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Genetic prognostication in uveal melanoma

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Chapter 8

Summary, Discussion, and Future Perspectives

SUMMARY AND GENERAL DISCUSSION

Uveal melanoma (UM) is a relatively rare malignancy that is fatal in up to 50% of patients.¹ Despite improvement in the treatment of the primary tumor over the last decades, the survival of UM patients has remained poor.² It may be that effective treatment of the primary tumor only prevents metastatic spread from small tumors, whereas patients with large tumors already have subclinical micrometastases at the time of diagnosis of their primary tumor (**Chapter 1**). The fact that the prognosis of UM patients has not improved despite excellent local control of the primary tumor implies that the survival of UM patients can be mainly enhanced by potent therapies targeting those metastases. The continued unraveling of the biology of UM, mainly through an increased understanding of the genetics and molecular pathways of UM, has contributed to the identification of novel promising targets for therapy and new clinical trials have commenced in recent years. Against the backdrop of the growing number of clinical trials, reliably determining a patients' risk of developing metastases has become increasingly important since only high-risk patients are enrolled in most trials. Accurate stratification of patients based on metastatic risk aids in the identification of patients who may benefit from experimental therapies and in whom the effects of experimental systemic treatments can best be evaluated.

Besides its importance for the enrollment of patients in clinical trials, reliable prognostication is relevant for reassuring patients at low-risk and providing the opportunity for life-planning in high-risk cases. Furthermore, it allows clinicians to tailor follow-up measures based on metastatic risk: surveillance can be offered for high-risk cases, while low-risk patients can be spared costly and possibly invasive examinations. Various demographic, anatomic, histologic, and genetic features of UM have been proven to be prognostic parameters in UM. Particularly certain recurrent and non-randomly occurring aberrations in chromosomes 1, 3, 6, and 8 are valuable prognostic markers (**Chapter 2**). The loss of chromosome 1p, monosomy 3, and gain of chromosome 8q are associated with an adverse clinical outcome, while gain of chromosome 6p is correlated to a favorable prognosis.³⁻⁶ The concomitant occurrence of chromosome 3 loss and gain of 8q conveys an even greater risk of metastatic death than either of the aberrations alone.⁷ Recently, UMs have been subdivided into two prognostic classes, class 1 of low-risk UMs and class 2 of high-risk UMs, based on gene expression profiling (GEP).⁸

GEP classes correspond strongly with chromosome 3 status.⁹ The discovery of aberrations in the genes *BAP1*, *SF3B1*, and *EIF1AX* has added a new dimension to genetic prognostication in UM.¹⁰⁻¹² Inactivating mutations in *BAP1*, which is located on chromosome 3p, are associated with formation of metastases, and rarely occur in non-metastasizing UM.¹⁰ Combining chromosome aberrations and GEP with gene mutations has been shown to refine genetic prognostication: disomy 3 cases with a wild-type *BAP1* gene have a 10-fold lower risk of developing metastases in case of a mutated *EIF1AX* gene, while *SF3B1*-mutant disomy 3 tumors are associated with an increased metastatic risk at the long-term.^{12, 13} Germline mutations in *BAP1* have been reported in approximately 20% of familial cases of UM and are associated with larger tumor size and ciliary body involvement, both of which are related to an unfavorable prognosis.^{14, 15} Moreover, patients with germline *BAP1* mutations are at higher risk of developing other cancers such as lung adenocarcinoma, renal cell carcinoma, meningioma, and malignant mesothelioma.¹⁶

Although 80% of UM patients who eventually develop overt metastases do so within 10 years post-treatment, the remaining substantial group of UM patients develops metastases many years after treatment of their primary tumor.¹ Besides variety in the effectiveness of host immune responses in controlling tumor growth, diversity in the aggressiveness of metastasizing tumor cells may influence the moment patients are diagnosed with clinical metastases. In **Chapter 3**, we compared demographic and tumor features between patients who died early (<three years post-enucleation) due to UM metastases and those dying at a later stage (between three to five years and more than five years post-enucleation). Additionally, we performed a competing risks regression analysis to evaluate factors that are associated with UM-related death in patients surviving more than five years after enucleation. We corroborated that patients dying at a late stage are more often females and younger at enucleation, and have smaller, AJCC stage I, tumors which exhibit a spindle-cell morphology. Monosomy 3 and gain of 8q were detected in 57% and 67%, respectively, of patients dying due to UM metastases after five years post-treatment. These figures were 68% and 71%, respectively, in patients dying between three to five years after enucleation, and 90% and 80%, respectively, in patients dying in the first three years following enucleation. In patients surviving more than five years following enucleation, female gender and gain of chromosome 8q were the only features independently associated with death due to UM metastases. This information is important for

the counseling of patients with an indolent UM type, such as the GEP class 1 tumors. In addition, reliably identifying patients who may die at a late stage can be useful for effectively targeting adjuvant therapy, since patients with slow growing metastases may benefit the most from such a treatment, contrary to patients with more aggressive metastases who die soon after treatment of their primary tumor. The identification of patients at risk of dying at a late stage due to UM metastases may be improved by future studies evaluating the combination of chromosome status or GEP and specific mutations such as *SF3B1* and *EIF1AX*. As mentioned above, these mutations have been demonstrated to stratify metastatic risk in indolent UM types.

However, survival is not only determined by genetic factors: combining several types of prognostic parameters may enhance risk stratification in UM. Damato and associates have developed a multifactorial algorithm which generates individual prognostic curves based on patient age and sex, tumor size, histologic features, and chromosome 3 loss.^{17, 18} Albeit much progress has been made in the genetic typing of UM and many genetic prognostic indicators have been identified, the value of traditional prognostic parameters such as tumor size for prognostication should not be neglected. Two recent studies have reported that tumor diameter provides prognostic information that is independent of GEP.^{19, 20} Several reports have proposed that combining the American Joint Committee on Cancer (AJCC) staging system, which uses the Tumor-Node-Metastasis (TNM) classification, and genetic features of UM may result in better risk stratification in UM.^{17, 18, 21} In 2014, Bagger et al. demonstrated that AJCC stage III and chromosome 3 and 8 aberrations are independent prognostic factors in a cohort of 153 Danish UM patients.²² In **Chapter 4**, we investigated the effect of combining the AJCC staging and information on the chromosome 3 and 8q status on prognostication in UM in a cohort of 522 patients, consisting of 275 UMs enucleated in the LUMC and 247 Danish cases. We show that combining the AJCC stage and chromosome 3 and 8q status of tumors enhances risk stratification in UM. The prognostic value of the AJCC staging system, which is an internationally recognized and validated prognostication method, is enhanced when information on the chromosome 3 and 8q status is added. Reversely, AJCC staging stratifies the risk of dying due to UM metastases in patients with tumors harboring monosomy 3 and/or chromosome 8q gain. The results of our study can be utilized to further improve the AJCC staging system, which has become more sophisticated in recent years but does not yet include genomic information.

Moreover, it corroborates that prognostication based on genetics may be improved by including non-genomic indicators, as proposed earlier.¹⁷⁻²² Since GEP correlates well with chromosome aberrations, combining the AJCC staging and GEP may refine prognostication, as Kivelä and Kujala suggested earlier.²¹ Although GEP has been claimed to be superior to chromosome testing,^{23, 24} GEP classes correspond strongly to certain chromosome aberrations and combining multiple chromosome alterations has been reported to substantially increase the prognostic accuracy of chromosome testing.^{5, 7, 9, 25-27} Traditionally, and partly due to the high costs associated with mRNA expression analyses, many ocular oncology centers use chromosome analyses for prognostication in UM. As most UMs are nowadays treated by radiotherapy, we wondered what the effects of irradiation are on chromosome testing in UM. In **Chapter 5**, we analyzed what the effect of prior irradiation is on the success rate of karyotyping and fluorescence in situ hybridization (FISH) and evaluated whether pre-enucleation irradiation results in specific chromosome aberrations. We demonstrate that prior irradiation is a limiting factor for successfully performing karyotyping as well as FISH. Karyotyping succeeded in 79% of non-irradiated UM (primary enucleations) whereas the success rate was only 25% in enucleations that had been treated by radiotherapy. Fluorescence in situ hybridization was successfully performed in 83% of primary enucleations and 49% of irradiated enucleations. Interestingly, the reason for secondary enucleation after prior irradiation seemed to influence the success rate of karyotyping and FISH. Both tests were more often successfully performed in irradiated tumors that were enucleated due to tumor recurrence, when compared to cases enucleated because of lack of response or occurrence of radiation-related complications. However, differences were not significant, which may most probably be due to the low number of cases. We propose that the higher success rate in tumors enucleated because of recurrence is probably related to the fact that the newly arisen tumor is unaffected by the radiobiological effects of irradiation, which has been reported to cause necrosis and fibrosis.^{28, 29} This supports the practice of taking biopsies for genetic testing before applying radiotherapy. Similar to our results, Horsman et al. reported a success rate of only 58% for karyotyping in irradiated cases.³⁰ Other genetic tests may be more successful in irradiated tumors, as shown by several groups that used CGH,³¹ MLPA, MSA,³² and GEP.³³ However, these studies were performed in small cohorts. Another intriguing finding of our study was the higher frequency of chromosome aberrations and the complex karyotypes in previously-irradiated enucleations.

This made us wonder whether genetic results obtained after irradiation are representative of the primary genetic status of the tumor. Compelling evidence on the reliability of genetic testing in irradiated cases is however lacking and, accordingly, the use of the DecisionDx-UM GEP in irradiated samples has been considered ineligible by the manufacturer of this commonly-used kit.³⁴ Future studies evaluating pre- and post-radiotherapy genetic results in larger cohorts with longer follow-up are necessary to determine the effects of irradiation on the reliability of genetic typing in UM.

Although radiotherapy is an effective eye-sparing method to treat primary UM, the therapy fails in a proportion of patients. A primary UM may be non-responsive to radiotherapy or a new tumor may arise after radiotherapy seemed successful initially. Although the evidence is inconclusive, several studies have reported on the association of genetic features such as chromosome 3 status and GEP class and tumor regression rate after irradiation.³⁵⁻³⁸ Since irradiation has its effects by causing DNA damage, resulting in tumor cells going into senescence, stopping dividing and becoming apoptotic, we hypothesize that DNA repair pathways may play a role in the responsiveness of a UM to radiotherapy. However, DNA repair is an underexposed subject in UM research and not much is known about the role of DNA repair pathways in the development and progression of UM. Apart from their role in the sensitivity of tumors to radiotherapy, aberrant DNA repair proteins play a role in the development of various cancers and may be a target for therapy.^{39, 40} The lack of knowledge regarding DNA repair in UM hampers such approaches in UM. In **Chapter 6**, we report on the results of a study evaluating the expression of DNA repair genes in UM. We hypothesized that there is a significantly differential expression of DNA repair genes between UM with a favorable prognosis and UM with an adverse clinical outcome. In an effort to test our hypothesis, we identified 121 genes encoding proteins involved in DNA repair pathways, based on the available literature. The expression of a group of 44 genes, that showed an acceptable level (standard deviation > 0.3) of variation in Illumina gene expression in a cohort of 64 UMs, was compared between disomy 3 and monosomy 3 tumors. After validation in 110 tumors from Genoa and Paris and in 80 UMs of The Cancer Genome Atlas (TCGA) project, four genes were repeatedly found to be significantly differentially expressed between UM with a favorable or poor prognosis. A low expression of *WDR48*, *XPC*, and *BAP1* was related to an adverse clinical outcome, while this was also the case for a high expression of *PRKDC*. Since *WDR48*, *XPC*, and *BAP1* are located on chromosome 3p, which is

frequently lost in poor prognosis UM, we propose that deficient DNA repair may be one of the consequences of the loss of chromosome 3. Inadequate DNA repair mechanisms may contribute to the accumulation of gene mutations and chromosome abnormalities that promote tumor growth and malignant transformation. The *PRKDC* gene is located on chromosome 8q, which is frequently amplified in poor prognosis UM. Intriguingly, the DNA-PKcs protein encoded by the *PRKDC* gene has been shown to be a driver of migration and metastasis in prostate cancer and a high expression has been related to a worse survival in hepatocellular carcinoma.^{41, 42} Kotula et al. have revealed that DNA-PKcs promotes invasion and migration.⁴³ In our study, inhibition of DNA-PKcs had a growth inhibitory effect in UM cell lines. Moreover, we show that DNA-PKcs inhibition suppresses the expression of *SNAIL1*, which has been shown to be involved in invasiveness of UM cells.⁴⁴ Considering the pro-metastatic functions of DNA-PKcs and its location on chromosome 8q, it is conceivable that this protein plays a role in metastatic outgrowth of UM and should therefore be investigated as a potential target for therapy in UM.

Epigenetics is another field that has recently gained a lot of attention in cancer research but about which little is known in UM. Epigenetics is the study of heritable changes that do not involve changes in a cell's nucleotide sequence.⁴⁵ Epigenetic modifications, mainly by DNA methylation and histone modification, result in the alteration of the expression of genes. There is increasing evidence for the importance of epigenetics in the pathogenesis of UM, and it has been shown that several genes such as the *RASSF1a* tumor suppressor gene on chromosome 3p, and the *pINK4a* gene involved in cell cycle regulation, are often silenced in UM by promoter DNA methylation.^{46, 47} Recently, hypomethylation of *PRAME* has been reported.⁴⁸ The TCGA project on UM showed that the methylation pattern of this tumor is associated with its chromosome 3/BAP1 status.⁴⁹ Besides promoter methylation, histone modification plays a role in UM development and progression. The *BAP1* gene, whereof the loss is associated with metastasizing UM, is a histone-modifying enzyme that encodes a deubiquitinating enzyme.¹⁰ Loss of BAP1 is associated with hyperubiquitination of histone 2A.⁵⁰ Since epigenetic regulators are seemingly important for UM development and progression, we wondered which epigenetic modifiers play a role in UM. In **Chapter 7**, we compared expression levels of 59 epigenetic modifiers between disomy 3 and monosomy 3 tumors. We observed a general downregulation of the expression of epigenetic modifiers in monosomy 3/GEP class 2 tumors. After analysis in a

validation set, we found a significantly lower expression of the epigenetic regulator genes *KAT2B*, *HDAC11*, *KMT1C*, *KDM4B*, *KDM6B*, and *BMI-1* in monosomy 3/GEP class 2 cases. In this study, we have shown the association of a low expression of epigenetic modifiers with adverse prognosis UM and corroborated the association of epigenetic dysregulation with UM development and progression.

CONCLUSIONS AND FUTURE PERSPECTIVES

In the last few decades, many advances have been made in the prognostication of UM. This has mainly been fueled by discoveries of prognostically relevant genetic markers. First, recurrent and nonrandom chromosome alterations such as monosomy 3, chromosome 6p gain, and chromosome 8q gain were identified. This was followed by the finding that UMs can be separated into two prognostic classes based on their gene expression profile. Recently, mutations in specific genes (*BAP1*, *SF3B1*, *EIF1AX*) which provide further risk stratification have been reported. These discoveries have not only improved prognostication, but also resulted in the increased understanding of the pathogenesis of UM, and contributed to the identification of new targets for therapy.

In this thesis, we explored ways of improving genetic prognostication in UM, evaluated the effect of irradiation on chromosome testing, and investigated the association of DNA repair genes and epigenetic regulators with prognosis in UM. We reveal that monosomy 3, chromosome 8q gain and histologic features characteristic of high malignancy are less often observed in tumors of patients who die due to UM metastases more than five years after enucleation, and show that gender and chromosome 8q status are the only parameters that independently influence disease-specific survival after five years following enucleation. This finding is important for the counselling of patients with an indolent UM type, such as those with a class 1 tumor who may still develop metastases many years after diagnosis. We demonstrate that combining the AJCC staging and information on the chromosome 3 and 8q status improves the prognostic value of both prognostic systems. The recently published 8th edition of the AJCC staging does not yet include information on genetics and could be improved by incorporating anatomic staging and genetics in the next edition, as supported by the findings of our study. Furthermore, our study shows that, despite considerable progress made in genetics of UM, traditional staging methods provide additional risk stratification and should be considered together with genetics for accurate prognostication in UM. We provide evidence that

supports the current practice of taking biopsies before applying radiotherapy, since chromosome testing may fail more often in irradiated cases or be non-representative of the primary genetic status of the tumor. Studies evaluating pre- and post-radiotherapy genetic testing in larger studies with longer follow-up are necessary to validate the use of genetic tests in irradiated tumor samples. Our studies show that the expression of certain DNA repair genes and epigenetic regulators is associated with prognosis. Future studies need to be conducted to explore their role in the pathogenesis of UM and their potential as therapeutic targets.

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