Targets for personalized treatment of conjunctival melanoma

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Chapter 7

Summary, Discussion, and Perspective

Summary and general discussion

Although conjunctival melanoma (CM) is not a common disease, many aspects need to be considered in terms of medical care: treating the primary malignancy and preventing local recurrences, regional and distant metastases. The subject of this thesis is to better understand the biology of CM and look for potential targets of personalized treatment, as conventional chemotherapy has little effect on metastatic CM. Instead of the conventional medical concepts of "one disease, one target" and "one-size-fits-all", personalized medicine monitors the individual's comprehensive profile, and facilitates the development of multiple therapeutic strategies which may provide the best option. Therefore, I first set out to do experiments to identify the potential targets within CM, and subsequently studied the efficacy and safety of some pharmacological compounds, which have already shown therapeutic effects in other malignancies, on CM in laboratory circumstances.

Understanding the molecular mechanisms and pathways of CM may unmask oncogenic targets and explain how tumor cells escape from immune surveillance, thereby providing new therapeutic strategies to prolong survival of patients with CM. It would be great if our pre-clinical laboratory studies will lead to new treatment options with clinical applications, although many challenges remain to be addressed.

Cancer is a genetic abnormality. On the one hand, mutations occur in proto-oncogenes and can activate the oncogenic signaling pathway, letting tumor cells grow out of control. On the other hand, mutations existing in tumor suppressor genes may reverse the downregulation of cell division, thereby promoting tumor cell growth. Recognizing and targeting the right mutations is of great importance for targeted therapy, and for minimizing undesirable side effects. In **Chapter 2**, by performing immunohistochemistry (IHC) with specific antibodies, we discover frequent *BRAF* V600E mutations in conjunctival nevi (19%) and CM (26%). On the contrary, primary acquired melanosis (PAM) with or without atypia in the conjunctiva did not show any *BRAF* V600E mutation. Since CM mainly derives from PAM with atypia rather than a nevus, one may assume that only the presence of a

BRAF V600E mutation is not enough for tumor formation, and that other mutations in oncogenes and/or tumor-suppressor genes are needed to form CM. In addition, phosphorylated-ERK and phosphorylated-AKT are expressed in conjunctival melanocytic lesions, indicating that the MAPK and PI3K/AKT pathways are constitutively activated and can serve as targets for CM. *In vitro* experiments show that the BRAF inhibitors Vemurafenib and Dabrafenib inhibit the growth of *BRAF* V600E-mutated cell lines, while the MEK inhibitor MEK162 and AKT inhibitor MK2206 inhibit the proliferation of all CM cell lines, regardless of their mutation status. Moreover, combining MEK162 and MK2206 shows a synergistic effect on cell viability and the cell cycle profile, and induces more cell death than the single compounds. Our findings suggest that clinical investigations should be carried out to test the efficacy and safety of those small molecule inhibitors in CM.

Besides genetics, epigenetics is of great importance in regulating cell cycle and survival. Epigenetics changes the genome without altering nucleotide sequences. Epigenetic modifications play an important role in cellular transformation to malignancy: tumor-suppressor genes are frequently silenced by epigenetic alterations in cancer. Targeting epigenetic alterations has been shown to affect cancer prevention, detection, and treatment [1,2]. Enhancer of zeste homolog 2 (EZH2) is a core subunit of the polycomb repressive complex 2 (PRC2) family. Overexpression of EZH2 has been shown in many cancers, and has been associated with poor survival [3]. Some drugs that target epigenetic changes have been developed and have shown a high potency *in vitro* and *in vivo* against some cancers [4]. In **Chapter 3**, similar to cutaneous melanoma and prostate cancer, we also observe frequent overexpression of EZH2 in primary CM, and show that EZH2 expression is associated with tumor thickness. In contrast, EZH2 is not expressed in normal conjunctival melanocytes. Subsequently, we carried out *in vitro* experiments using genetic knockdown or pharmaceutical inhibition, and reveal that both types of inactivation of EZH2 lead to a significant decrease of cell viability, colony formation, EZH2-mediated protein expression and induce cell cycle arrest. EZH2 inhibitor GSK503 shows similar apoptosis induction as EZH2 depletion does, however,

another EZH2 inhibitor UNC1999 does not induce apoptosis in all CM cell lines, suggesting additional effects of the first drug. Furthermore, GSK503 inhibits growth of CM cells in zebrafish at a well-tolerated concentration. Our findings suggest that EZH2 may provide a new therapeutic option for CM patients.

Animal models have many advantages for studying cancer. Researchers can control and/or exclude potential confounding factors which may affect the results. Especially regarding such a rare malignancy as CM, the lack of human tissues limits the material available for further investigations. Animal models, therefore, may provide more opportunities to study tumor behavior and therapeutic strategies for CM. Currently, there are only a few proper animal models for CM research. While the treatment of primary CM has gained success in past years, we aim to explore potential therapies for distant metastasis. In **Chapter 4**, we first subconjunctivally injected three primary CM cell lines into nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) IL-2 rγ^{null} mice. Primary tumor growth was successfully detected in all cases subsequently, whereas no metastasis developed. Therefore, we next applied an *in vivo* passaging technique: cells derived from subconjunctival xenografts were implanted into the subconjunctival space of the same strain of immunodeficient mice, and this method eventually resulted in frequent lung metastases. This murine model mimics the natural route of human metastasis as CM often spreads to the lung. Another benefit of this model is that corresponding xenografts maintain their mutation status present in the cell lines. This is especially important for testing new drugs targeting these mutations. Our research can provide novel options to prevent metastatic dissemination, and furthermore, to treat lung metastases. However, we should be aware that the lack of a functional immune system in immunodeficient mice limits the possibility to study the immunology of CM.

The discovery of immune checkpoints inhibitors has opened a new era of personalized treatment for advanced cutaneous melanoma, non-small cell lung cancer, bladder and renal cancer [5]. Considerable efforts have taken place to translate these exciting investigations towards clinical treatment. As targeted

therapies may induce resistance, current efforts of drug development pay a lot of attention to boost T-cell function, thereby killing tumor cells. The inhibitory immune checkpoint molecules cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death 1 (PD-1) play an essential role in inhibiting T cell signaling. Antibodies blocking these two molecules have achieved great success in treating advanced malignancies and have led to good clinical responses [6]. The successful achievement of blocking PD-1/PD-L1 axis may be dependent on PD-L1 expression on the tumor cell membrane [7,8]. Recently, the predictive value of PD-L1 expression by tumor-infiltrating lymphocytes (CTL), which constitute the tumor microenvironment, for the clinical effect of the inhibitors has also been addressed [9]. In **Chapter 5**, we wonder whether treatment of immune checkpoint blockade can be applied to CM, and we study the expression of PD-L1 in CM, and the relation to the tumor microenvironment. Our results show that PD-L1 is expressed on tumor cells of 19% primary CMs and stromal cells (mainly $CD68^{+}CD163^{+}$ M2 macrophages) of 59% tumors. The tumoral PD-L1 expression is correlated with distant metastases and worse prognosis. Interestingly, the density of TILs is negatively associated with tumor size: large tumors contain less TILs than small tumors, suggesting tumor cells grow out when there are not enough infiltrating immune cells. These results suggest that advanced CM patients could be recruited for PD-1/PD-L1 axis blockade therapies.

Cancer utilizes multiple methods to escape immune recognition and attack. Currently, seven parameters have been characterized in relation to cancer-immune interactions, including general immune status, tumor foreignness, tumor sensitivity to immune effectors (human leukocyte antigen (HLA) expression and IFN-γ sensitivity), absence of inhibitory tumor metabolism, absence of soluble inhibitors, and absence of checkpoints [11]. The activation of cytotoxic T lymphocyte (CTL) responses by antigen-presenting cells demands the presence of HLA class I-associated peptides. HLA expression is often impaired on tumor cells, thus avoiding "visualization" by the immune system. No studies have as yet shown the correlation between HLA expression and clinical outcomes of anti-PD-1 or anti-CTLA-4

immunotherapies. However, studying tumor sensitivity will be helpful to understand the mechanisms of resistance of immunotherapy and to find out which patients are less likely to respond to T-cell mediated therapies. Furthermore, downregulation of HLA class I expression is considered a biomarker of poor prognosis in many cancer types [12]. In **Chapter 6**, we therefore examine HLA class I expression, and analyzed its correlation with some clinical parameters in CM. The immunofluorescence staining shows frequent downregulation of HLA class I expression, and that its expression is associated with the presence of some TILs but not with PD-L1/PD-1 status. We also observe that thick tumors often present weak or no HLA class I expression. T cell effector cytokines, especially IFN-γ, are a crucial promotors of HLA expression. The association of HLA class I expression and TILs may suggest that in thick tumors, less TILs produce IFN-γ, thereby impairing HLA expression, decreasing immune sensitivity and allowing tumors to grow unrestrained. We tested the influence of IFN-γ *in vitro* by incubating CM cell lines with IFN-γ to mimic the tumor environment and study the molecular changes. The mRNA and membranous protein expression of HLA class I and components of the antigen presentation machinery indeed were increased upon IFN-γ treatment. *In vivo* results show that HLA class I expression was maintained in a murine xenograft. In summary, our findings suggest that downregulation of HLA class I may contribute to the mechanisms of immune escape in CM. And it is necessary to enhance tumor HLA class I expression and improve the efficacy of T cell-based immunotherapy.

Conclusions and future perspectives

CM is a disease with its own characteristics: it is often mismanaged by family doctors at its early stages because of its rarity; ophthalmologists treat the primary malignancy and cooperate with pathologists to determine the accurate diagnosis and to schedule the necessary therapeutic strategies. Frequent local recurrences reduce the quality of life and the development of metastases may result in death. Therefore,

more collaborations should be made by ophthalmologists, pathologists, oncologists, geneticists and biochemists to provide better medical options to prevent and treat metastases in CM patients. Understanding tumor biology and immunology is a tedious but essential task. Nowadays, still, conventional chemotherapies may not show an effect in some malignancies and cause severe side effects; more attention therefore needs to be paid to personalized treatments and many studies indeed are showing promising clinical outcomes. One is encouraged to evaluate the patients' condition and make precise decision, intervention and treatment strategies for patients with cancer.

In this thesis, we have explored biological and immunological mechanisms in CM. We reveal that HLA class I expression is frequently downregulated in CM, which may contribute to tumor immune evasion. Our studies demonstrate that *BRAF* and *NRAS* mutations, EZH2 overexpression and PD-L1 expression can be targets for personalized therapies. We show that the establishment of a murine metastatic CM model mimics the human malignancy and can facilitate further pre-clinical analysis for CM. Our findings will support knowledge-based treatments of this disease. It is no doubt that more and more potential molecular targets will be uncovered by basic science and oncology research. Future studies will most likely investigate the combination treatment targeting multiple pathways and activating the function of immune cells. We hope our attempts may provide novel therapeutic options and prolong survival of CM patients.

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