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Uveal melanoma: Towards a molecular understanding

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A B S T R A C T

Uveal melanoma is an aggressive malignancy that originates from melanocytes in the eye. Even if the primary tumor has been successfully treated with radiation or surgery, up to half of all UM patients will eventually develop metastatic disease. Despite the common origin from neural crest-derived cells, uveal and cutaneous melanoma have few overlapping genetic signatures and uveal melanoma has been shown to have a lower mutational burden. As a consequence, many therapies that have proven effective in cutaneous melanoma -such as immunotherapy- have little or no success in uveal melanoma. Several independent studies have recently identified the underlying genetic aberrancies in uveal melanoma, which allow improved tumor classification and prognostication of metastatic disease. In most cases, activating mutations in the Gα11/Q pathway drive uveal melanoma oncogenesis, whereas mutations in the *BAP1*, *SF3B1* or *EIF1AX* genes predict progression towards metastasis. Intriguingly, the composition of chromosomal anomalies of chromosome 3, 6 and 8, shown to correlate with an adverse outcome, are distinctive in the *BAP1*^{mut}, *SF3B1*^{mut} and *EIF1AX*^{mut} uveal melanoma subtypes. Expression profiling and epigenetic studies underline this subdivision in high-, intermediate-, or low-metastatic risk subgroups and suggest a different approach in the future towards prevention and/or treatment based on the specific mutation present in the tumor of the patients. In this review we discuss the current knowledge of the underlying genetic events that lead to uveal melanoma, their implication for the disease course and prognosis, as well as the therapeutic possibilities that arise from targeting these different aberrant pathways.

1. Introduction

Uveal melanoma (UM) is the second most common form of melanoma, arising from melanocytes located in the uveal tract of the eye. It is a highly aggressive disease, with a strong tendency to metastasize from the eye to other organs, such as the liver. The primary tumor can be treated successfully using several options, such as enucleation, stereotactic radiotherapy, brachytherapy and proton therapy (Damato, 2018; Singh et al., 2011). At the time of diagnosis of the primary tumor, only 4% of patients show detectable metastases; however, up to half of all UM patients will eventually develop metastatic disease despite earlier successful local treatment of the primary tumor. This implies that UM already develops micro-metastases early during tumorigenesis and that these micro-metastases may remain dormant for several months or even years (Eskelin et al., 2000). Once these micro-metastases become overt, the prognosis is poor and disease-related death usually occurs within one year (Augsburger et al., 2009). Metastases are often detected within a few years after diagnosis, but they can also be observed several decades after the initial diagnosis (Coupland et al., 1996; Singh et al., 2004; Zimmerman et al., 1978). In general, UM can be subdivided into three metastatic risk groups: high, intermediate and low risk.

Several clinical and histological features can predict high metastatic risk, such as large tumor size, extraocular extension, high mitotic activity and an epithelioid cell type, whereas the spindle cell type is associated with low metastatic risk (Fig. 1) (McLean et al., 1982; Shields et al., 2009; Yavuzyigitoglu et al., 2016a). Genetic features associated with metastatic disease include loss of chromosome 3 and mutations in the *BAP1* and *SF3B1* gene. BRCA-associated protein 1 (*BAP1*) mutations are observed in approximately half of all UM and usually result in metastasis within 5 years (Harbour et al., 2010). Our group has shown that tumors with an *SF3B1* mutation also frequently metastasize, but this can take up to 15 years and these tumors are therefore considered to have an intermediate metastatic risk (Yavuzyigitoglu et al., 2016b). UM that harbor a mutation in the *EIF1AX* gene seldom metastasize (Martin et al., 2013). None of the 45 UM patients in our cohort with only an *EIF1AX* mutation developed metastasis.

Recurrent chromosomal alterations are frequently observed in UM and the majority occur in the context of a specific mutation. Most of the *BAP1*-mutated UM show loss of chromosome 3, as well as gain of 8q. Tumors with a mutation in the *EIF1AX* or *SF3B1* gene often show gain of chromosome 6p (Yavuzyigitoglu et al., 2017). Since there is little heterogeneity in UM, it is likely that although these mutational and chromosomal events can occur sequentially, both are mandatory in the

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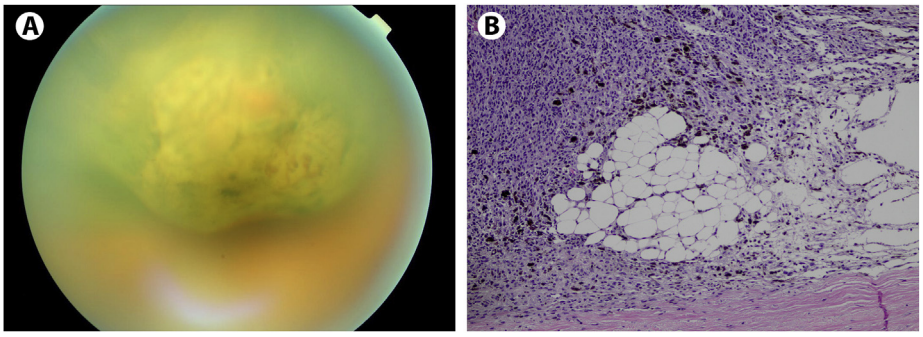


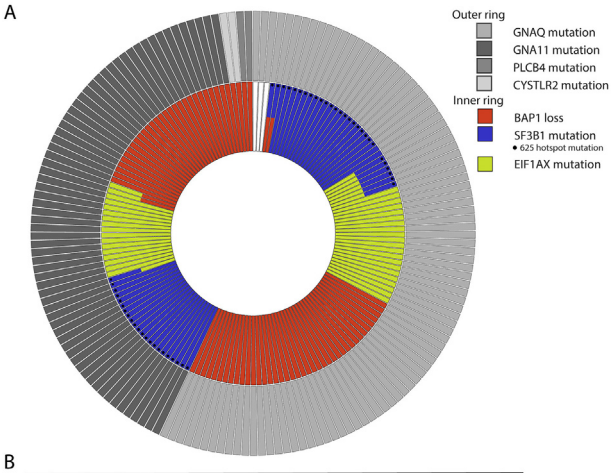
Fig. 1. The fundoscopic and histologic appearance of UM. A) A dome-shaped pigmented mass in the posterior pole. **B)** Hematoxylin-eosin (HE) staining shows that the tumor consists of spindle-type UM cells and several adipocytic appearing cells (100x). Reproduced with permission from Yavuzyigitoglu et al. (2016a), via Copyright Clearance Center.

development of UM to result in tumor growth (Field et al., 2018). Downstream mRNA expression can also be determined to predict metastatic risk. As previously described by Onken et al., UM patients can be classified into a low or high metastatic risk group based on the expression profile of 15 genes (Onken et al., 2004). These two groups are known as class 1 and class 2, with class 2 having the worst prognosis; class 1 can be divided into 1a and 1b. A more recent subdivision which is based on chromosomal data from The Cancer Genome Atlas (Robertson et al., 2017), separates the tumors into categories A-D (Jager et al., 2018).

Despite extensive research, the survival of metastasized UM patients did not improve over the last three decades (Singh et al., 2011). Whereas treatments such as immunotherapy and BRAF-inhibitors show promising results in patients with cutaneous melanoma (CM), UM seem to be unresponsive despite their shared origin as neural-crest derived melanocytes. This indicates that different mechanisms play a role in tumorigenesis. Oncogenic mutations in *BRAF* and *NRAS* are the main drivers in CM, but these mutations have only been found in iris melanoma and do not occur in posterior UM (Rimoldi et al., 2003; van Poppelen et al., 2018). Most CM (80%) exhibit a mutational signature specific to DNA damage caused by ultraviolet radiation, characterized by C > T transitions at the 3′ end of pyrimidine dinucleotides. Even though population studies suggest a geographic predisposition, there is no molecular evidence for this signature in UM (Robertson et al., 2017). UM has a remarkably low mutational burden; with a rate of < 1 single nucleotide variations (SNVs) per Mb, this mutation burden is much lower than observed in most cancer types. Only 35% of the observed SNVs in UM are C > T transitions and there is no enrichment of these lesions at the 3' position of pyrimidine dinucleotides, further showing that CM and UM have a different etiology (Furney et al., 2013). So far, UM clinical trials have focused on treatment modalities copied from CM. However, despite these therapeutic options, the prognosis of patients with metastatic UM has not improved, which emphasizes the need to explore and develop UM-specific therapies. In this review, we highlight several scientific findings and studies that provide us with insight into the mechanisms of oncogenesis of *GNAQ*, *GNA11*, *BAP1*, *SF3B1* and *EIF1AX* mutations. Elucidating the development of UM and obtaining a better understanding of the complex interaction between genetic factors, molecular signaling and potential targets will aid in developing new therapies specific for UM.

2. Genes involved in the development of UM

An updated mutational overview of our previously published ROMS cohort containing over 900 UM patients, shows initiating hotspot mutations in *GNAQ* in 57% of the UM tumors and in *GNA11* in 41% (Fig. 2A) (Yavuzyigitoglu et al., 2016b). Samples that do not contain a *GNAQ* or *GNA11* mutation are usually found to harbor a mutation in another gene linked to the Gα11/Q pathway*: PLCB4* and *CYSTLR2*. *GNAQ* and *GNA11* mutations are already observed in most nevi (Vader et al., 2017). In addition, in UM, one can often discern a mutation in one of the three secondary driver genes (*BAP1*, *SF3B1*, *EIF1AX*): forty-



B

Gene	Mutation rates Our study	Other studies	References
GNAQ	56%	42 - 46%	*Raamsdonk 2010; Dono 2014; Ewens 2014; Robertson 2017*
GNA11	40%	32 - 35%	*Raamsdonk 2010; Dono 2014; Ewens 2014; Robertson 2017*
BAP1	43%	31 - 50%	*Harbour 2010; Dono 2014; Ewens 2014; Robertson 2017*
SF3B1	26%	10 - 23%	*Martin 2013; Harbour 2013; Ewens 2014; Robertson 2017*
EIF1AX	21%	13 - 21%	*Martin 2013; Dono 2014; Robertson 2017*

Fig. 2. Driver mutations in UM. A) Donut chart showing the mutation status of 165 UM patients of the ROMS cohort. The outer ring shows initiating mutations in *GNAQ*, *GNA11*, *PLCB4* and *CYSTLR*. The inner ring indicates co-mutations in *BAP1*, *SF3B1* or *EIF1AX*. The black dots indicate *SF3B1* Q625 hotspot mutations **B)** The mutation rate of each gene observed in our study and other studies. Adapted from (Yavuzyigitoglu et al., 2016b).

four percent of our 175 UM samples showed a *BAP1* mutation, 26% a mutation in *SF3B1* and 21% a mutation in *EIF1AX*. We and others have noticed that even with next generation sequencing (NGS) technology mutations in *BAP1*, especially deletions encompassing whole exons, can be missed and more sophisticated calling algorithms, in combination with RNA sequencing have to be applied to detect these *BAP1* mutations (Field et al., 2018). We are using a combined BAP1-immunohistochemistry (IHC) and targeted NGS approach which is also suitable for small biopsies and formalin-fixed, paraffin-embedded (FFPE) tissue samples (Smit et al., 2018). The *BAP1* gene acts as a classic tumor-suppressor gene and in combination with loss of chromosome 3, no active nuclear BAP1 protein is present in the tumor cells (Farquhar et al., 2018). Missense mutations in *SF3B1* or indels in *EIF1AX* mutations are in-frame and create a small change in the respective proteins, albeit with a large effect on many cellular RNAs and proteins (Martin et al., 2013). Missense mutations in *SF3B1* usually arise at amino acid R625, a UM hotspot; however, some samples also show mutations outside this hotspot region, such as at amino acids K666 or H662 or in related spliceosome complex genes as *SRSF2* and *U2AF1* (Robertson et al., 2017). Although not actually a hotspot, *EIF1AX* in-frame mutations are located in the first 10–15 AA of this gene which limits the region to be analyzed. A complicating factor in NGS analysis is the closely-related pseudogene *EIF1AXP1* on

chromosome 1. When possible, we use a SNP array to detect chromosomal aberrations to confirm the UM subclass. Our results and the observed percentages do not deviate substantially from previously observed mutation rates (Fig. 2B) (Dono et al., 2014; Ewens et al., 2014; Harbour et al., 2010, 2013; Martin et al., 2013; Van Raamsdonk et al., 2009, 2010; Yavuzyigitoglu et al., 2016b). The secondary mutations are in general mutually exclusive, although 1–2% of the tumors did harbor (hotspot) mutations in two of these metastasis-associated genes. In these latter cases it would be interesting to see whether these mutations arise in the same cells.

2.1. Activation of the Gα11/Q pathway drives neoplastic growth of uveal melanocytes

The first gene reported to be mutated in UM was the guanine nucleotide-binding protein alpha Q (*GNAQ*) gene (Van Raamsdonk et al., 2009). Most of the UM samples that do not harbor a mutation in *GNAQ* carry a mutation in its paralogue *GNA11* (Van Raamsdonk et al., 2010). Both proteins are involved in the Gα11/Q pathway, which regulates several cellular processes such as proliferation and cell growth. In this pathway, leukotrienes activate the G protein-coupled receptor CYSTLR2 located at the cell surface. Guanine nucleotide-binding proteins (G-proteins) consist of three subunits: alpha, beta and gamma. GNAQ and GNA11 are alpha subunits (Gα) bound to a guanosine diphosphate (GDP). Upon binding of the ligand to CYSTLR2, G proteins are activated by exchanging GDP for guanosine triphosphate (GTP) (O'Hayre et al., 2013). GTP binding initiates a conformation change in the G-protein, which allows the G protein to be released from the CYSTLR2 receptor and to activate a large number of downstream effectors, such as PLCB4 and ARF6 (Moore et al., 2018; Yoo et al., 2016) (Fig. 3).

Activation of ADP-ribosylation factor 6 (*ARF6)* by GNAQ or GNA11 initiates several processes, such as β-catenin release from the cytoplasm to the nucleus and activation of the growth-inducing gene *YAP1* (Feng et al., 2014). YAP1 is critical for growth and is therefore often found in the nucleus of proliferating cells. Inhibiting YAP1 strongly limits the proliferation of UM cells (Feng et al., 2014; Yu et al., 2014a). Activated PLCB4 causes a rise in cytoplasmic Ca^{2+}, thereby activating several calcium-regulated pathways (Griner and Kazanietz, 2007). PLCB4 also indirectly activates the mitogen-activated protein kinase (MAPK) and AKT/mTOR pathway through the downstream effector RasGRP3 (Chen et al., 2017; Moore et al., 2018). MAPK and AKT/mTOR promote cell growth and proliferation and are often upregulated in cancer.

Over 95% of the UM contain a mutually exclusive mutation in *GNAQ* or *GNA11*. Mutations in *GNAQ* and *GNA11* affect residues Q209 and R183, which are required for the GTPase activity (Van Raamsdonk et al., 2009, 2010). In non-malignant cells, this activation is intrinsically terminated by a guanosine triphosphate (GTPase); oncogenic GNAQ or GNA11 on the other hand are constitutively activated and therefore result in over-activation of the aforementioned signaling pathways. UM that do not harbor a mutation in *GNAQ* or *GNA11* usually have a somatic mutation in *CYSTLR2* (3%) or *PLCB4* (2%) (Johansson et al., 2016; Moore et al., 2016). Wildtype CYSTLR receptors become active after binding of the ligand and transition to the inactivated state is initiated upon release of the ligand. However, mutated receptors stay active even after ligand dissociation and thereby constitutively activate GNAQ and GNA11 (Moore et al., 2016). This confirms the requirement of aberrant Gα11/Q signaling in the development of UM.

Introducing *GNAQ* and *GNA11* mutations (Q209L) in zebrafish results in increased proliferation, signaling and migration (Perez et al., 2018). However, most UM carry a *GNAQ* and *GNA11* mutation regardless of their tumor stage. This suggests that *GNAQ* and *GNA11* mutations are necessary to initiate tumorigenesis, but are insufficient to induce full malignant transformation, as is also shown by our finding that these mutations are also present in nevi (Vader et al., 2017). The aggressiveness of UM is determined by secondary driver mutations but treatments targeting oncogenic GNAQ and GNA11 signaling or one of their many downstream targets might reduce the proliferative potential of UM and can therefore be promising for future therapy modalities as will be discussed later in this review.

2.2. Loss of BAP1 is linked to metastatic UM

The majority of the metastasizing UM harbor -in addition to a *GNAQ* or *GNA11* mutation- a mutation in the *BAP1* gene located on chromosome 3 (Harbour et al., 2010). We and others observed, using immunohistochemical analysis, (partial) loss of the BAP1 protein in *BAP1*-

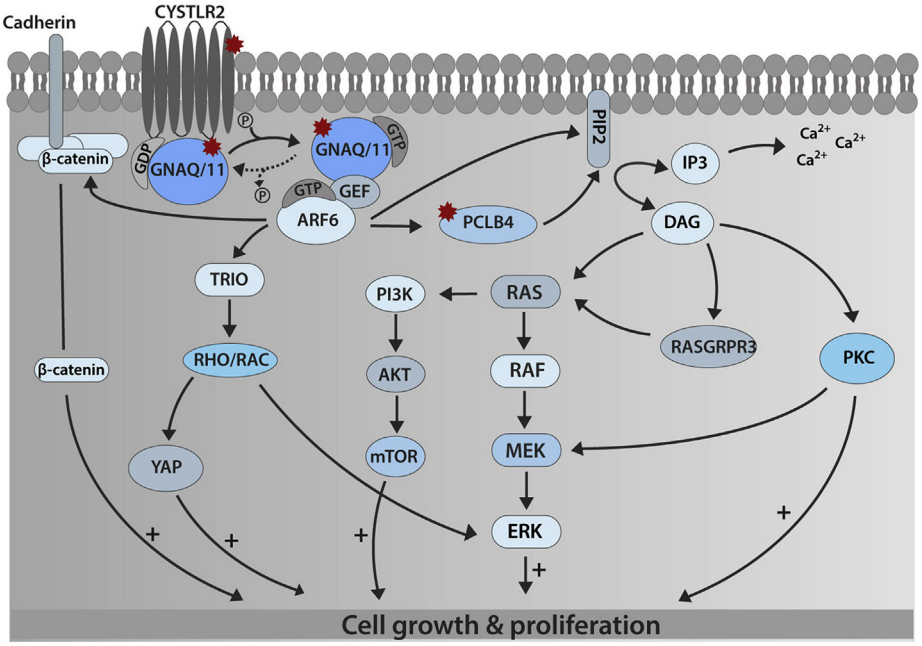


Fig. 3. Schematic overview of the Gα11/Q pathway and their downstream effectors. Activated GNAQ binds to a guanine nucleotide exchange factor (GEF), which in turn activates ARF6. ARF6 initiates β-catenin release, which promotes gene transcription (Grossmann et al., 2013). GNAQ and GNA11 can also activate the protein TRIO and the TRIO dependent RHO-GTPases; RHOA and RAC1. Once activated, RHO and RAC1 trigger the release of YAP and stimulate YAP-dependent transcription. ARF6 also activates PCLB4 which initiates hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP2) and produces 2 s messengers; inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). The production of IP3 causes a rise in cytoplasmic Ca2+, which stimulates several calcium-regulated pathways. With the help of DAG, protein kinase C (PKC) is activated and subsequently stimulates several processes, such as cell proliferation. DAG and PKC together also activate RAS guanyl-releasing protein 3 (RASGRP3), by binding and phosphorylation. RASGRPR3 is a GEF, that integrates GNAQ and GNA11 to the MAPK- and PI3K/AKT pathway by activating RAS. GEFs stimulate the release of GDP and the subsequent binding of GTP, thereby yielding active RAS (RAS-GTP) (Chen et al., 2017; Moore et al., 2018; Yoo et al., 2016; Yu et al., 2014a,b,c; Feng et al., 2014).

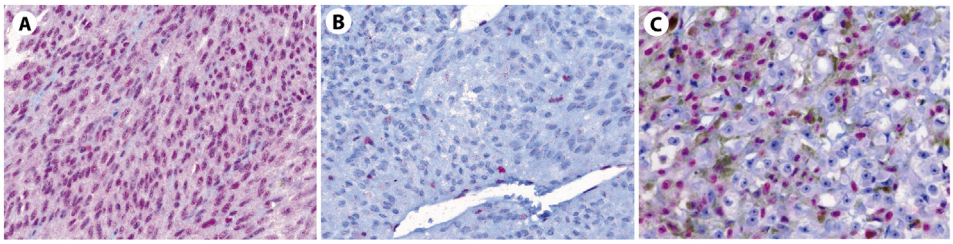


Fig. 4. BAP1-stained sections from three UM. Immunohistochemistry (IHC) staining of BAP1 protein (red) in **A)** A UM with positive BAP1 expression **B)** A UM with negative BAP1 expression and **C)** A UM showing a heterogeneous distribution of BAP1 throughout the tumor (400x). Reproduced with permission from: Koopmans et al. (2014).

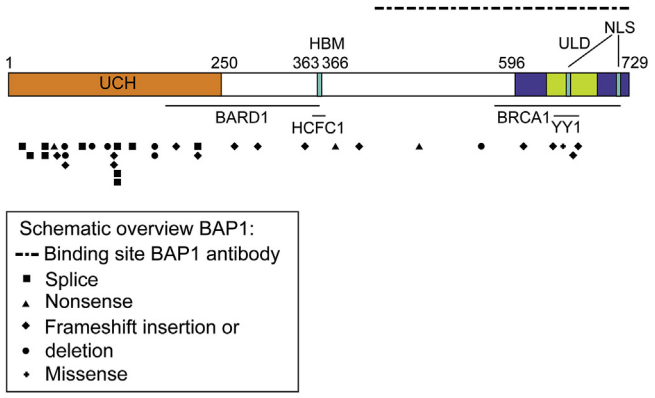


Fig. 5. Overview of the BAP1 protein and its functional and interacting domains. The *BAP1* mutations we observe in our ROMS cohort are depicted below the protein and classified according to their mutation type and location. The N-terminal UCH domain ranges from amino acid 1–250, the HCF1-binding domain (HMB)-like motif from amino acid 363–366, the UCH37-like domain (ULD) from 634–396 and the two nuclear-localization signaling (NLS) from amino acid 656–661 and 717–722. BAP1 also shows binding domains for BARD1, HCFC1, BRCA1 and YY1. The binding site of the BAP1 IHC antibody is indicated with the dashed line. Reproduced with permission from: Koopmans et al. (2014).

mutated UM (Fig. 4) (Kalirai et al., 2014; Koopmans et al., 2014; Shah et al., 2013; Stalhammar et al., 2019a; Szalai et al., 2018). Other studies show that loss of BAP1 staining is strongly correlated to GEP class 2 and chromosome 3 loss and that loss of BAP1 protein expression is often associated with lower BAP1 mRNA expression (van Essen et al., 2014). This implies that these mutations are loss-of-function mutations, requiring the loss of the other allelic copy harboring the wildtype gene (monosomy 3). Surprisingly, some *BAP1*-mutated/immunohistochemically BAP1-negative tumors still show expression of BAP1 mRNA, suggesting that negative nuclear staining for BAP1 protein is not solely caused by nonsense-mediated RNA decay but rather by an as yet unexplained different mechanism (unpublished data). Mutations in *BAP1* are found throughout the entire gene and are not restricted to a specific domain, although we did observe a skewed distribution towards the N-terminal region (Fig. 5) (Koopmans et al., 2014). In the ROMS cohort, we observed a large variety of mutations, such as large out-of-frame deletions, but also missense mutations. In a preliminary analysis we did not find a significant association between mutation-type or location with disease-free survival.

Despite extensive research exploring the function of the BAP1 protein, it is as yet unclear how BAP1 loss in UM promotes the development of UM metastasis. BAP1 belongs to a specific group of proteases, called deubiquitinating enzymes (DUB), which function as a critical regulator of ubiquitin signaling by removing ubiquitin from proteins. Initially, BAP1 was identified because of its interaction with breast cancer 1 (*BRCA1)*, a tumor suppressor gene involved in homology directed DNA-repair. The absence of BAP1 inhibits homology directed DNA-repair and thereby forces cells to rely on the more error-prone non-homologous end joining (NHEJ) (Yu et al., 2014c). Surprisingly, the mutational load in UM is significantly lower than in other cancer types, suggesting that BAP1 loss in UM does not heavily impair DNA-damage repair mechanisms. Other proteins that interact more frequently with BAP1 could therefore be a more interesting target.

Some new proteins identified in these studies as interactors with BAP1 are the forkhead transcription factors FOXK1 and FOXK2, the histone acetyltransferase HAT1, the histone lysine demethylase KDMB1B, the polycomb group proteins ASXL1 and ASXL2, host cell factor C1 HCF1, and the ubiquitin-conjugated enzyme UBE20 (Ji et al., 2014; Machida et al., 2009; Mashtalir et al., 2014; Peng et al., 2018; Sowa et al., 2009; Yu et al., 2010). Most of these proteins are involved in the regulation of chromatin-associated processes such as transcription. This large number of interacting proteins implicates that absence of BAP1 can have a plethora of downstream effects.

One protein that predominantly interacts with BAP1 is HCF1, a protein involved in regulating the cellular localization of BAP1 through the formation of multiprotein complexes with transcription factors such as Yin Yang 1 (YY1) and FOXK1/2 (Dey et al., 2012; Yu et al., 2010). HCF1 plays an important role in stem cell maintenance by regulating genes involved in RNA processing and the cell cycle (Machida et al., 2009). RNAi-mediated depletion of BAP1 expression triggered a primitive, stem-like phenotype in UM cells. Genes involved in the maintenance of stem cells and developmental processes were upregulated and melanocyte-specific genes, such as *MITF*, were downregulated (Matatall et al., 2013). Thus, loss of BAP1 dysregulates transcriptional programs which are essential in the maintenance of the differentiated melanocytic phenotype. The acquisition of a stem cell like-phenotype is a common event in cells undergoing the epithelial-to-mesenchymal (EMT) transition required for metastasis. EMT programming may contribute to the highly metastasizing potential of *BAP1*-mutated UM cells by enabling cells to physically disseminate from the primary tumor. It also provides cells with the self-renewal capability that is crucial for clonal expansion at the site of dissemination (Mani et al., 2008).

The interaction of BAP1 with ASXL1 and ASXL2, important catalytic subunits of the polycomb repressive deubiquitinase (PR-DUB) complex, could influence the regulation of homeobox genes by deubiquitinating histone H2A (Chittock et al., 2017; Scheuermann et al., 2010). Histone H2A plays a role in several cellular processes, such as stem cell maintenance and cell proliferation (Bommi et al., 2010). The ubiquitination of histones alters the chromatin structure and thereby regulates the accessibility of the DNA for the transcriptional machinery (van Leeuwen and van Steensel, 2005). Knock down of *BAP1* by RNA interference induced an increase in H2A ubiquitination in UM cells, implying that H2A ubiquitination might be an interesting therapeutic target in high-risk UM.

Besides the aforementioned nuclear roles of BAP1, it also plays a role outside the nucleus. BAP1 can be localized in the endoplasmic reticulum (ER), where it stabilizes the type 3 inositol-1,4,5-triphosphate receptor (IP3R3). IP3R3 is involved in promoting apoptosis by tightly regulating the release of $Ca2^{+}$ from the ER into the cytosol. Loss of BAP1 reduces the amount of stable IP3R3, resulting in reduction of Ca^{2+} influx and thereby preventing cell apoptosis (Bononi et al., 2017). However, recent work from Farquhar et al. questioned the role of cytoplasmic BAP1 in the metastasis of UM, since they did not find a correlation between disease-free survival of UM patients and the cytoplasmic expression of BAP1 (Farquhar et al., 2018; Szalai et al., 2018).

This large number of potentially relevant proteins makes it difficult to determine the exact function of BAP1 due to the complex interaction networks. BAP1 assembles into a multiprotein complex, which contains several transcription- and co-factors. It is not clear yet which of these many transcription factors plays the crucial role in the metastasis of UM, showing that additional research regarding BAP1 in UM is necessary. For this, it is interesting to study the outliers in the *BAP1*-mutated group. For example, in our cohort we observed seven patients with *BAP1*-mutated tumors who remained metastasis-free for over 10 years. It has also been described that patients who carry a germline *BAP1* mutation do not have a substantially earlier age-of-onset of UM than other UM patients (Walpole et al., 2018). Additionally, these UM patients with germline mutations in *BAP1* have a better prognosis than patients with somatic mutations in *BAP1*. This suggests that in these patients, mechanism(s) have developed that could temporarily counteract the metastasis-promoting effect of BAP1 loss. Elucidating which mechanisms would be capable of doing that will contribute significantly to the development of a therapy targeted against BAP1 loss in UM.

2.3. *SF3B1* mutations result in aberrantly spliced mRNA

Metastasizing UM that do not show a *BAP1* mutation often harbor a mutation in the gene *SF3B1* (Furney et al., 2013; Harbour et al., 2013; Martin et al., 2013; Yavuzyigitoglu et al., 2016b). *SF3B1* encodes subunit 1 of the splicing factor 3b, which is responsible for proper branchpoint recognition during splicing of pre-mRNA. Correct RNA splicing is crucial for cell survival and allows cells to produce multiple proteins from one single gene. Somatic mutations in components of the spliceosome have been observed in several malignancies, such as breast, pancreatic and hematologic cancers (Bailey et al., 2016; Ciriello et al., 2015; Wang et al., 2011). The prevalence of cancer-associated mutations in spliceosome genes suggests that dysregulation of splicing can efficiently lead to the development of cancer.

SF3B1 mutations can result in aberrant splicing and it has been shown that these mutations in UM result in alternative splicing at the 3' end of exon borders (Alsafadi et al., 2016). These aberrantly spliced transcripts can be degraded by nonsense-mediated RNA decay, resulting in a loss of expression, but they can also be translated into unique, aberrant proteins (Paolella et al., 2017) (Fig. 6). Several genes have been shown to be affected in UM, such as ubiquinol-cytochrome C reductase complex chaperone (*UQCC*) and the multidrug resistance-associated protein ABCC5 (Furney et al., 2013).

The SF3B1 protein consists of an N-terminal hydrophilic region and a C-terminal region consisting of 22 non-identical HEAT (Huntington, elongation factor 3, protein phosphatase 2A, targets of rapamycin) repeats. UM-associated mutations in *SF3B1* are found almost exclusively in the fifth HEAT-repeat at codon position arginine (R) 625. In other cancer types, such as breast cancer and leukemia, mutations in *SF3B1* are more prevalent in the sixth and seventh HEAT-repeat at lysine residues K666 and K700, respectively. These lineage-specific mutations can be explained by several factors, such as tissue-specific interaction partners, the mutation rate of the gene and the activity of several pathways in a specific tissue that might confer survival advantage (Seiler et al., 2018a). However, since these residues are predicted to be spatially close to one another, it is not surprising that these mutations have a similar functional impact on transcription. RNA-sequencing data from *SF3B1*-mutated UM and breast cancer samples show some unique aberrant transcripts but the majority of the aberrant transcripts is observed in both malignancies (unpublished data). Samples harboring a mutation outside the HEAT-domains do not show aberrant splicing, implying a different effector on splicing or no effect at all (Darman et al., 2015).

SF3B1 is the most frequently mutated spliceosome gene in UM, but mutations in *U2AF1* and *SRSF2* have also been described (Robertson et al., 2017). *U2AF1* and *SRSF2* are both involved in the assembly of the spliceosome and it has been shown that mutations in these genes produce alternative transcripts in hematological malignancies. Similar to the *SF3B1* gene, particular *SRSF2* mutations are more prevalent: we observed that 4 of the 5 *SRSF2* in-frame deletions involve the same protein residues (AA 92–99), indicating that this particular activity of SRSF2 creates a specific effect on splicing, required in UM etiology (van Poppelen et al., 2019). Whether the downstream effects of these mutations are similar is unclear but these observations are intriguing and are the subject of further research by us and others.

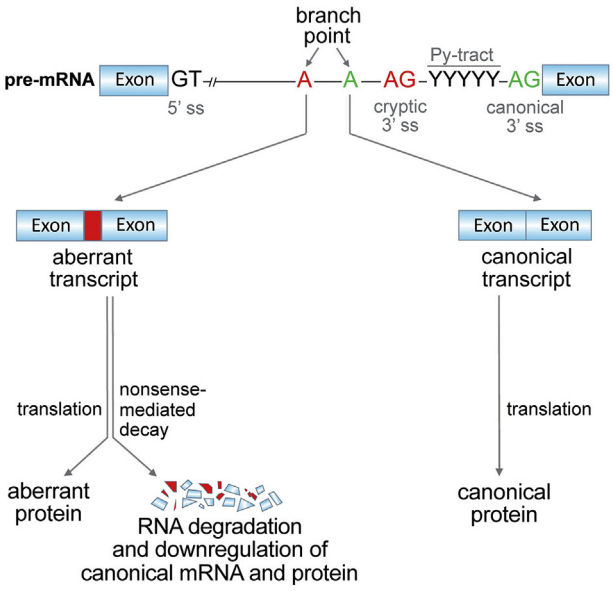


Fig. 6. Splicing of pre-mRNA into mature mRNA. Wildtype SF3B1 binds to the branchpoint (BP) of the pre-mRNA, which is usually an adenosine located ~25 nt upstream of the 3′ splice site (3′ ss). This allows a correct assembly of the spliceosome on the pre-mRNA, resulting in mature mRNA and a canonical protein. Whereas mutant SF3B1 recognizes an alternative BP (BP′), resulting in mis-spliced mRNA. This mis-spliced mRNA can be translated into an aberrant protein or degraded by nonsense-mediated RNA decay, resulting in downregulation of the RNA and protein. Reproduced with permission from Darman et al. (2015), via Copyright Clearance Center.

Most patients in our cohort with *SF3B1*-mutated UM eventually developed metastasis, but have a longer disease-free survival than *BAP1*-mutated UM, implying that *SF3B1*-mutated micro-metastases remain longer in their dormant state than *BAP1*-mutated micro-metastases. In our own patient cohort, the effect of SF3B1 is probably masked by the bulk of UM patients with *BAP1* mutations, as we did not observe a significant difference in prognosis for patients with or without a *SF3B1*-mutated UM (Fig. 7A). However, in the disomy 3 group, *SF3B1* mutations do show an association with a worse prognosis. The overall survival curve of all UM, stratified by mutation subtype, confirms that *SF3B1*-mutated UM are a distinct subclass associated with late metastasis. It can also be observed that the overall survival can vary greatly between *SF3B1*-mutated cases, as some develop metastases within 5 years whereas others after a decade (Fig. 7B) (Yavuzyigitoglu et al., 2016b). What causes this difference in metastatic potential is not clear and we did not find a specific segregation with the other well-known clinical-pathological or genetic prognostic markers using principle component analysis. However the number of patients in the early metastasizing subgroups was small which could have prohibited a proper analysis. We are in the process of collecting data of more patients with *SF3B1*-mutated tumors to survey RNA expression and epigenetic differences between these early and late metastasizing tumors, which might help us understand these different effects of aberrant splicing on metastatic risk.

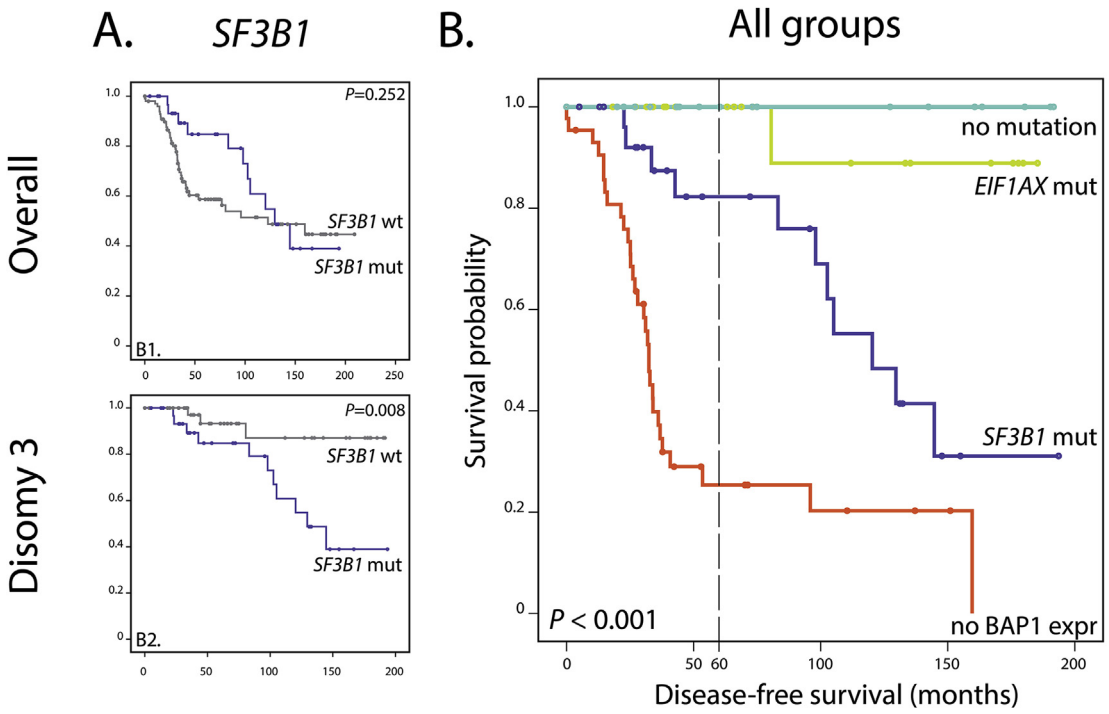


Fig. 7. Disease-free survival of UM patients. A) Kaplan-Meier curve showing the disease-free survival of the *SF3B1*^{mut} UM in the overall or disomy 3 group. **B)** In the overall survival curve containing all UM; it can be observed that every mutation status corresponds with a distinct survival pattern. Reproduced with permission from Yavuzyigitoglu et al. (2016b) via Copyright Clearance Center.

2.4. *EIF1AX* plays an important role in the initiation of translation

The *EIF1AX* gene is mutated in approximately 20% of UM and is involved in the initiation of gene translation in eukaryotic cells (Martin et al., 2013). Ribosomes bind the 5' end of the mRNA in a relatively unstable state, which allows scanning of the mRNA for the start codon. Several eukaryotic initiation factors (EIFs) support the ribosome in this process and subsequently stabilize the ribosome once it reaches a start codon. EIF1A consists of a globular domain and two unstructured tails, the N- and C-terminal tail, which are involved in the scanning of the mRNA and the accurate recognition of the start codon.

UM patients harboring only an *EIF1AX* mutation (in addition to a *GNAQ* or *GNA11* mutation) hardly metastasize; in our cohort, none of the patients with a pure *EIF1AX* mutation developed metastases: the only *EIF1AX*-mutated patient who developed metastases had a concurrent *BAP1* mutation (Fig. 7B). Remarkably, UM-associated mutations in *EIF1AX* -indicated in Fig. 8 by the red dots-occur exclusively in the N-terminal tail of the protein, a highly conserved region in eukaryotes. Mutations in the N-terminal tail inhibit the scanning process by stabilizing the ribosome. This promotes the utilization of less optimal start codons and thereby alters gene expression in UM (Martin-Marcos et al., 2017). Experiments in yeast show that *EIF1A* mutations alter the relative use of start codons in mRNA encoded by tumor suppressor genes or oncogenes. Immunohistochemical staining of EIF1AX in samples harboring a mutation showed a positive staining throughout the cytoplasm of the cell, showing that mutations in the *EIF1AX* gene do not cause loss of the protein (Martin et al., 2013). *EIF1AX* mutations have also been observed in other cancer types, such as breast cancer, prostate cancer, adenocarcinoma and glioma (indicated in Fig. 8 by the grey dots) (Tate et al., 2019). Surprisingly, these mutations are found throughout the entire protein-coding DNA, as opposed to UM, where mutations are only observed in the N-terminal tail. This raises the question if the N-terminal region of EIF1AX executes specific functions or engages with specific binding partners in UM. Change of function mutations in the *EIF1AX* gene might make melanocytes more malignant and stimulate their division, but not enough to initiate metastasis. Whether UM cells do not spread at all or whether micro-metastases are present in distant organs but remain dormant, is unknown.

3. Chromosomal abnormalities and RNA expression in UM

UM progression involves several chromosomal gains and losses. Chromosome 8q gain and complete loss of chromosome 3 frequently occur in high-risk UM, whereas low metastatic risk UM carry two copies of chromosome 3 and often show gains of chromosome 6p (UM type A) and distal 8q (UM category B) (Damato et al., 2011; Jager et al., 2018; Prescher et al., 1996; van den Bosch et al., 2012). Thirty percent of UM patients also have a deletion of chromosome 1p, which is associated with a higher metastatic risk (Kilic et al., 2005). Aberrations on other chromosomes have been observed, but are less frequent and show no correlation to metastatic risk. Cytogenetic analyses are useful but very time and labor consuming. Culturing UM tumor cells is hampered by overgrowth of fibroblasts and only short time cultures can be used to obtain an accurate karyotype. Nowadays, a Single Nucleotide Polymorphism (SNP) array technology is used to determine copy number variations (CNV) in tumor specimens. Apart from the observation that

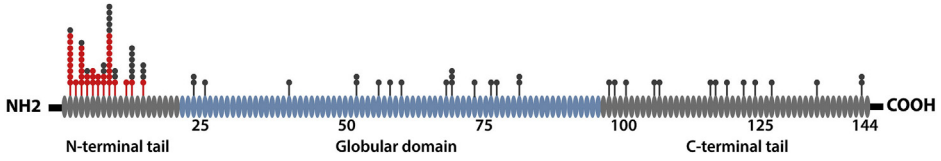


Fig. 8. *EIF1AX* mutations. Malignant mutations in the EIF1AX protein observed in UM (red) and other cancer types (grey). All observed mutations are in-frame mutations (Tate et al., 2019).

CNV analysis as such is an independent prognostic test and does contribute to prognostication models (Drabarek et al., 2019; Eleuteri et al., 2012), we have observed earlier intriguing differences between these CNVs in metastasizing UM which did urge us to go back to the results obtained with karyotype analysis. We noticed that whereas *BAP1*-mutated tumors did harbor in general a few whole chromosome anomalies resulting in isochromosome formation (e.g. (i)8q or (i)6p), in *SF3B1*-mutated tumors smaller gain or losses of the terminal parts of chromosome 6p and 8q are more prevalent (Yavuzyigitoglu et al., 2017). Whether this is a consequence of the underlying *BAP1* or *SF3B1* mutation causing a different route to generate these chromosomal aberrations or that the resulting genetic changes sort out the most optimal effect in combination with the specific mutated gene, is not clear. Most, if not all UM, present with both the mutation and the matching set of CNVs, but we occasionally observe these mutations without the corresponding CNV patterns or vice versa. A scrutinized genetic survey of these rare cases, preferably with –when available- also the subsequent metastatic tissues may shed more light on this causality dilemma. Alternatively, site-directed mutagenesis of these genes using CRISPR/Cas9 in melanocytic cells could help us to answer the chicken and egg story and analyze other intriguing differences regarding the altered pathways and route towards metastasis between *BAP1* or *SF3B1*-mutated tumors.

Nevertheless, these chromosomal abnormalities contribute to the development of UM by altering gene expression. Specific gene expression profiles (GEP) are associated with low metastatic risk (class 1a), intermediate metastatic risk (class 1b) or high metastatic risk (class 2) (Field et al., 2016; Onken et al., 2004; Tschentscher et al., 2003; Zuidervaart et al., 2003). Interestingly, we described in a previous publication that differential expression only partially correlated with chromosomal abnormalities (Fig. 9). For example, a large part of the genes located on chromosome 3p were significantly downregulated in class 2 UM, whereas genes located on chromosome 3q were not (van Gils et al., 2008). In addition, parts of chromosome 8q and 6q showed upregulation. This indicates that other mechanisms, such as methylation, might compensate for chromosomal abnormalities.

The oncogene *MYC* is located in the frequently amplified chromosome 8q24 region. Several analyses show that the presence of extra copies of chromosome 8 is associated with a worse prognosis (Cassoux et al., 2014; Damato et al., 2011; Dogrusoz et al., 2017; Versluis et al., 2015). Although MYC signaling has been shown to be involved in UM development, no direct association has been observed between MYC expression and metastatic death (Robertson et al., 2017). Expression of the adjacently-located long non-coding RNA PVT1 (plasmacytoma variant translocation gene) does show a direct association with metastasis. This indicates that gene expression regulation is complex and that other processes, in addition to copy number status, are involved in regulating expression levels. *PVT1* is often amplified in several cancer types and acts as an oncogene by regulating transcriptional activity and acting as a miRNA sponge by binding to complementary miRNAs, thereby preventing the miRNAs from exerting their role in gene expression. Another oncogene located on the amplified 8q region is development and differentiation enhancing factor 1 (*DDEF)*. High-risk UM show higher *DDEF* expression than low-risk UM. DDEF regulates the remodeling of the cytoskeleton, which is necessary for cell motility. Overexpression of *DDEF* in low-risk UM cells increases their motility, suggesting that upregulation of *DDEF* contributes to the invasive phenotype of high-risk UM. However, most of these studies do not discriminate between *SF3B1* and *BAP1*-mutated tumors, so these observations might not be valid for the often small 8q amplified regions of *SF3B1*-mutated tumors. Hence, repeating these analyses in well-defined UM subgroups based on genetic changes in the secondary driver genes might result in a different set of classifier genes. Furthermore, amplification of an isochromosome 8q (8q gain in combination with 8p loss), as seen frequently in *BAP1*-mutated tumors is also present in other tumor types. In a recent study on hepatocellular carcinoma performed by The Cancer Genome Atlas (TCGA) network, 14 of 23 *BAP1*-mutated samples did show signs of isochromosome 8q, suggesting a similar common and perhaps more universal underlying genetic mechanism (Cancer Genome Atlas Research Network. Electronic address and Cancer Genome Atlas Research, 2017). It would be interesting to determine whether other *SF3B1*-mutated tumors harbor similar *SF3B1*–CNV patterns in addition to the observed overlap in altered expressed RNAs.

Gene expression can also be used to predict disease-free survival of UM patients. Unsupervised clustering of primary UM based on mRNA

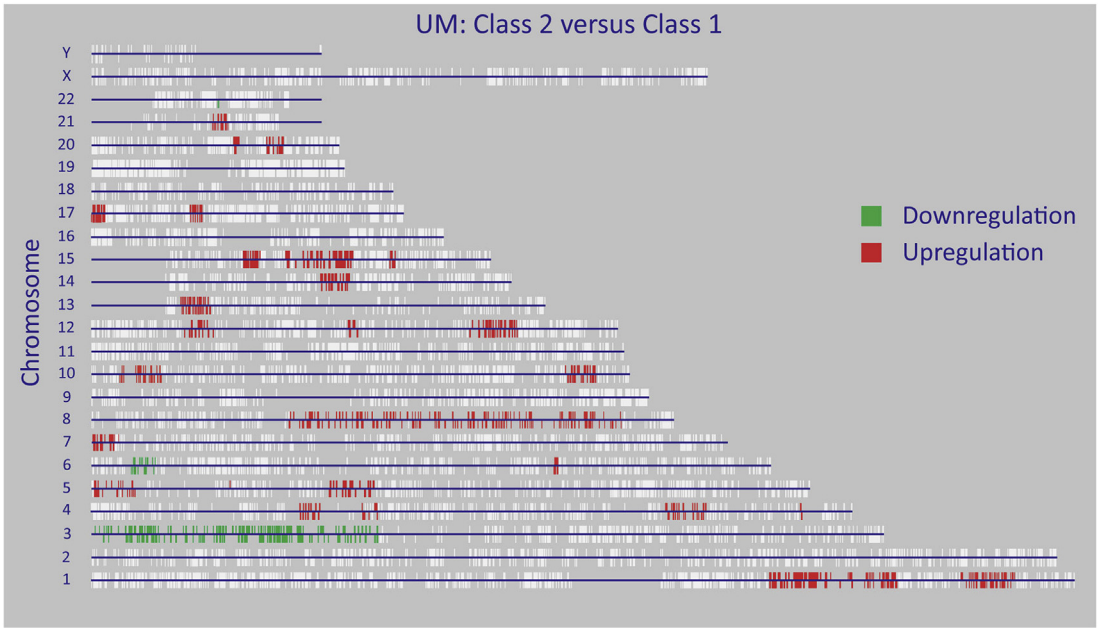


Fig. 9. Differential gene expression between class 1 and class 2 UM. Several chromosomal regions contain differentially expressed genes identified by a locally adaptive statistical procedure (LAP)-analysis. White bars indicate locations of the microarray-probes, whereas red (upregulation) or green (downregulation) indicate a differentially expressed gene. Adapted from van Gils et al. (2008).

expression shows two distinct classes as shown by Onken et al. (2004). Class 1 consists of *EIF1AX* and *SF3B1*-mutated UM that show the transcriptome of a differentiated melanocyte, whereas class 2 contains monosomy 3/*BAP1*-mutated tumors characterized by a stem cell-like expression profile. Functional annotation of these differentially-expressed genes revealed involvement in development, cell communication, cell growth, cell motility and apoptosis. Interestingly, most of the identified developmental genes are known to be implicated in neural crest development (Onken et al., 2004).

TCGA contains the expression data of 80 primary UM. Monosomy 3 TCGA samples showed increased transcription of *MAPK*, *AKT* and the transcription factors *FOXA1* and *FOXM1*, indicating increased proliferation in this group (Robertson et al., 2017). Several long non-coding RNAs were found to be higher expressed in monosomy 3 samples, such as the aforementioned *PVT1* gene, as well as the oncogenes *CYTOR* and *BANCR*. The expression of multiple immunological genes was also significantly elevated in the poor prognosis clusters. This indicates an activation of the immune system, which is in contrast to what has been observed in other cancer types, where an activated immune system is typically seen in tumors with low metastatic risk. An association between monosomy 3 and an inflammatory phenotype has been described previously (Maat et al., 2008). An important difference may be that UM metastasizes hematogeneously, and that the presence of infiltrating macrophages contributes to intratumoral vessel growth (Bronkhorst et al., 2011; Brouwer et al., 2019; Jager et al., 2011; Ly et al., 2010).

Within the monosomy 3 TCGA samples, two separate clusters were observed, categories C and D (Jager et al., 2018; Robertson et al., 2017). Surprisingly, one cluster showed an activation of the DNA damage response (DDR) pathway, which, however is not reflected in the mutational load of UM. MYC signaling and HIF1a were also upregulated in this cluster, which is consistent with aberrant *BAP1* expression. The other cluster is characterized by elevated levels of MAPK and AKT, two effectors of the Gα11/Q pathway. This implies that BAP1 loss may enhance the effect of oncogenic GNAQ and GNA11.

4. UM metastases

Most UM research focuses on primary UM, although the metastases cause death in UM patients and not the primary tumor. Metastatic outgrowth of a tumor is a complicated, multi-step process that is often difficult to unravel. Only a few UM patients with metastases undergo liver resection, and diagnostic biopsies usually do not provide sufficient material for additional research. Moreover, metastases samples can contain a mixture of UM cells, admixed reactive cells as well as hepatocytes, making a proper description of the genomic profile of UM metastases challenging.

4.1. Metastatic spread of UM

The metastatic capacity of cancer cells is mainly determined by the interaction with the microenvironment. In order to allow UM cells to grow in distant organs, several steps have to be taken; they must lose contact with neighboring cells, home and survive in the host organ, become established and finally also be able to grow into macro-metastases (Lambert et al., 2017). Therefore, it could be hypothesized that UM cells need to acquire several additional genetic aberrancies in order to successfully grow in distant organs.

In 1882, the ophthalmologist Ernst Fuchs described the predisposition of uveal melanoma to metastasize to the liver and postulated an organ-specific predisposition for metastases (Fuchs, 1882). Seven years later, Paget formulated the famous Seed and Soil hypothesis (Paget, 1889) which suggests that metastasis is not random and cancer cells (the seeds) show a preference when metastasizing to distant organs (the soil). Since the large majority of UM metastasize to the liver, it could be implied that there is a favorable microenvironment in the liver for UM cells. Whether the primary tumor stimulates this microenvironment by promoting the development of pre-metastatic niches remains unclear. Unraveling which factors in the liver contribute to this favorable microenvironment might provide us with possible therapeutic targets. One factor that is thought to play an important role in creating a pre-metastatic niche in specific organs are exosomes. These small (~100 nm) lipid bilayer-delimited vesicles are released from cells and carry several functional biomolecules that can be transferred to recipient cells. A specific repertoire of integrines on the exosome-surface dictates the adhesion of exosomes to specific cell types; Hoshino et al. have shown that tumor-derived exosomes preferentially interact with cells at the future metastatic site (Hoshino et al., 2015). After these exosomes are taken up by the target cells, several signaling pathways and inflammatory responses are initiated which are necessary to complete the development of the pre-metastatic niche (Liu and Cao, 2016). Since a study by Angi et al. has shown that primary cultured UM cells secrete almost half of their secreted proteins via exosomes, one may speculate that exosomes play a role in metastasis formation in UM (Angi et al., 2016).

UM patients who have undergone successful treatment of the primary tumor still develop metastases. This implies that UM cells have already spread into the circulation before the primary tumor was diagnosed and treated. A study by Eskelin et al. calculated tumor doubling times for UM and showed that primary UM metastasize already several years before treatment (Eskelin et al., 2000). With this in mind, one would expect that circulating tumor cells (CTCs) could be detected at time of diagnosis. However, CTCs are mainly detected in blood of patients with metastatic UM, whereas patients with primary UM often show no CTCs (Beasley et al., 2018). Whether this is due to the low number of CTCs in blood at the time of diagnosis or the seeding of CTCs by metastatic lesions is unknown. Isolating these rare CTCs both at diagnosis and during the metastatic phase could aid our understanding of the metastatic process in UM. Another promising biomarker that could identify dissemination of UM is cell-free DNA (cfDNA). CfDNA are small fragments of DNA that are released in the circulation by (tumor) cells and are present in increased amount in cancer patients. Several studies have showed that *GNAQ* and *GNA11*-mutations could be detected in cfDNA from metastatic UM patients and that their presence showed an association with metastases-volume and overall survival (Beasley et al., 2018; Bidard et al., 2014; Metz et al., 2013).

4.2. Chromosomal alterations in UM metastases

A wide spectrum of chromosomal alterations can be identified in UM metastases, such as gain of 8q, 6q, 1q and alterations in chromosome 3. These chromosomal alterations are also commonly found in primary UM, however, the frequencies are different (McCarthy et al., 2016). UM metastases showed more copies of 8q than the corresponding primary UM (Shain et al., 2019). Given this high prevalence of 8q amplifications in UM metastases, it could be hypothesized that the 8q region contains gene(s) that potentially promote metastasis. However, further investigation is necessary in order to determine whether upregulation of oncogenes such as *MYC* and *PTP4A3* have a direct effect on metastasis, or that this is just a consequence of extra copies of chromosomal region 8q. As expected, the majority of UM metastases contained alterations in chromosome 3. Whereas primary UM mainly show monosomy 3, UM metastases frequently showed isodisomy 3 and large regions of homozygosity (McCarthy et al., 2016; Rodrigues et al., 2019). Monosomy 3 at first stimulates tumor progression through BAP1 inactivation; however, haploinsufficiency of some chromosome 3 genes could result in a reduced expression of genes such as *MITF*, *MBD4* and *CTNNB1* and thereby limit UM progression (Onken et al., 2004; van Gils et al., 2008). In response to this limitation, duplication of chromosome 3 could be a compensating mechanism in metastasizing UM cells.

4.3. Mutational analysis in UM metastases

As described in the introduction, UM shows a remarkable low mutational burden compared to other malignancies. Rodrigues et al. sequenced 15 UM trio's (germline, primary UM and UM metastases) and did not observe a significant increase in SNVs between primary UM and its metastases (median 13 SNVs vs 16 SNVs) (Rodrigues et al., 2019). All UM metastases contained a mutually exclusive *GNAQ* or *GNA11* mutation that matched with the primary UM, confirming that these mutations arise early in the development of the disease. Interestingly, Shain et al. also observed LOH of mutant *GNAQ* in multiple metastases samples, suggesting that *GNAQ*-mutated UM require a second hit later in UM progression. LOH of mutant *GNAQ* shifts the allelic balance towards mutant *GNAQ*, which activates the GαQ signaling even further, thus allowing cells to become fully malignant (Shain et al., 2019). This corresponds with previous studies suggesting that *GNA11*-mutations are more potent oncogenes, since an over-representation of mutations in *GNA11* was observed in UM metastases (Griewank et al., 2014). Secondary driver mutations in *BAP1* and *SF3B1* were observed in high frequencies in UM metastases as well, indicating early occurrence. Surprisingly, additional mutations in new oncogenes were also observed in UM metastases, which however, occurred in much lower frequencies than the secondary driver mutations. This indicates that these tertiary driver mutations occur later in UM progression, after mutational activation of *BAP1* and *SF3B1*. Tertiary driver mutations were found in oncogenes, such as *PTEN*, *EZH2*, *CDKN2A*, *TP53* (Rodrigues et al., 2019; Shain et al., 2019). These newly-identified mutations might offer new opportunities in UM therapeutics. However, it is still early for the field to develop therapies targeting these genes. Firstly, it remains unclear to what extent each of these mutations contributes to the malignant phenotype. Secondly, novel targeted treatment may possibly only have an effect on metastatic UM subclones harboring particular actionable mutations.

5. Therapeutic options

We have gained considerable insight into the genetic background of UM, but this has not yet resulted in successful treatments of metastatic UM. Treatment of metastatic UM with classic chemotherapy has been disappointing, with low response rates (Carvajal et al., 2014b; Chattopadhyay et al., 2016). Over the past 35 years, survival of patients with metastatic UM has not improved (Rantala et al., 2019). However, now that we know the genes and the pathways they might be involved in, we can start developing new therapeutic modalities for UM.

5.1. Targeting the Gα11/Q pathway

Since the majority of UM contain mutations that deregulate the Gα11/q pathway, drugs targeting this pathway might be effective in the majority of UM, regardless of their further mutational background. Inhibiting GNAQ and GNA11 themselves might be difficult, because of high GTP levels in the cytoplasm. Several studies have therefore focused on interfering with the critical downstream effectors, such as MAPK, PKC, PI3K and AKT signaling. Clinical trials with the MEK1/2 inhibitor selumetinib resulted in promising preliminary results (Carvajal et al., 2014a). UM patients treated with selumetinib had an improved progression-free survival of up to 15 weeks, compared to patients treated with chemotherapy. Unfortunately, selumetinib did not improve overall survival in UM patients. This indicates that selumetinib can inhibit metastatic growth only for a limited time: once the tumor acquires resistance to MEK inhibition, it grows even more aggressively than non-treated metastatic tumors. A combination of the chemotherapeutic drug dacarbazine and selumetinib did not give improvement in survival (Carvajal et al., 2018). Similar results were obtained with the MEK inhibitor trametinib and the Akt inhibitor GSK795: no improved survival rate was observed in 40 metastatic UM patients (Shoushtari et al., 2016).

These disappointing results could be explained by acquired resistance, which also causes CM patients to become resistant to the BRAF inhibitor vemurafenib (Nazarian et al., 2010). Another reason for this limited response could be that these inhibitors act far downstream of oncogenic GNAQ and GNA11. As shown by Mouti et al., progression of UM in zebrafish is dependent on YAP activation, rather than activated extracellular signal-regulated kinases (ERK). Only a subset of the malignant uveal melanocytes showed activation of ERK, while knockdown of *GNAQ* or *PLCB4* did not affect the levels of activated ERK, suggesting that MAPK signaling only partially contributes to the development and maintenance of UM (Mouti et al., 2016). Inhibiting only one arm of an oncogenic network is likely less efficient than interfering with nodes that act closer to GNAQ and GNA11, such as ARF6.

ARF6 regulates multiple downstream signaling pathways and might therefore be a more suitable target for treatment of UM. Knockdown of *ARF6* induces the re-localization of GNAQ and GNA11 from the cytoplasm to the plasma membrane, resulting in a decrease of all Gα11/q -mediated pathways. Yoo et al. showed that inhibition of ARF6 in the *GNAQ*- and *SF3B1*-mutated UM cell line, Mel 202, resulted in a lower tumor incidence and size when injected into immune-compromised mice (Yoo et al., 2016). A significant reduction in the levels of downstream activated ERK, RAC/RHO, p38, JNK and C-JUN was observed as well. Treating cells and xenograft mouse models with NAV-2729, a direct inhibitor of ARF6, resulted in similar results as the knockdown experiments. Until now, the results have been promising and no toxicity was observed; however, this treatment is not yet FDA-approved and additional studies have to be performed to investigate whether pharmacological inhibition of ARF6 is an effective treatment for UM.

Other targets for UM treatment could be PLCB4 and YAP. YAP can be successfully inhibited by the well-tolerated compound verteporfin. Treatment of xenograft mouse models with this drug showed a reduction in UM growth (Yu et al., 2014b). However, these compounds too only target one arm of the oncogenic Gα11/q network and it is therefore likely that they will only show a limited effect unless they are used in combination with another drug.

5.2. HDAC inhibitors to reverse the effect of BAP1 loss

Several studies have described histone deacetylase inhibitors (HDACI) as promising anti-cancer drugs. HDACIs interfere with HDACs, which are frequently upregulated in cancer (Halkidou et al., 2004; Song et al., 2005; Zhang et al., 2005). These HDACs remove acetyl groups from histones, which changes the structure and accessibility of chromatin and thereby affects gene expression (Li et al., 2010). UM cell lines or xenograft models treated with HDACI show a reduced proliferation and an induced cell cycle arrest. A study by Landreville and colleagues (Landreville et al., 2012) described that BAP1-deficient cells have an increased sensitivity for HDACI. HDACIs initiated morphologic and transcriptomic changes consistent with melanocyte differentiation and reduced proliferation through G1 cell cycle arrest. BAP1-deficient cells might be more sensitive to HDAC inhibition because of their increased H2A ubiquitination. It has been shown that distinct histone modifications act together to regulate chromatin structure and gene expression: for example, the deubiqituitinase enzyme H2A-DUB not only regulates deubiquitination of histones, but also acetylation (Zhu et al., 2007). Interfering with the acetylation of histones in BAP1-deficient UM might reverse the biochemical deficit caused by BAP1 loss by shifting the cell to a less aggressive, more differentiated state. HDACIs could therefore prolong survival of UM patients by keeping micro-metastases in a quiescent, differentiated state.

5.3. Spliceosome inhibitors

SF3B1-mutated UM require a different approach. As mentioned before, tumors with an *SF3B1* mutation show aberrant splicing of pre-

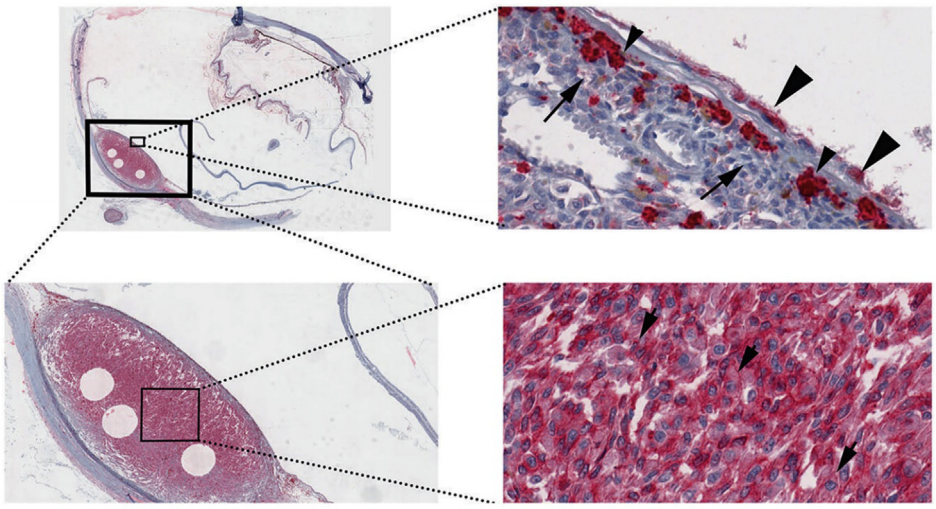


Fig. 10. Heterogenous PD-L1 expression in primary UM. The lowest magnification (0.3x) shows the flat choroidal tumor, whereas the medium magnification (1.4×) shows diffuse membranous expression of PD-L1. This is confirmed at higher magnifications; the top right and lower right picture (20× and 40x respectively) show positive membranous PD-L1 expression in UM cells (small arrowheads), positively-stained retinal pigmented epithelium cells (large arrowheads) and tumor-infiltrating macrophages (small arrows). Reproduced with permission from Zoroquiain et al. (2018) via Copyright Clearance Center.

mRNA resulting in an increased rate of transcripts containing premature termination codons. Mutations in splicing factors always occur in a heterozygous state and have never been observed to coincide with another splicing-factor mutation. The spliceosome is essential for survival and cancer cells require wildtype splicing to survive. Inhibiting the spliceosome in cancer cells with spliceosome inhibitors has shown exciting results in several different malignancies and might therefore also be a promising treatment for UM.

Several components have been identified that are able to successfully inhibit the spliceosome assembly at an early stage, such as sudemycin (Fan et al., 2011), E7107 (Kotake et al., 2007) and spliceostatin A (Kaida et al., 2007). E7107 and spliceostatin A bind non-covalently to SF3B1 and thereby prevent exposure of the branchpoint-binding region of U2 snRNP. This results in defective formation of the spliceosome early in the splicing process. Mutations in the *SF3B1* gene result in resistance to E7107, as shown by long term treatment of human colorectal cancer cell lines with E7107 (Yokoi et al., 2011), indicating that only wildtype (WT) SF3B1 is affected. Since *SF3B1*-mutated UM require wildtype splicing in order to survive, interference of WT SF3B1 by E7107 will result in cell death.

In vivo treatment of isogenic murine myeloid leukemias that harbor an *SRSF2* mutation with E7107, reduced the leukemic burden by inducing preferential cell death of cells bearing an *SRSF2* mutation (Lee et al., 2016). Inhibition with this compound showed the same effects as RNAi-mediated silencing of SF3B1, such as an accumulation of unspliced mRNA in the nucleus. A subset of this unspliced mRNA leaked into the cytoplasm, which resulted in the production of aberrant proteins, including an unusually stable form of the cell cycle inhibitor p27 (Kotake et al., 2007). Unfortunately, clinical trials with E7107 in patients with metastatic solid cancer had to be suspended due to an unexpected side effect in bilateral optic neuritis, resulting in loss of vision. However, in most patients the drug was well tolerated and inhibition of splicing was observed (Eskens et al., 2013; Hong et al., 2014). Additional compound screens will be necessary to identify spliceosome inhibitors that act on the spliceosome assembly at a later stage. Recently, a phase 1 trial was started with the new spliceosome inhibitor H3B-8800 (Seiler et al., 2018b).

While targeting the spliceosome will probably have most potential in *SF3B1*-mutated UM, it might also be beneficial to treat *BAP1*-mutated UM with these compounds, as it has been shown that cancer cells with an increased MYC activity might also be more vulnerable to spliceosome inhibition (Hsu et al., 2015). Since a subset of the *BAP1*-mutated tumors show an upregulation of *MYC*, treatment of these tumors with spliceosome inhibitors might be a promising option.

A possible problem with spliceosome-inhibitors might be their lack of specificity. Another method to alter splicing in cells is through oligonucleotides. Oligonucleotides bind to RNA in a sequence-specific manner and prevent interaction between the spliceosome and pre-mRNA by steric hindrance. Aberrantly-spliced genes, that contribute significantly to the malignant phenotype of UM, can thereby be specifically targeted and inhibited. Oligonucleotides have been shown to regulate the presence of aberrant splice variants and restore the production of essential proteins (McClorey and Wood, 2015). Unfortunately, no oligonucleotide-based treatment has yet been approved for the treatment of cancer patients.

5.4. Immunotherapy in UM

Metastasis can arise several years after successful removal of the primary tumor. This long latency period can be explained by the presence of dormant UM cells, and dormancy may be due to immunological inhibition. Once a cancer cell is able to overcome the immune response, micro-metastases can start to proliferate, which will result in a fatal outcome.

A new, exciting area of cancer drug development is immunotherapy. One example of immunotherapy is the use of monoclonal antibodies against CTLA-4, PD-1 and PD-L1. CTLA-4 and PD-L1 act as natural immune checkpoints in T cells, to tune down and thereby avoid exaggerated immune responses. It has been shown that cancer cells suppress immune responses by upregulating the ligand PD-L1. The monoclonal antibodies used in immunotherapy block the checkpoints and subsequently unblock and thus activate T cells, which results in the removal of cancer cells. Many of these monoclonal antibodies have already been approved for clinical use, such as nivolumab targeting PD-1. Immunotherapy treatment of advanced melanoma, lung cancer and renal cancer patients showed remission and in some cases even eradicated metastatic disease. Unfortunately, these antibodies show only limited activity in UM patients (van der Kooij et al., 2017; Wierenga et al., 2019; Zimmer et al., 2015). Dual immune-checkpoint blocking resulted in a response rate of 38% in CM, but no response was observed in UM patients (Kirchberger et al., 2018). These disappointing results cannot solely be explained by the fact that UM do not express PD-L1, since two studies show heterogeneous expression of PD-L1 (> 5% positivity) in approximately 50% of the UM (Fig. 10) (Wierenga et al., 2019; Zoroquiain et al., 2018). However, a potentially more promising strategy would be to inhibit checkpoints that have been shown to be consistently highly upregulated in metastatic UM, such as indoleamine-pyrrole 2,3-dioxygenase (*IDO*) and T cell immunoreceptor with Ig and ITIM domains (*TIGIT*) (Robertson et al., 2017; Stalhammar et al., 2019b). Interestingly, Rodrigues et al. describe an unexpected high

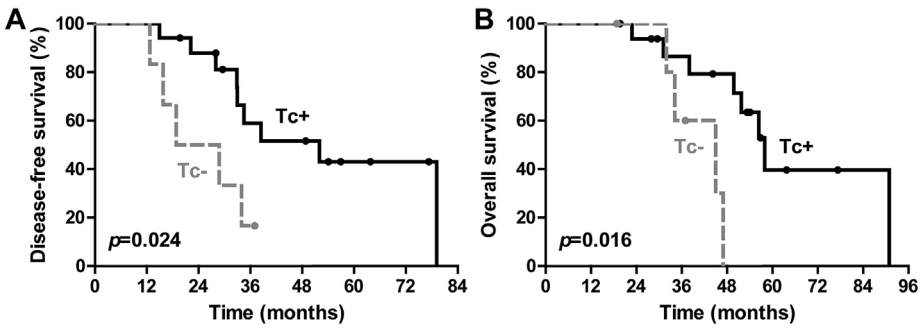


Fig. 11. Survival of high risk UM patients after dendritic cell treatment. Kaplan-Meier curve showing the disease-free survival **(A)** and overall survival **(B)** in correlation with the presence of tumor antigen-specific T cells after receiving dendritic cell vaccination. Reproduced with permission from Bol et al. (2016), via Copyright Clearance Center.

sensitivity to the PD-1 inhibitor pembrolizumab in one UM patient (Rodrigues et al., 2018). DNA sequencing identified a germline mutation in *MBD4*, a gene located on chromosome 3 and involved in base excision repair. Mutations in this gene result in an unusual high mutational load, thereby sensitizing the tumor to PD-1 inhibition. A similar UM patient was recently described (Johansson et al., 2019), while within the TCGA-dataset, two other UM patients with *MBD4* mutations were identified, indicating that a small fraction of the UM patients could directly profit from PD1-inhibition.

A more classic approach to immunotherapy is inducing an immune response by making use of activated dendritic cells (DCs). A collaborative study in The Netherlands by Bol et al. treated UM patients with autologous DCs loaded with antigens derived from gp100 or tyrosinase, two melanocyte-specific proteins (Bol et al., 2016). No severe toxicities were observed after the vaccinations and 74% (n = 17) of the patients showed the presence of tumor-specific T cells after DC vaccination, indicating an activation of the immune system. These patients showed a significantly longer disease-free and overall survival than patients that did not show an immune response (58 months vs 45 months respectively) (Fig. 11). However, no significant difference in the overall survival rate was observed compared to the control group (Bol et al., 2016). A new approach uses a novel molecule, tebentafusp, to initiate an immune response in UM patients. Tebentafusp acts as a bridge between UM and cytotoxic T cells and thereby ultimately results in T-cell activation and subsequent killing of UM cells (Damato et al., 2019; Komatsubara and Carvajal, 2017). Preliminary results indicate that biopsies, which were taken after the injection, confirmed the influx of lymphocytes and an increase in PD-L1 expression.

Still, the results obtained from widely used immunotherapy unfortunately present less promising results in UM patients than in CM patients. As the eye is an immune-privileged site, immune responses may not develop as easily as in other locations, and may even be inhibited actively (Niederkorn, 2009). In most cancer types, immune infiltration is associated with good prognosis and sensitivity to immunotherapy. However, especially the prognostically infaust monosomy 3 UM show a dense immune infiltrate and increased HLA Class I expression (Bronkhorst et al., 2012; de Lange et al., 2015; Jager et al., 2011). This different response rates could be explained by the presence of immunosuppressive cells, such as regulatory T cells and macrophages, which dampen the immune response. A study of low risk and high risk tumors indicated that extra copies of chromosome 8q were associated with influx of macrophages, while loss of BAP1 was linked to higher numbers of T cells (Bronkhorst et al., 2012; Gezgin et al., 2017). These data show that genetic changes are related to the development of the inflammatory phenotype in UM, but they do not explain why the immune system is so unresponsive in most patients. However, all these results imply that future immunotherapy agents should mainly focus on overcoming the immune suppression in UM. By determining the precise immune landscape in every tumor, we might be able to predict which UM would be sensitive to immunotherapy. Another factor that could explain the disappointing response rate to immunotherapy is the low mutational load of UM cells. As previously described, CM show a high mutational burden which correlates with a plethora of neo-antigens and thus renders them particularly suitable for immunotherapy. However, *SF3B1*-mutated UM could very well be more sensitive to immunotherapy if this is directed against the proteins produced by aberrant splicing. Mass spectrometry analysis of the proteome secreted by *SF3B1*-mutated UM could identify and characterize these aberrant proteins.

These aforementioned treatments can be given systemically in order to remove any metastases in distant organs; however they can also be given locally to UM patients with liver metastases by isolated hepatic perfusion (IHP). In case metastatic disease is confined to the liver, the liver can be isolated from the systemic circulation, which allows a much higher concentration of therapeutics to be used. Fifty to seventy-five percent of metastatic UM patients responded to IHP with the chemotherapeutic agent melphalan. The most common adverse effects were hematological events – such as thrombocytopenia, anemia, and neutropenia – which were clinically manageable, indicating that IHP with melphalan could be a promising treatment for UM patients with liver metastasis (Artzner et al., 2019; Meijer et al., 2019; Vogel et al., 2017). Currently IHP is performed for established metastatic disease, but future drugs with less side-effects might allow targeted adjuvant treatment in high-risk UM patients (Olofsson et al., 2014).

6. Future directions and conclusions

Even though our understanding of UM has advanced in the last decade, UM remains one of the very few malignancies for which there is no treatment available for metastatic disease. In recent years, there has been a tendency to transpose treatments shown to be effective in CM to UM, such as immunotherapy and MEK-inhibitors. However, as described in multiple studies, the biological behavior of these two malignancies is completely different and therefore they require a different approach. A better understanding of the complex genetic and immunologic background of UM will allow a more personalized approach which is necessary for effective treatment. Treatments targeting oncogenic GNAQ and GNA11 signaling could be applied to all UM patients, although it remains unclear if this will be sufficient for effective UM treatment. GNAQ and GNA11 are relatively weak oncoproteins and can only become truly malignant when combined with co-mutations in secondary driver genes. Therefore, additional research into agents targeting these deregulated processes, such as spliceosome inhibitors and HDAC inhibitors, is necessary.

The genetic background could also play a role in the treatment outcome. *SF3B1*-mutated tumors could benefit from different therapeutic agents than *BAP1*-mutated tumors. This might require a more personalized approach, where the genetic background of each UM patient is investigated in the diagnostic setting and determines the best treatment option (Fig. 12). In the case of an *EIF1AX*-mutated UM, local treatment of the primary tumor could already be sufficient. However, *SF3B1* and *BAP1*-mutated UM will require a more rigorous treatment protocol. For these patients it is known that in many cases micro-metastases are already present at the time of diagnosis, meaning that

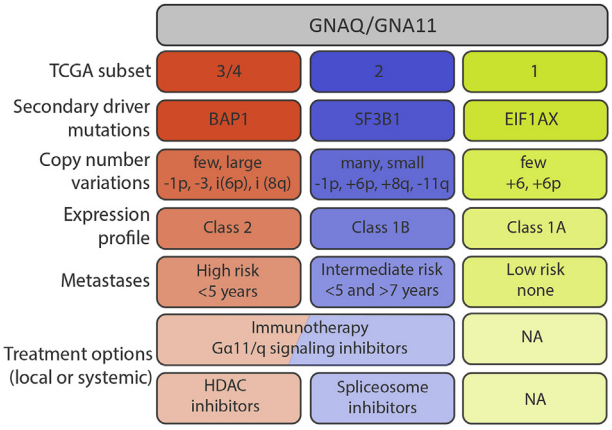


Fig. 12. Schematic overview of the different UM subtypes. Each UM subtype is characterized by a TCGA subset, a specific mutation in one of the secondary driver-genes, several copy number variations and a different expression profile. Both intermediate and high risk UM could profit from immunotherapy or therapies that interfere with oncogenic GαQ signaling. Additionally, high risk, *BAP1*^{mut} UM could profit from treatment with HDAC inhibitors, whereas intermediate risk, *SF3B1*^{mut} UM could be more sensitive to spliceosome inhibitors. These treatments can be administered systemically, but also locally by isolated hepatic perfusion.

besides treatment of the primary tumor, the dormant micro-metastases have to be targeted as well to improve overall patient survival. We therefore hypothesize that a combinatorial treatment approach in which local treatment of the primary tumor is combined with systemic treatment targeting the micro-metastases in high-risk cases would have the most potential in UM therapeutics. However, it remains controversial which specific characteristics are necessary to define high-risk UM patients, since different research groups use different prognostic parameters. In order to synchronize UM prognostication and treatment, a universal prognostication model using a combination of clinical, histological and genetic parameters should be considered to reliably identify high-risk UM patients (Drabarek et al., 2019; Eleuteri et al., 2012; Vaquero-Garcia et al., 2017). A number of important questions still remain open and research into these questions will dramatically aid the development of treatments for metastatic UM: does the entire GNAQ and GNA11-signaling network contribute to the development and progression of UM or is it only one arm of the network? Which gene on chromosome 8q plays a role in the development of metastases? Which functions of the BAP1 protein contribute mostly to the aggressive phenotype observed in UM? What is the role of macrophages versus infiltrating T lymphocytes in high risk tumors, or are both only bystanders? And what stimulates dormant UM micro-metastases in the liver to suddenly proliferate and give rise to fatal metastatic foci? The complexity and rarity of this type of cancer has made research into this malignancy difficult, but the recent progress in our understanding of UM will bring us step-by-step closer to effective treatments.

Declaration of competing interest

None.

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