetic and immunologic profile of UM, which are very different from what is seen in cutaneous melanoma and CoM (**chapter 3.1**). We focus on activation of the growth-related YAP1 pathway as potential predictor of metastases and as therapeutic target for UM (**chapter 4.1**). Next we study angiogenesis as a factor defining UM behaviour and as link between tumour genetics and clinical outcome (**chapter 4.2**). In a patient setting using new imaging devices, we study vasculature in both uveal and conjunctival lesions to differentiate benign and malignant disease (**chapters 5.1, 5.2**).

The genetic and immunologic background of UM are different from cutaneous and conjunctival melanoma

UM has a remarkable genetic profile and immunologic background, very different from what is seen in cutaneous melanoma and CoM (**chapter 3.1**). UM's are characterized by early mutations in *GNAQ/11*, and secondary mutations in *BAP1*, *EIF1AX* and *SF3B1*.[57] There is no role for UV radiation in the etiology of *posterior* UM, while new insights show that *anterior* UM occasionally demonstrate typical UV-induced genetic signatures.[58] The presence of immune infiltrate is unfavorable in UM, suggesting that immune cells fail to destroy the tumour; a possible explanation is found in the expression of immune inhibitors such as *Indoleamine 2,3-dioxygenase* (IDO1) and *T cell immunoreceptor with Ig and ITIM domains* (TIGIT), limiting immune responses.[59] Newly-introduced targeted and immunotherapy are currently not successful in UM, which is again attributed to the altered immune response compared to what is seen in extraocular CoM and cutaneous melanoma.[60]

The YAP1-pathway is involved in tumour growth and provides a new approach to UM therapy

Cell growth is regulated by several stimuli, including the YAP1 pathway.[61] Interestingly, YAP1 is activated by the *GNAQ/11* mutation that is commonly identified in UM,[62,63] and the YAP1 pathway received recent interest as player in UM behaviour and as candidate for therapy; it can be inhibited by the readily available ophthalmic drug verteporfin.[64]

In **chapter 4.1** we study the YAP1 pathway in both UM and CoM. We show that YAP1 expression is higher in UM with an unfavorable genetic profile and tends to be associated with worse clinical outcome. In vitro tests with verteporfin show a response in several UM cell lines, but only a limited response in CoM cell lines and (slow growing) BAP1-negative UM cell lines, demonstrating that not only the studied genetic background but also traits such as cell growth rate underlie drug sensitivity. While verteporfin may not be best as a single-use drug for UM, targeting the YAP1 pathway may be part of an approach for UM and beneficial to overcome drug resistance with other agents.

Angiogenesis relates to tumour genetics and worse clinical outcome in UM

Angiogenesis is important for the development and behavior of UM.[45,65] Vessels provide nutrients and oxygen to a tumour, and provide a route for metastatic cells to disseminate. Angiogenesis is influenced by the tumour micro environment as immune cells can produce pro-inflammatory and pro-angiogenic cytokines. It was recently demonstrated that genetic events in UM relate to the presence of immune cells[66] and we therefore wondered whether genetic events relate to (markers of) angiogenesis. In **chapter 4.2** we show that vascular density relates to the genetic profile, with an increased vascular density in M3/BAP1-loss UM. Status of chromosome 8q (of which gain is an early event)[67] was not related to the vascular density, indicating that true increased angiogenesis is a later event. Increased vascular density was associated with expression of ANGPT2, VWF and remarkably less VEGF-B, a cytokine that needs further elucidation (in contrast to the better-known VEGF-A).

A key regulator of angiogenesis is HIF1a.[68] Drugs targeting HIF1a are currently under investigation in UM[69] and we wondered which patients could benefit most. We showed that higher expression of HIF1a was observed in BAP1-loss UM. This provides information on the development of UM and suggests that tumours with M3/BAP1-loss may be the best candidates for HIF1a targeting.[70]

Clinical assessment of retinal oximetry differentiates between choroidal melanoma and nevi

Tumour vessels are currently assessed in clinical practice to differentiate benign from malignant ocular lesions. This can be done using fluorescein angiography, with injection of dye and assessment of vascular patterns and leakage.[71,72] Drawbacks to the technique are the invasive nature and limited use in anterior segment lesions particularly of the conjunctiva as dye easily leaks from conjunctival

vessels.[73] As proliferating tumour cells are expected to have an increased metabolism, we studied oxygenation of retinal vessels in eyes with a choroidal melanoma or nevus using a relatively new imaging device (Oxymap T1) (**chapter 5.1**). While choroidal nevus eyes had no alterations, we found different oxygen values in choroidal melanoma eyes, including in retina not-overlying tumour tissue. The observed alterations may be due to a different oxygen metabolism, inflammation, and relocation of flow in melanoma eyes. As a diagnostic technique, other techniques may currently be more specific, but retinal oximetry adds to this knowledge and also allows for future monitoring of treatment-related (radiation) effects.

OCT-Angiography is feasible for CoM and UM of the anterior segment but currently limited by imaging and software techniques

A new non-invasive imaging technique to depict the structure of vessels of the eye is OCT-Angiography (OCTA). While being developed to study retinal vessels,[74] we applied this technique to the anterior segment with the aim of visualizing tumour vessels in the iris and conjunctiva (**chapter 5.2**). We show that vessels can be depicted, but that obtaining good-quality images is highly dependent on patient and tumour characteristics such as cooperation and pigmentation status. Within nevi as well as melanoma, we found tortuous vascular patters, distinct from healthy iris and conjunctiva. We did not observe differences in vascular density or patterns between benign and malignant lesions, however, possibly hampered by a small sample size and the reported limitations of current imaging techniques.

Future Perspectives

The search for treatment of (disseminated) UM continues, and several targets are under investigation. Multi-pathway blocking may overcome issues with current drugs, and targeting the YAP1 pathway is a promising route as part of treatment for UM. Verteporfin, as a readily-available ophthalmic drug, may also demonstrate other usage such as slowing down tumour growth while waiting for (radiation) therapy. The immune privilege of the eye, and the position of UM, needs better understanding to possibly introduce drugs that revolutionized therapy of cutaneous, and conjunctival, melanoma.

New imaging techniques are promising in the non-invasive approach to diagnose ocular lesions. For the assessment of tumour vessels, developments in imaging resolution and analysis software are beneficial to overcome artefacts of tumour pigment and lesion thickness. Oximetry of retinal vessels may perhaps not be an addition for diagnostic purposes, but a candidate to monitor treatment response, in combination with structural imaging using OCTA. The latter has proven suitable to detect minor vascular aberrations in UM eyes and may be implemented more with the renewed studies into radiation retinopathy following the application of anti-VEGF therapy.

CONCLUDING REMARKS

Over the last two centuries, much has changed in the field of ocular oncology. The implementation of the ophthalmoscope (to visualize intraocular lesions in patients), and histological assessment (to visualize individual melanoma cells) were only the beginning of a path that led to advanced diagnostic procedures and therapeutic possibilities. A variety of imaging techniques is currently available to study melanocytic lesions, and cell traits can be studied on a genetic level identifying subclones within single tumours. Surgery, radiotherapy and conventional chemotherapy have been complemented by individualized (targeted/immune) therapy for specific tumour cells.

Why then, two centuries of study later, is ocular melanoma still a deadly condition and is the call for better management still urgent? As we demonstrate in this thesis, a first explanation may be that 'ocular melanoma' is not a homogenous field of study, and that in fact it comprises a variety of tumour types. Not only UM and CoM have different traits, but as knowledge continues, subgroups within UM and within CoM are being identified, all requiring a different approach. Second, the rarity of these entities does not allow for large-scale trials. Collaborations, internationally, are therefore further needed to answer the pending questions with sufficient numbers. In line with rarity is lack of exposure for many (general) ophthalmologists, calling for specialized structures of healthcare. And third, perhaps the era of digital imaging and personalized medicine has only just started. For CoM, some major advances coming from cutaneous melanoma have been introduced and it is expected that this will largely benefit patients in the coming years. For UM, a personalized approach needs further study of possible targets, but it is not unlikely that new drugs will follow shortly. Technological advances develop by the day, and as we look upon how much technology has changed in a decade, who knows what imaging techniques will be developed. This thesis, naturally, can only aim to be a piece in that large puzzle, and hopefully adds to the path of making ocular melanoma a disease of the past.

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