In search of synergy: novel therapy for metastatic uveal melanoma

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Chapter 6

General Discussion

UMCure 2020

The work presented in this thesis is a part of the Horizon UMCure 2020 project, which has had the advantage of collaborations between distinct disciplines, including basic as well as clinical researchers, oncology specialists and patients in order to find effective approaches to treatment of metastatic uveal melanoma. At the beginning of the project, the main biological mechanisms responsible for progression of UM, such as most recurrent mutations in *GNAQ/GNA11*, *BAP1* and chromosomal abnormalities, had been identified and reported. However, the understanding of the underlying biological processes has not converted yet into an effective treatment. The clinical trials of various classes of therapeutic agents, such as DNA-damaging agents, targeted inhibitors and immune checkpoint inhibitors demonstrated limited improvement of overall survival of the patients, often at cost of severe adverse effects.

Within the scope of this thesis we investigated the signaling landscape of metastatic UM and searched for novel avenues of therapy.

Preclinical Evaluation of Trabectedin in Combination with Targeted Inhibitors for Treatment of Metastatic Uveal Melanoma

In Chapter 2 we report that the multitarget drug Trabectedin inhibits growth and induces apoptosis of UM cells *in vitro* and reduces tumor growth of UM Patient-Derived Xenografts (PDX) in *in vivo* models. Trabectedin has been reported to demonstrate efficiency in pre-clinical studies for various types of solid tumors and has been designated by FDA as a second line treatment for soft tissue sarcomas [1-5].

Lurbinectedin, a less toxic modification of Trabectedin, demonstrated significant effect for patients with small cell lung cancer and has been approved by FDA as a second line treatment. The combinations of Lurbinectedin with other chemotherapeutics, such as DNA-damaging agents doxorubicin and paclitaxel, Pembrolizumab (mAb against PD-1), Durvalumab (mAb against CD274) are currently under investigation in the clinic for solid tumors, particularly small cell lung cancer [6-9].

We demonstrate that combination of Trabectedin with either the Casein Kinase 2/CDC-like kinases double-inhibitor Silmitasertib or with the c-Met/TAM (TYRO3, AXL, MERTK) receptor inhibitors Foretinib and Cabozantinib synergistically inhibits proliferation and enhances apoptosis in UM cell lines. Due to the multitarget nature of Trabectedin as well as the other applied drugs, it is difficult to pinpoint a precise mechanism of the observed synergistic effect. At the same time, we demonstrate that in case of Foretinib and Cabozantinib, attenuation of activity of the TAM receptors, particularly MERTK, but not of c-Met, is essential to inhibit proliferation of UM cells and synergize with Trabectedin.

To our knowledge, this is the first report about a putative role of MERTK and TYRO3 in proliferation of UM cells *in vitro*. The role of TAM receptors in pathology of UM has not been previously discussed, except for a report by van Gienkel et al., who found up-regulation of AXL in UM cells compared to uveal melanocytes [10]. It is important to note, that the investigators used an atypical uveal melanoma cell line, MEL290, which lacks a mutation in *GNAQ* or *GNA11*. Interestingly, we found essentially no expression of AXL in all tested UM cell lines containing a *GNAQ* or *GNA11* mutation, while, indeed, MEL290 and another cell line without *GNAQ/GNA11* mutation, MEL285, show high expression. So, although we could verify the expression of AXL in MEL290, we question the importance of this study, since most cell lines lack AXL expression while MERTK and/or TYRO3 are highly expressed all of them.

The recent publication of Kaler et al. highlights the role of MERTK not on UM cells, but on adjacent CD163+ macrophages [11]. Mechanistically, BAP1-negative UM cells demonstrated elevated expression of one of TAM receptors' ligand, PROS1, which interacted with MERTK on CD163+ macrophages, causing MERTK phosphorylation and activation, what eventually resulted in polarization of the macrophages into a suppressive M2 state. Expressed PROS1 was shown to localize on the membrane of cancer cells, although PROS1, as well as the other TAM receptor ligand, GAS6, can be secreted. We found that also *GAS6* mRNA expression is significantly up-regulated in BAP1-negative tumors, and *GAS6* expression negatively correlates with survival of the patients. In this respect, it might be interesting to investigate if autocrine activation of TAM receptors takes place in BAP1 negative UM cells.

Novel Treatments of Uveal Melanoma Identified with a Synthetic Lethal CRISPR/Cas9 Screen

In chapter 3 we show that targeting either IGF1R or DNA-PKcs in in combination with inhibition of mTOR by everolimus, synergistically slows down proliferation of UM cell lines. Although the combinations of everolimus with either DNA-PKcs inhibitor KU-57788 or IGF1R inhibitor OSI-906 did not demonstrate tumor regression of an UM PDX in an *in vivo* model, we could find significant activity of the dual DNA-PKcs/mTOR inhibitor CC-115 on this UM PDX.

The initial report by Amirouchene-Angelozzi et al., who first tested everolimus on UM cell lines, indicated that apoptosis is not induced upon the treatment even at relatively high doses, although the proliferation of the cells slows down [12]. In their follow-up work the authors suggested that combining everolimus with PI3K inhibitor GDC0941 switched the mode of action to apoptosis induction, thus preventing the activation of the putative compensatory mechanisms of mTOR inhibition [13].

In our work we exploited pooled CRISPR-Cas9 dropout screening in order to search for effective synergistic combination of everolimus in an unbiased way. The library we used consisted of sgRNAs targeting over 500 kinases, allowing us to assay their activity in one experiment. However, the hits of the screen, namely DNA-PKcs and IGF1R, when combined with everolimus, induce fast onset of cell cycle arrest rather than apoptosis in UM cell lines. Probably, the dropout screen setup that was used facilitated identification of the sgRNAs causing cell cycle arrest rather than cell death. In order to find the hits associated with apoptosis induction, the application of another read-out, for example activity of caspases 3/7, might be advantageous.

Elevated IGF1R activity is associated with tumor progression in several cancer types; in addition, activation of IGF1R signaling is a known mechanism of resistance to mTOR inhibition [14-16]. Various inhibitors of IGF1R signaling have been developed and clinically investigated as monotreatment or in combination with, among others, mTOR inhibitors. The initial enthusiasm over the outcome of first clinical trials has quickly shifted to understanding that IGF1R blockade might be beneficial only for selected cohorts of patients [17]. The combinational treatment by mABs targeting IGF1R and mTOR inhibitors demonstrated significant, but short lasting, clinical responses in patients with Ewing sarcoma [18]. On the contrary, in patients with metastatic colorectal cancer, treatment with everolimus in combination with OSI-906 indicated no clinical activity [19]. For the patients with metastatic UM, limited clinical benefit was achieved by everolimus combined with pasireotide, a drug that indirectly modulates IGF1R signaling. These studies together with our pre-clinical data indicate existence of a resistance mechanisms for targeting mTOR/IGF1R axis.

Simultaneous inhibition of mTOR and DNA-PKcs has mainly been studied in the context of prostate cancer, where both these kinases, featuring some structure similarities, fuel in androgenic receptor signaling - the main driver of prostate tumor progression [20]. The phase I trial of dual mTOR/DNA-PKcs inhibitor CC-115 in combination with an inhibitor of androgen receptor signaling, Enzalutamide, in patients with metastatic castration-resistant prostate cancer showed high activity and good tolerability [21]. CC-115 was concluded to be a promising anticancer treatment, based on the outcome of phase I study in patients with advanced solid tumors [22]. Our studies demonstrate antiproliferative activity of CC-155 on PDX UM in *in vivo* model.

Combined Mcl-1 and YAP1/TAZ inhibition for treatment of metastatic uveal melanoma

In Chapter 4 we show that genetic depletion YAP1/TAZ together with Mcl-1 inhibition synergistically reduces the viability of UM cell lines. YAP1 emerged as a putative therapeutic target in UM after publications by Li et al., who demonstrated that YAP1 signaling plays role in UM progression, and by Feng et al., who showed that YAP1 in UM is regulated primarily by a cascade dependent on mutated Gαq/11, rather than by canonical Hippo-pathway [23-25].

It worth noting, that signaling of YAP1 is mainly studied in these publications, while TAZ is recognized as a paralog of YAP1 with by default overlapping functions. However, our preliminary experiments indicate some discrepancies in the functioning of YAP1 and TAZ in UM. For example, genetic depletion of YAP1 results in G1 arrest, while knockdown of TAZ results in accumulation of cells in G2 cell cycle phase. Moreover, we noticed slightly stronger growth inhibition caused by TAZ knockdown compared to YAP1 knockdown. At the same time, double knockdown of YAP1 and TAZ inhibits growth of UM cells stronger than either of single knockdowns, indicating partly overlapping functions.

Our data, as well as the published reports, suggest that genetic interference with YAP1 or TAZ signaling inhibits the growth of UM cells, but does not fully stop proliferation. Therefore, blocking additional pathways will be necessary in order to abrogate UM progression. As follows from our small-scale drug screen, inhibition of Bcl-2 family members, particularly Mcl-1, synergize with YAP1 and TAZ knockdown. *Mcl-1* has been designated as a transcriptional target of YAP1, what might explain the observed synergistic effect [26,27]. However, in contrast to the publications mentioned above, we noticed upregulation of Mcl-1 on protein level upon prolonged (5 days) knockdown of TAZ, but not after 3 days, which suggests that this upregulation might be indirect response to stress, rather than transcriptional regulation (data not shown).

Mcl-1 gene has been found to interact with *WWTR1* gene in a functional tRNA-CRISPR screen, and simultaneous pharmacological inhibition of both targets has been suggested as potential treatment for non-small cell lung cancer [28]. In that particular study, as well as many others, verteporfin was used to interfere with interaction of YAP1 and TAZ with co-factors TEAD1-4 and block YAP1/TAZ dependent transcription. Since verteporfin engage multiple targets and its precise mechanism of action in unknown, we decided to utilize an inhibitor of the mevalonate pathway as an alternative approach to indirectly attenuate YAP1/TAZ signaling [29-31]. Geranyl-geranyl transferase inhibitor GGTI-298 synergized with Mcl-1 inhibition to antagonize UM cell proliferation. We realize that GGTI-298, as well as verteporfin, interferes with a number of cellular processes, what might be not optimal for clinical application, and utilization of specific small molecule inhibitors of YAP1 and TAZ, which are still under

development, might be more advantageous in the future. On the other hand, when we employed K-975, an inhibitor of palmitoylation of TEADs, on UM cell lines, and therefore also preventing the interaction of YAP1/TAZ with the TEADs, we could detect less effect on cell proliferation and less synergy in combination with Mcl-1 inhibition. Additionally, we noticed that effects of YAP1/TAZ inhibition, either genetic of pharmacological, vary substantially per cell line. Together with recent reports questioning the importance of YAP1/TAZ activity on UM patient prognosis, our studies could suggest that specific targeting of this pathway might not be the first choice for therapy of metastatic UM. However, we did not investigate the possible role of YAP1 and TAZ in migration of UM cells, what might still remain of interest in respect of prevention of metastatic spread.

Comparative analysis of phospho-proteome of primary and metastatic uveal melanoma cell lines

The recent studies of UM proteome are mainly centered around differences in protein expression between high and low risk primary tumors, or primary tumors and metastases [32, 33]. These studies bring further our understanding of molecular mechanisms of tumor progression. In our work we deliberately focused on phosphoproteomics, since protein kinases represent a major class of drug targets in oncology [34]. In Chapter 5 we compared the phosphoproteome of two metastatic UM cell lines and a primary tumor cell line derived from the tumor material from the same individual using mass-spectrometry. Mass spectrometry highlighted several differently phosphorylated sites on a number of proteins, such as ARHGEF2 and DLG5. The analysis of the upstream kinases of differentially phosphorylated sites revealed MARK3 as a putative target kinase.

Microtubule affinity-regulating kinase 3 (MARK3) negatively regulates microtubule growth by phosphorylating microtubule-specific proteins [35]. Phosphorylation of these proteins causes dissociation from microtubules and microtubule destabilization, what in turn dysregulates cell migration and might promote tumor invasion and metastasis [36]. In particular, MARK3 has been shown to phosphorylate ARHGEF2 (Rho/Rac Guanine Nucleotide Exchange Factor 2), and thus stimulate activation of RhoA, which is an essential up-regulator of YAP1 activity in UM [37]. The role of YAP1 and TAZ signaling in UM has been discussed in Chapter 4. Notably, we found that combination of YAP1/TAZ knockdown with the geranyl-geranyl transferase inhibitor GGTI-298, which is known to attenuate the activity of Rho proteins, synergistically slows down growth of UM cell lines. Similarly, combination of MARK3 knockout with GGTI-298 significantly enhances the effect on transcription of YAP1/TAZ target genes, and synergistically inhibits the growth of some UM cell lines. However, the synergistic effect is not very strong and cell line-dependent, therefore, the perspective of MARK3 as a therapeutic target in UM remains in question.

The association between *MARK3* expression and risk of death has been reported, however, this correlation can be either positive or negative depending on tumor type. We demonstrate that high *MARK3* expression is associated with increased risk of death in UM and that *MARK3* expression is significantly higher in the tumors harboring monosomy 3, correlating with poor prognosis.

Concluding remarks

The efforts of many investigators, including those participating in the UMCure 2020

consortium, have shed more light on the biology of UM, putative risk factors and targets. Still, many pieces need to be added to complete the puzzle.

One of the characteristic features of uveal melanoma is an ability to metastasize almost exclusively to the liver bypassing local lymph nodes. The underlying mechanism remains unclear, and important to investigate, since targeting the link between the tumor cells and a metastatic niche might prevent metastases formation.

The other unsolved riddle is the function of BAP1 in UM progression. Loss of BAP1 is a strong predisposing factor to metastases development, and it is not clear whether it is possible to prevent this effect. Since BAP1 functions as a transcriptional repressor, its loss triggers changes in expression of a plethora of genes, and the main downstream actors of BAP1, responsible for aggressive UM phenotype are yet to be identified. The revealed putative targets, such as *PROS1* or *GAS6*, genes associated with suppressive immune responses, and cell adhesion molecules, need further investigation [11, 38, 39].

Additional barrier, limiting therapeutic options, is the limited infiltration of immune cells into the tumor metastases - UM is referred to a class of immunologically “cold” tumors. Encapsulation by fibrous collagens might prevent the immune cells from infiltration and interaction with tumor cells[40]. Relatively low mutational burden of UM might be one of the factors of weak immune activation and poor response to immune checkpoints inhibitors. Moreover, heterogeneity of metastatic UM makes it very difficult to target all subclones by a uniform approach. Nevertheless, some success in application of immunotherapy approach to metastatic UM treatment has been achieved. The case study of the patient with extraordinary response to PD-1 inhibitor emphasizes the importance of patient stratification and personalized approach to treatment. The recent clinical success of Tebentafusp, a drug that directs T cells to glycoprotein 100–positive tumor cells, highlights one of the possible directions of future therapy development.

These general considerations needed to be addressed for further treatment development, which might take long before implementation in clinical care. In this thesis we focused on the known targets and the drugs that have been approved by regulators or have entered clinical trials. We suggested several synergistic drug combinations that demonstrated efficacy *in vitro*, for example Trabectedin with Cabozantenib, or GGTI-298 in combination with MIK665, or the dual inhibitor CC-115, as promising candidates for further clinical investigations.

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