

Characterizing cutaneous and uveal melanoma differences

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Abbreviations

AKT, protein kinase B; ARF, alternative reading frame; BAP1, BRCA1-associated protein; BRAF, brain rapidly accelerated fibrosarcoma; CDH1, E-cadherin; CDKN2A; cyclin-dependent kinase inhibitor 2A; CM, cutaneous melanoma; CTLA4, cytotoxic T-lymphocyte-associated protein 4; CYSLTR2, cysteinyl leukotriene receptor 2; DAG, diacylglycerol; DCT, dopachrome tautomerase; EIF1AX, eukaryotic translation initiation factor 1A, X-Linked; ERK, extracellular regulated kinase; GEF, guanine nucleotide exchange factor; GNA11, guanine nucleotide-binding protein subunit alpha-11; GNAQ, guanine nucleotide-binding protein G(q) subunit alpha; GPCR, G protein-coupled receptor; GTP, guanosine triphosphate; HDM2, human double minute 2; HGF, hepatocyte growth factor; INK4a, inhibitor of kinase a; IP3, inositol trisphosphate; LTD4, leukotriene D4; MAP2K1, mitogen-activated protein kinase kinase; Mb, megabase; MC1R, melanocortin-1 receptor; MITF, microphthalmia-associated transcription Factor; NF1, neurofibromin 1; OIS, oncogene-induced senescence; OM, ocular melanoma; PD1, programmed cell death 1; PDL1, programmed cell death ligand 1; PI, phosphatidylinositol; PI3K, phosphatidylinositol-3-kinase; PIP3, phosphatidylinositol-3 phosphate; PKC, protein kinase C; PLC β , phospholipase C beta; PRAME, PReferentially Expressed Antigen in Melanoma; PREX2, phosphatidylinositol-3,4,5-triphosphate dependent Rac exchange factor 2; PTEN, phosphatase and tensin homolog; RAC1, Ras-related C3 botulinum toxin substrate 1; RAS, Rous sarcoma; RASA2, RAS p21 protein activator 2; SF3B1, splicing factor 3B subunit 1; SNP, single nucleotide polymorphism; snRNP, small nuclear ribonucleoprotein; TERT, telomerase reverse transcriptase; TYRP1, tyrosinase-related protein-1; UM, uveal melanoma; UVR, ultraviolet radiation; YAP, yes-associated protein

Abstract

Cutaneous melanoma (CM) and uveal melanoma (UM) derive from cutaneous and uveal melanocytes that share the same embryonic origin and display the same cellular function. However, the etiopathogenesis and biological behaviors of these melanomas are very different. CM and UM display distinct landscapes of genetic alterations and show different metastatic routes and tropism. Hence, therapeutic improvements achieved in the last few years for the treatment of cutaneous melanomas have failed to ameliorate the clinical outcomes of patients with uveal melanomas.

The scope of this review is to discuss the differences in tumorigenic processes (etiologic factors and genetic alterations) and tumor biology (gene expression and signaling pathways) between CM and UM. We will develop hypotheses to explain these differences, which might provide important clues for research avenues and the identification of actionable vulnerabilities suitable for the development of new therapeutic strategies for metastatic UM.

Physiological function of melanocytes

Melanocytes are cells responsible for the synthesis of melanin pigments within organelles called melanosomes through an enzymatic cascade involving tyrosinase, tyrosinase-related protein-1 (TYRP1), and tyrosinase-related protein 2/dopachrome tautomerase (DCT). Two types of pigment are produced, the brown/black pigment eumelanin and the orange/yellow pigment pheomelanin; the latter is formed in the presence of cysteine or glutathione. The proportion of these two types of melanin defines the variation in skin and iris color. The ratio of eumelanin/pheomelanin is significantly greater in both dark brown skin and eyes than in pale skin and eyes with light-colored irises (hazel, green, yellow-brown and blue in color) (Rees 2004; Wakamatsu et al. 2008).

Melanocytes derive from neural crest cells. These undifferentiated cells, called melanoblasts, migrate to their final location where they synthesize melanin. They are found in various parts of the human body, such as skin, eyes, meninges, heart and cochlea. The role and function of melanocytes are well established in skin but not in other anatomical locations.

In the epidermis, melanocytes transfer melanosome-containing melanin to neighboring keratinocytes to ensure homogeneous pigmentation and to provide efficient skin protection against the harmful effects of ultraviolet radiation (UVR) from solar light (Brenner and Hearing 2008).

In the eyes, melanocytes can be found (i) in the conjunctiva, a non-keratinized epithelium that covers the anterior part of the sclera and the internal surface of the eyelids, and (ii) in all areas of the uvea: the iris, ciliary body and choroid. The role of melanocytes in the conjunctiva remains unknown. The quantity and quality of

melanin pigment in the iris determines eye color. However, in contrast to the skin, the iris color remains stable after exposure to sunlight. Furthermore, the presence of melanin in uveal melanocytes is thought to contribute to eye protection against ocular diseases that can cause blindness, including age-related macular degeneration and uveal melanoma (Sarna 1992). However, how melanin mediates this protection remains mostly unknown.

The presence of melanocytes in organs that are not exposed to UVR indicates that these cells might have functions other than those solely related to photoprotection. Melanocytes in the stria vascularis of the cochlea are involved in the generation of endolymph-mediated action potentials necessary for normal hearing (Barrenas and Lindgren 1990; Tachibana 1999) and in equilibrium function (Takeda et al. 2007). Brain melanocytes are associated with neuroendocrine functions and may also protect against oxidative damage (Zecca et al. 2008). Heart melanocytes play a role in the mechanical properties of the valves (Carneiro et al. 2015) and have been shown to be involved in atrial arrhythmia (Levin et al. 2009).

In this review, we provide several hypotheses to explain why cells sharing the same embryonic origin and cellular functions (*i.e.*, melanin synthesis) are subjected to different tumor transformation processes. We discuss the biological and genetic differences between skin and eye melanomas and, based on these differences, how treatment and clinical outcomes are affected (Table 1).

Classification and prognosis of cutaneous and ocular melanoma

Both cutaneous (CM) and ocular melanoma (OM) arise from melanocyte transformation and represent deadly forms of cancer. Their rate is higher among

Caucasians compared with African-Americans (McLaughlin et al. 2005; Jovanovic et al. 2013). In most cases, they both occur *de novo*, but they can also develop from preexisting melanocytic lesions such as nevi or primary acquired melanocytosis (Tsao et al. 2003; Jovanovic et al. 2013).

The incidence of CM, which develops from cutaneous melanocytes, has dramatically increased in white populations over the past several decades to reach 230,000 new cases worldwide each year (World Health Organization) and accounts for 1.6% of all diagnosed cancers.

A clinico-anatomical classification (Clark's classification) based on the site of cancer occurrence and histological morphology distinguished the following five types of CM: superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, mucosal melanoma and acral melanoma (Clark et al. 1986). Superficial spreading melanoma presents as an enlarging patch during the radial growth phase, which subsequently extends downwards through the skin in the vertical growth phase. Nodular melanoma, which presents as a nodule, has a propensity to grow vertically and to display aggressive behaviors. Lentigo maligna melanoma grows slowly in diameter over many years. It is associated with cumulative sun exposure and thus is found most often in the elderly. Acral melanoma involves the non-pigmented palmoplantar and subungual areas, and mucosal melanoma can occur in all mucosal surfaces. These lesions have been termed acral lentiginous melanoma because they share several features and often present a lentiginous component (Arrington et al. 1977).

Very early skin-localized stage melanoma (Breslow <1 mm) can be cured by wide surgical excision and has a 5-year survival rate of over 98%. By contrast, when diagnosis is delayed, CM becomes increasingly more devastating and individuals

display an increased risk of developing lymph node and visceral metastases. CM is believed to spread mainly via the lymphatic route, although hematogenous diffusion has also been reported (Zbytek et al. 2008). Almost all organs can be involved, but the most common sites for distant CM metastases are the lungs, liver, bones and brain. Until 2012, studies have shown that patients with distant metastatic CM had a median survival rate typically ranging from six to ten months and a 5-year survival rate of approximately 15% to 20% (Tas 2012).

OM, which originates from eye melanocytes, is the most common primary malignancy in the adult population. OM is classified based on the anatomic site of origin as conjunctival or uveal melanoma (UM). The large majority of OM originates from the uvea (95%), comprising the posterior uvea (choroid 90% and ciliary body 5%) and the anterior uvea (iris 5%). The UM staging system is based on the largest basal tumor diameter, ciliary body involvement and extraocular involvement (Kujala and Kivela 2005). Approximately 8,000 new cases of UM and 800 new cases of conjunctival melanoma are diagnosed worldwide each year. CM is 20-30 times more common than UM and 360-900 times more common than conjunctival melanoma (Singh and Topham 2003; Wong et al. 2014). In contrast to the incidence of UM, which has remained stable over last three decades, the incidence of conjunctival melanoma is increasing (Triay et al. 2009). In the early stages, UM usually presents as a pigmented choroidal nodular mass in the eye fundus, growing towards the vitreous space with a typical mushroom shape. It can extend-through sclera or the optic nerve in advanced stages. Symptoms of UM include blurred vision and seeing flashing lights and shadows, but most UMs are initially completely asymptomatic and are diagnosed by an ophthalmologist during a routine sight test, accounting for their

frequent late-stage diagnosis. Despite successful treatment of the primary tumor at diagnosis, only 1-3% of patients have detectable extraocular lesions, and up to 50% of patients develop metastases. Consequently, micrometastases appear to be established several years before the diagnosis of UM. UM spreads mainly via the bloodstream (*i.e.*, hematogenously) (Dithmar et al. 2000). In 80-90% of UM cases, the liver is the most common metastatic site, with the second most common site being the lung (Rietschel et al. 2005; Singh et al. 2005). Importantly, a non-liver first metastasis has been correlated with improved survival. At the metastatic stage, long-term survival is rare. Patients with liver metastases have a median survival time of 2 to 8 months and 80% of patients die within 1 year (Diener-West et al. 2005). Conjunctival melanoma is a completely different entity and generally presents as a pigmented nodular lesion that is usually on the bulbar conjunctiva and often involves the limbus (Shields et al. 2011b). Most conjunctival tumors do not cause symptoms and are diagnosed during a routine eye examination by an ophthalmologist. Approximately 20%-30% of people will develop metastatic disease. Conjunctival melanoma disseminates via the lymphatics and the bloodstream to invade the lungs, brain, liver, skin, bones, and gastrointestinal tract, but it can undergo direct extension to the eyeball and orbit (Kenawy et al. 2013). The melanoma-specific survival rate is 86% at 5 years and 71% at 10 years (Missotten et al. 2005).

Risk factors and genetic predisposition

The etiology of melanoma is complex and heterogeneous because it involves environmental, phenotypic and genetic risk factors. The major risk factors for CM include a personal and familial history of CM, a large number of nevi/dysplastic nevi, sun exposure and skin reactions to sun exposure according to the phototype.

Approximately 10% of CM is estimated to exhibit familial inheritance. Mutations in cyclin-dependent kinase Inhibitor 2A (*CDKN2A*) are found in up to 40% of cases of familial melanoma (Hussussian et al. 1994). *CDKN2A* encodes completely distinct proteins from two alternatively spliced transcripts, p16^{INK4a} (inhibitor of kinase a) and p14^{ARF} (alternative reading frame). p16^{INK4a} inhibits the cyclin-dependent kinases 4 (CDK4) and 6 (CDK6), thus preventing phosphorylation of the retinoblastoma tumor suppressor RB1 and blocking E2F transcriptional activation. p14^{ARF} inhibits human double minute 2 (HDM2), leading to p53 stabilization and increased expression of its target gene p21^{Cip1}.

The second high-risk CM susceptibility gene, for which only a few families have been reported to carry mutations, is *CDK4*. Germline mutations in *CDK4* contain arginine at position 24 instead of cysteine (p.R24C) or histidine (p.R24H) and prevent its interaction with p16^{INK4A} (Zuo et al. 1996).

Additionally, germline inactivation of *RB1* predisposes carriers to CM, at least those who survive their retinoblastoma, a rare cancer of the eye (Fletcher et al. 2004). Hence, multiple mechanisms operate in CM to overcome the RB-dependent G1 arrest, thereby favoring improper progression from G1 to S phase and allowing uncontrolled cell proliferation. Furthermore, RB plays a pivotal role in the induction and maintenance of senescence. Therefore, all the above-described alterations in the RB pathway favor senescence bypass, which is a mandatory step toward melanoma progression (Sherr and McCormick 2002).

In recent years, other high-risk genes have been discovered and may explain approximately 1-2% of familial CM. Although not discussed in detail herein, these candidates are associated with genes implicated in DNA repair, such as the gene encoding *BRCA-1 associated protein (BAP1)* (Wiesner et al. 2011), and in telomere

maintenance, including *POT1*, *ACD*, *TERF2IP* and *TERT* (reviewed in (Aoude et al. 2015)). Thus, the process of senescence appears to be central to the development of melanoma because the melanoma susceptibility genes mentioned above are also linked to cellular senescence.

In addition to these rare but highly penetrant mutations, which confer a high risk of CM, more common single nucleotide polymorphisms (SNPs) represent low-to-intermediate CM susceptibility alleles. Two susceptibility genes with medium penetrance, melanocortin-1 receptor (*MC1R*) and microphthalmia-associated transcription factor (*MITF*), have also been implicated in the risk of CM. *MC1R* encodes the melanocyte-stimulating hormone receptor that acts by activating the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) pathway to control *MITF* expression and the pigmentation process (Bertolotto et al. 1998a; Bertolotto et al. 1998b). *MC1R* variants reduce the ability to stimulate eumelanin production, causing melanocytes to favor pheomelanin synthesis, and are responsible for the red hair color (RHC) phenotype (Schioth et al. 1999). Furthermore, *MC1R* interacts with *PTEN* (phosphatase and tensin homolog) and protects it from degradation, allowing moderation of the downstream phosphatidylinositol-3-kinase (*PI3K*) signaling pathway. Interestingly, the *MC1R* RHC variants do not interact with *PTEN* (Cao et al. 2013) and therefore might favor sustained activation of the *PI3K* pathway, which supports senescence bypass in the context of *BRAF*^{V600E} melanoma cells (Dankort et al. 2009; Vredeveld et al. 2012). Moreover, *MC1R* is also linked to DNA repair mechanisms (reviewed in (Herraiz et al. 2017)). Therefore, alterations of *MC1R* functions in photoprotective melanin synthesis, DNA repair and senescence bypass, by RHC variants might explain the increased risk of melanoma in carriers.

MITF is a master regulator gene of melanocyte development and differentiation

(Steingrimsson et al. 2004) and has also been associated with melanoma development and progression (Garraway et al. 2005). Recently, we and others identified a recurrent germline mutation in MITF (p.E318K) that predisposes carriers to melanoma (Bressac-de-Paillerets et al. 2002; Bertolotto et al. 2011; Yokoyama et al. 2011; Ghiorzo et al. 2013). Additionally, variants of pigmentation genes (*MC1R*, *ASIP*, *MATP*, *TYRP1*, *SLC45A2* and *OCA2*) or of non-pigmentation genes (*MTAP*, *PARP1* and *CASP8*) represent low penetrance mutations (reviewed in (Aoude et al. 2015)). Although both medium-to-low penetrance genes *per se* have a weak impact on melanoma predisposition, they can act as modifiers of high-risk genes and somatic mutations, and can dramatically impact melanoma development.

UM also occurs in a familial setting in 1-2% of cases (Krygier et al. 2001). Major risk factors include fair skin and light eye color, a large number of dysplastic nevi, the presence of oculodermal melanocytosis or nevus of Ota, variation in the *HERC2/OCA2* region that influences the human pigmentation phenotype (Sturm and Larsson 2009; Ferguson et al. 2016) and infrequent mutations in the tumor predisposition syndrome gene *BAP1* (Harbour et al. 2010; Gupta et al. 2015). UM patients have a significantly increased risk of cutaneous melanoma (Scelo et al. 2004), but the mechanisms underlying this risk remain unexplained. Variants in *MC1R* that were shown to influence the quality and quantity of melanin production do not play a role in the susceptibility to developing UM (Metzelaar-Blok et al. 2001; Hearle et al. 2003a; Hearle et al. 2003b; Vajdic et al. 2003). Likewise, current data argue against an important role of the *CDKN2A* gene in UM susceptibility. However, methylation of the *p16^{INK4A}* gene and inhibition of its expression or cyclin D overexpression have been reported (van der Velden et al. 2001). Moreover, although

RB and p53 are infrequently mutated in UM, their respective pathways may be functionally inactivated (Brantley and Harbour 2000a; Brantley and Harbour 2000b). Tumor rarity and the few population-based studies restrict robust conclusions regarding potential risk factors for conjunctival melanoma.

Acquired risk factors: Genetic alterations

- Cutaneous melanoma

Recently, Boris Bastian updated the classification of melanoma by integrating the huge amount of data revealing the genetic alterations in melanoma in association with specific clinical or histopathological characteristics and with different environmental factors such as UVR, thereby providing an integrated taxonomy of melanocytic neoplasia (Bastian 2014). These acquired genetic alterations are depicted below (Figure 1). In this review, we mainly refer to driver mutations, which by definition confer a selective growth advantage to the cells in which they occur (Vogelstein et al. 2013).

Whole-genome and whole-exome sequencing of large CM series confirmed the presence of *BRAF* (50%) and *NRAS* (20-25%) mutations that had been previously identified using candidate gene approaches, and they also revealed a panel of novel frequent somatic genetic alterations that activate oncogenes or inactivate tumor-suppressor genes (Berger et al. 2012; Hodis et al. 2012; Krauthammer et al. 2012; Network 2015).

The Ras family consists of the three isoforms *HRAS*, *NRAS*, and *KRAS*, each encoding a membrane-localized small GTPase that triggers the activation of RAF family serine/threonine kinases (ARAF, RAF1 and BRAF) and the downstream ERK pathway. The ERK pathway plays a very important role in tumor development,

particularly in melanoma development, because it is involved in the control of several key cellular processes including migration, survival and proliferation (Dhillon et al. 2007).

Somatic NRAS mutations are concentrated within two hotspots that occur most frequently in exon 1 leading to the substitution of glycine at position 12 (p.G12V), or in exon 2 leading to the substitution of glutamine at position 61 (Q61K/L/R) (Platz et al. 2008). The mutations prevent GTP hydrolysis and lock NRAS in a permanently active state that continually activates downstream effectors such as BRAF.

Ninety percent of the hotspot somatic mutations in the serine/threonine protein kinase BRAF cause the amino acid substitution p.V600E in exon 15 (Davies et al. 2002). This mutation disrupts the normal intra-molecular interaction that holds BRAF in an inactive conformation, thereby constitutively activating BRAF (Garnett and Marais 2004). Mutations in *BRAF* and *NRAS* occur in a mutually exclusive pattern. Remarkably, among the new recurrent driver mutations that have been identified, two genes, neurofibromin 1 (*NF1*) and *RASA2*, which are mutated in approximately 15% and 5% of CM, respectively, function as RAS-GAP. *NF1* and *RASA2* undergo loss-of-function mutations that increase the level of active RAS-GTP and the activation of downstream ERK and PI3K signaling pathways (Hodis et al. 2012; Krauthammer et al. 2012; Arafeh et al. 2015; Krauthammer et al. 2015).

Mutations in mitogen-activated protein kinase kinase *MAP2K1* (*MEK1*) and *MAP2K2* (*MEK2*), which function downstream of BRAF, have also been identified in 8% of cases and confer resistance to MEK and BRAF inhibitors (Emery et al. 2009; Nikolaev et al. 2011; Villanueva et al. 2013).

Mutations in the genes discussed above are found in more than 80% of CM patients and result in constitutive activation of the ERK signaling pathway.

It is noteworthy that these driver mutations do not necessarily translate into tumor induction since *NRAS* and *BRAF* mutations are frequently found in congenital (Bauer et al. 2007) and acquired nevi (Pollock et al. 2003), respectively. The nevus is thought to be a pre-tumoral lesion that displays blunted progression towards melanoma by senescence (Michaloglou et al. 2005; Denoyelle et al. 2006; Zhuang et al. 2008). Additional epigenetic or genetic alterations of *CDKN2A* or in the PI3K pathway are required to allow melanoma development (Ackermann et al. 2005; Dankort et al. 2009; Dhomen et al. 2009; Vredeveld et al. 2012). Consistent with these findings, recurrent somatic mutations in *BRAF* are frequently associated with a deletion in *PTEN* (7%) and/or *CDKN2A*, which also occurs at a somatic level in a large proportion of melanomas (30%). A recent study from our laboratory supports this idea, demonstrating that the *MITF*^{E318K} variant functions by inhibiting cell cycle inhibitors including p16^{INK4A} to delay the implementation of *BRAF*^{V600E}-mediated oncogene-induced senescence (OIS) (Bonet et al. 2017).

Hence, the PI3K pathway and *CDKN2A* represent important modulators of ERK-dependent melanoma tumor progression (Tsao et al. 2004; Janku et al. 2011; Shull et al. 2012) by favoring OIS bypass and CM development.

PI3K also plays an instrumental role in melanoma development. PI3K activation stimulates the downstream kinase AKT and engages pleiotropic cellular responses, including the regulation of cell motility, survival and proliferation. In CM, the PI3K pathway can be activated as a consequence of constitutive *NRAS* activation and as a consequence of activating mutations or amplification in the catalytic subunit of *PI3K* (*PIK3CA* 4% and *PIK3CG* 3%) (Janku et al. 2011; Shull et al. 2012) (<http://cbioportal.org>) as well as AKT (1-4%) (Davies et al. 2008). However, the inactivation of *PTEN* that occurs in 20 to 30% of CM cases due to mutations (8%),

deletion (6%) or epigenetic silencing is the main cause of PI3K pathway activation (Wu et al. 2003; Mirmohammadsadegh et al. 2006). Supporting the key role of the PI3K pathway in melanoma, mutations in other effectors of the PI3K pathway have also been reported. The AKT family can activate β -catenin, another factor that is important for melanoma formation, which can also be altered by mutations (7% in the TCGA cohort) (reviewed in (Larue and Delmas 2006; Bennett 2008). β -catenin activation can impair OIS (Damsky et al. 2011) and through p16^{INK4A} inhibition can promote senescence evasion and immortalization in mouse melanocytes (Delmas et al. 2007). PTEN loss can also act through a PI3K-independent and caveolin-dependent pathway, to trigger nuclear β -catenin shuttling, p16^{INK4A} repression and senescence bypass (Conde-Perez et al. 2015).

Also operating downstream of AKT, the mTOR signaling pathway is commonly affected (>15% of melanoma cases) by mutations in *MTOR*, *TSC1*, *TSC2*, *RICTOR* and *RPTOR*, as observed in the TCGA melanoma cohort (<http://cbioportal.org>) (Cerami et al. 2012; Gao et al. 2013). However, further studies are required to determine their potential contribution to CM development.

Mutations in *RAC1*, one of the key targets/effectors of PI3K, occur at a frequency of approximately 4-7%. *RAC1* belongs to the Rho family genes that include more than twenty members and encode GTP hydrolases, which are known to affect the cell cytoskeleton and motility. Additionally, aberrant activation of PREX2 (phosphatidylinositol-3,4,5-trisphosphate-dependent RAC exchange factor 2), a member of the DBL family of guanine nucleotide exchange factors (GEF) specific for *RAC1*, has been identified in 14% of cases. PREX2 has also been reported as a PTEN-interacting protein and negative regulator of its phosphatase activity (Fine et al. 2009; Berger et al. 2012). However, whether PREX2 is a true melanoma driver

gene has recently been questioned and thus remains to be formally determined (Horrigan et al. 2017).

Of note, 50% of CM harbors genetic alterations in genes encoding PTEN, catalytic subunits of PI3K, AKT, and RAC1, which function in the PI3K signaling pathway

CM can also display mutations or amplification in the transmembrane receptor tyrosine kinase *KIT* (5-8% of cases). These alterations appear to be more frequent in acral, mucosal or lentigo maligna melanomas (Curtin et al. 2006; Beadling et al. 2008). Moreover, they lead to stem cell factor (SCF/*KIT* ligand)-independent activation of *KIT* and its associated downstream signaling cascade, including MAPK/ERK, PI3K and phospholipase C (Carvajal et al. 2011; Allegra et al. 2014).

Most importantly, whole genome/exome studies of CM have also led to the identification of frequent mutations in other genes including the tumor suppressor genes *TP53* (19%), the subunit of the PBAF chromatin-remodeling complex *ARID2* (7%) or the serine/threonine phosphatase *PPP6C* (12%) involved in the control of the cell cycle. The presence of recurrent somatic mutations in the *TERT* promoter (approximately 75% of metastases and 33% of primary lesions) has been reported in CM (Patel et al. 2016). All these mutations might also function to inhibit or delay OIS. A large panel of other (not discussed in this review), less frequently mutated genes, has been revealed, all of which might represent CM drivers or modifying genes and are potential targets for new therapeutic approaches (Bertolotto 2013; Shtivelman et al. 2014; Zhang et al. 2016; Weyden et al. 2017).

Thus, this mutational landscape emphasizes the importance of the ERK and PI3K signaling pathways, as well as the bypass of senescence in CM development, progression and resistance to therapies.

- Uveal melanoma

Similar large-scale whole-genome and whole exome sequencing studies performed in UM validated previously occurring mutations mainly in *GNAQ* or *GNA11* (83%), in *BAP1* (40%), in *SF3B1* (20%), and in *EIF1AX* (8%) (Decatur et al. 2016; Johansson et al. 2016; Moore et al. 2016). These studies also pinpointed other driver genes that provide a basis to identify molecular frameworks for the design of therapeutic strategies.

The G α subunit of the heterotrimeric G proteins, *GNAQ* or its paralog *GNA11*, share 90% sequence homology and represent the most frequently mutated genes in UM. Ninety-seven percent of the hotspot somatic mutations cause the amino acid substitution p.Q209L (the most common) or p.Q209P in exon 5. The other 3% of mutations cause a p.R183C amino acid change in exon 4. The Q209 mutation triggers a complete loss of intrinsic GTPase activity and renders *GNAQ/GNA11* constitutively active to prolong its downstream signaling. In contrast, the R183 mutation is weakly activating because it causes only a partial loss of intrinsic GTPase activity. The Q209 and R183 mutations occur in a mutually exclusive pattern in UM. Mutations in *GNAQ* or *GNA11* are found in 45% and 32% of primary UM, respectively, and in 22% and 57% of metastatic UM, respectively (Onken et al. 2008; Van Raamsdonk et al. 2009; Van Raamsdonk et al. 2010). Although differences between *GNAQ* and *GNA11* signaling have not been elucidated, these findings suggest that *GNA11* mutant tumors have a greater tendency to metastasize. GTP-*GNAQ/11* and beta-gamma subunits transfer the signal from the receptor to downstream effectors that stimulate diverse signaling pathways, including the MAP kinase pathway, possibly via DAG-mediated activation of protein kinase C isoforms, the ADP-ribosylation factor 6 (ARF6)-TRIO-RHO/RAC implicated in cytoskeletal

organization, and Yes-Associated Protein 65 (YAP), a key component of the HIPPO signaling pathway (Feng et al. 2014; Yu et al. 2014; Yoo et al. 2016). ARF6 acts as a proximal node of oncogenic GNAQ signaling and contributes to activation of the downstream pathways (Yoo et al. 2016). Of note, no ARF6 mutations have been discovered to date in UM or in other cancers.

The importance of GNAQ in UM is highlighted by the observation that mutations in upstream regulator and downstream effectors were recently identified. Cysteinyl leukotriene receptor 2 (CYSLTR2) is a seven transmembrane G protein-coupled receptors (GPCRs) and a member of the rhodopsin-like family that responds to purinergic or pyrimidineric nucleotides (P2Ys). As a member of the GPCR family, CYSLTRs signal through activation of GNAQ/11 (Mong et al. 1988). Recurrent p.L129Q, as well as p.R136H mutations in *CYSLTR2* have been discovered in UM patients (Moore et al. 2016). Only p.L129Q *CYSLTR2*, which favors the transition towards an active conformation, is oncogenic. CYSLTRs are activated by cysteine-containing leukotrienes (LTC₄, LTD₄, and LTE₄), which are lipid mediators and potent inflammatory mediators. LTC₄ and LTD₄ are potent mitogens of normal human epidermal melanocytes (Morelli et al. 1989). Leukotrienes also have roles in multiple diseases including cancer. LTD₄, a *CYSLTR2* agonist, facilitates cell survival and proliferation of intestinal epithelial cells through β -catenin activation (Mezhybovska et al. 2005). LTD₄ also regulates the survival and migration of human colon cancer cells by regulation of an anti-apoptotic member of the BCL2 family and activating integrin, respectively (Massoumi et al. 2003; Wikstrom et al. 2003a; Wikstrom et al. 2003b). Collectively, these data are consistent with a role for LTD₄ in cancer, including UM. Additionally, in response to a pathobiological event, *CYSLTR2*

can mediate an increase in vascular permeability in some tissues (Beller et al. 2004), a process that might contribute to UM blood dissemination.

Among the downstream effectors of GNAQ/11 are members of the phospholipase C (PLC) family, mainly PLC Beta, which hydrolyze PIP2 to generate IP3 and DAG. IP3 triggers the release of calcium ions from the endoplasmic reticulum, whereas DAG activates the protein kinase C (PKC) signaling cascade. A recurrent mutation in *PLCB4*, which encode p.D630Y, was recently identified in UM patients (Johansson et al. 2016; Moore et al. 2016). This mutation is located in the Y-domain of the highly conserved catalytic core of *PLCB4*, which is activated by direct interaction with GNAQ (Lyon and Tesmer 2013). A novel mutation in *PLCB3* encoding p.K898N has also been discovered (Johansson et al. 2016). Again, this mutation lies in a domain, the CTD linker, which is linked to GNAQ activation (Lyon and Tesmer 2013). However, the role of *PLCB3* as a UM driver gene remains to be demonstrated. Mutations in *CYSLTR2*, *GNAQ*, *GNA11* and *PLCB4* are mutually exclusive, suggesting that they operate in the same pathway

UM metastases are also associated with inactivating somatic mutations in *BAP1* in approximately 80% of cases, which generally cause protein truncations and are associated with a poor prognosis (Harbour et al. 2010). *BAP1* is a chromatin-associated deubiquitinase that induces poly(ADP-ribose)-dependent recruitment of the polycomb deubiquitylase complex PR-DUB to sites of DNA damage and is required for efficient assembly of the homologous recombination (HR) factors *BRCA1* and *RAD51* (An et al. 2014). Consequently, its mutation impairs its function in DNA double-strand break repair (Ismail et al. 2014). Moreover, *BAP1* impacts histone H2A ubiquitination and regulates transcriptional programs, which support the maintenance

of melanocytic cell identity, and blocks their transition towards a stem-like phenotype (Landreville et al. 2012; Matatall et al. 2013).

Dysregulation of the activity of two other genes, splicing factor 3B, subunit 1 (*SF3B1*) and eukaryotic translation initiation factor 1A, X-Linked (*EIF1AX*), have prognostic value in UM.

EIF1AX is a component of the 43S pre-initiation complex (PIC), which mediates the recruitment of the small 40S ribosomal subunit to the 5' cap of messenger RNAs. It is not clear how these mutations might promote cancer, but the dysregulation of mRNA translation is a frequent feature of neoplasia (Bhat et al. 2015). Recurrent mutations in *EIF1AX* are mainly found in low metastatic risk tumors with no ciliary body involvement (Furney et al. 2013; Martin et al. 2013; Johansson et al. 2016), meaning they are associated with a good prognosis.

SF3B1 encodes a core component of the U2 small nuclear ribonucleoprotein (snRNP) complex of the spliceosome involved in 3'-splice site recognition during RNA splicing (Furney et al. 2013; Zhang and Manley 2013; Alsafadi et al. 2016). Alternative splicing contributes to structural transcript variation and proteome diversity, a process involved in disease progression (Sveen et al. 2016). The *SF3B1* missense hotspot mutations (p.R625C, p.R625H, p.K666T and p.K700E) are associated with low risk for metastasis (Furney et al. 2013; Harbour et al. 2013; Martin et al. 2013; Alsafadi et al. 2016). Recently, Dr. Harbour's group has shown that *SF3B1* mutations often occur in tumors expressing the oncogene PReferentially expressed Antigen in MElanoma (*PRAME*), which is an independent biomarker for metastasis. *PRAME* expression appears to be inversely associated with *EIF1AX* mutations (Field et al. 2016a; Field et al. 2016b).

BAP1, *SF3B1*, and *EIF1AX* mutations are almost mutually exclusive with each other (Martin et al. 2013; Decatur et al. 2016; Royer-Bertrand et al. 2016; Yavuziyigitoglu et al. 2016).

Finally, whole genome/exome studies have revealed somatic missense or truncating mutations in a panel of other genes, but their roles in UM remain to be elucidated (Johansson et al. 2016; Royer-Bertrand et al. 2016).

Interestingly, GNAQ/11 mutations have also been identified in approximately 5% of skin melanomas, but oncogenic driver mutations similar to those identified in UM (Q209P or Q209L) are found in only approximately 2% of CM cases (<http://cbioportal.org>). One Q209 mutation was found in a CM from chronically sun-damaged skin among the 74 samples analyzed (Van Raamsdonk et al. 2010). Furthermore, *PLCB4* is recurrently mutated with a high frequency (21% to 28%) in CM (Wei et al. 2011; Hodis et al. 2012; Cancer Genome Atlas 2015; Krauthammer et al. 2015). However, none of these mutations is identical to those found in UM and no hotspot mutations can be found, indicating that they are unlikely to function as driver mutations. It should be noted that *TRIO* is also affected in 10% of CM. In 5% of the cases, these alterations seem to be passenger mutations, but in the remaining 5%, *TRIO* is amplified (<http://cbioportal.org>) and might participate in CM development. Nevertheless, elucidation of the role of GNAQ/11 and its downstream effectors, *PLCB4* and *TRIO*, in CM, requires additional investigation.

Finally, *BAP1* is affected in 40% of UM cases, and the majority of the alterations are truncating mutations. In contrast, *BAP1* is affected in 3% of CM cases, but truncation mutations are a rare event (<http://cbioportal.org>).

Little is known about the genetic perturbations in conjunctival melanomas. Nevertheless, primary and metastatic conjunctival melanomas harbor BRAF, mainly p.V600E (27-50%) and NRAS p.Q61K/R/L (18%) mutations (Griewank et al. 2013). Mutations in the promoter of *TERT* are also detected in conjunctival melanomas (32%) but not in UM (Griewank and Murali 2013). They harbor a UV signature identical to those found in CM that mediates increased expression of TERT by generating new binding motifs for Ets transcription factors (Horn et al. 2013). Therefore, from a genetic perspective, conjunctival melanoma seems to have more similarities to CM than to UM.

Cytogenetic alterations

Before the use of deep sequencing approaches, the genetic modifications in CM and UM were determined by cytogenetic studies. Comparative genomic hybridization was used to map copy number abnormalities. Below are reported the frequent chromosomal aberrations:

CM exhibits complex cytogenetic alterations (for review (van den Bosch et al. 2010)). They are characterized by frequent losses involving chromosomes 4, 5, 6q and 8p, 9p, 10q, 11q, 12q, 14, 15, 16, 21, and 22, whereas gains most often occurred at 1q, 6p, 7, 8q, 18 and 20q (Bastian et al. 1998; Pirker et al. 2003). It is worth mentioning the rearrangement in chromosome 1, where *NRAS* (1p13 region) and *AKT3* (1q44 region) are located, of chromosome 7 harboring the *BRAF* gene (7q34) and of chromosome 9 with *CDKN2A* (9p21).

In UM, chromosomal aberrations include mainly monosomy 3 (50%) as well as 6p and 8q gain. UM tumors with monosomy 3 and polysomy 8q correlate with high metastatic risk and a poor prognosis (de Lange et al. 2015; Versluis et al. 2015).

Monosomy 3 occurs in 50% of the analyzed cases, is rather specific for UM because this chromosomal aberration is rarely encountered in other cancer types and is the most widely used predictor of metastatic disease (van den Bosch et al. 2012). Chromosome 3 likely hosts tumor suppressor genes. One of the most studied is *BAP1*. Conversely, genes that can contribute to tumor progression are located in the 8q region, such as *MYC* (8q24) (Muller et al. 2010) or *ASAP1* (*DDEF1*) (8q24) (Meyer and Penn 2008). Interestingly, ArfGAP with the SH3 domain, ankyrin repeat and PH domain (*ASAP1*) is a GTPase-activating protein for ARF1 as well as ARF6 (Furman et al. 2002). Although the gain of chromosome 8q is also found in 25% of CM, co-occurrence of both monosomy 3 and the gain of 8q is rare in CM. UM tumors with such a 6p gain are less likely to show chromosome 3 loss and are associated with better survival. Moreover, chromosome 10, which contains *PTEN*, is also altered in UM (27%) but to a lower frequency compared with CM (60%). *PTEN* down-regulation seems to occur in UM lesions with high genomic instability, supporting a role late in tumor progression (Ehlers et al. 2008).

Several other DNA copy number alterations, including the gain of 1q or loss of 8p, 1p and 6q, also characterize UM (Aalto et al. 2001; Trolet et al. 2009; Damato et al. 2010; Royer-Bertrand et al. 2016).

Collectively, CM tumors display very complex karyotypes that cannot be used to provide valuable prognostic information. In contrast, UM presents a relatively “simple” karyotype, with recurrent chromosomal anomalies, which has valuable prognostic impact for patients. Consequently, high-risk patients may benefit from accurate surveillance, including that of the liver, which is the most common metastatic site, or may enter clinical trials investigating adjuvant therapy.

The gene expression program

Cytological and histochemical methods have long been recognized as useful for analyzing the inter- and intra-tumor heterogeneity in CM. More recently, high throughput approaches (DNA arrays and RNA-Seq) have confirmed that CM displays a high degree of inter-tumor heterogeneity, even in the same individual (Kemper et al. 2015). Intra-tumoral heterogeneity was validated at the single cell level (Ennen et al. 2015; Tirosh et al. 2016). Tumor phenotypic heterogeneity may be caused by genetic heterogeneity but also may be generated by the impact of the tumor microenvironment, without any requirement for new or additional genetic events. Understanding and deciphering tumor heterogeneity remains a challenge to cancer therapy.

CM cells can be classified into at least two major states, *i.e.*, proliferative and invasive (Hoek et al. 2008). A model derived from these findings, the “phenotype-switching” model, predicts that melanoma cells are plastic and may switch between these two states to generate intratumoral heterogeneity (Hoek and Goding 2010). This model postulates that high MITF activity triggers a differentiation phenotype, whereas low MITF activity is associated with mesenchymal transition and an invasive phenotype (Carreira et al. 2006; Cheli et al. 2011; Ohanna et al. 2011). More recently, a larger gene repertoire, linked to activation of the HIPPO-YAP pathway (Muller et al. 2014; Verfaillie et al. 2015) and to metabolic stress responses (Falletta et al. 2017), has been established as an indicator of this plasticity. Previous reports have demonstrated that phenotype switching towards a more mesenchymal cell state is associated with intrinsic and acquired resistance to targeted therapies (Johannessen et al. 2013; Van Allen et al. 2013; Konieczkowski et al. 2014; Muller et al. 2014). Melanoma phenotype switching also negatively impacts immune

checkpoint blockade and impairs the efficiency of immunotherapy (Landsberg et al. 2012; Riesenberger et al. 2015; Falletta et al. 2017).

Intertumor heterogeneity has also been described in UM. Molecular analyses classify UM into two prognostically significant molecular classes (Onken et al. 2004; Onken et al. 2006; Onken et al. 2010a). Class 1 UM tumors, which have been further divided into well-defined class 1A and class 1B, retain a differentiated melanocytic phenotype and have a low to intermediate metastatic risk, respectively. *EIF1AX* and *SF3B1* are associated with class 1, with *SF3B1* showing a particular association with class 1B (Harbour et al. 2013). Class 2 UM tumors exhibit a dedifferentiated stem cell-like and epithelioid phenotype that is associated with monosomy of chromosome 3 and a high metastatic risk (Field and Harbour 2014). Interestingly, the class 2 expression program is mainly a consequence of monosomy 3 and loss of function of BAP1. Indeed, depletion of BAP1 in cultured class 1 UM cells induced a loss of the melanocyte differentiation markers and acquisition of a class 2 gene expression profile (Landreville et al. 2012; Matatall et al. 2013).

The gene expression profile capable of distinguishing class 1 and 2 primary UM has been further restricted to a set of 12 genes (Table 2), which has been shown to be more accurate than all other clinical and pathologic factors, such as chromosome 3 status (monosomy 3), cytopathology and tumor size, to predict the development of metastases (Onken et al. 2010b).

Importantly, co-existence of the spindle and epithelioid cells in some UM tumors revealed by histopathological analysis suggests the existence of intratumor heterogeneity in addition to inter-tumor heterogeneity.

Furthermore, UM metastasis and poor patient outcome are associated with

monosomy 3 and with the loss of differentiation markers. Remarkably, MITF, which controls melanocyte differentiation (Bertolotto et al. 1998a; Cheli et al. 2010) is located in 3p13. Moreover, in skin melanocyte cells, MITF controls the expression of a repertoire of genes involved in DNA repair and replication (Giuliano et al. 2010; Strub et al. 2011). Consequently, one might hypothesize that in UM, monosomy 3 triggers a reduction of both BAP1 and MITF levels and dampens accurate DNA repair, favoring chromosomal instability and UM progression.

In support this idea, tumors with disomy 3, which rarely metastasize and thus are associated with a better survival rate and contain fewer chromosomal abnormalities (Onken et al. 2010a; Shields et al. 2011a), which might be explained by the maintenance of MITF. Moreover, MITF haploinsufficiency might favor UM cell switching from a differentiated to undifferentiated, metastatic prone phenotype, as observed for CM, and therefore might contribute to metastasis development. Collectively, these observations suggest that MITF, which is a nexus in CM pathology, might also play a critical role in UM.

Activation of the transcriptional coactivator YAP, a critical downstream effector of the HIPPO signaling pathway, has been reported in both CM and UM (Feng et al. 2014; Nallet-Staub et al. 2014; Yu et al. 2014). YAP is activated in UM cells downstream of the oncogenic mutation of GNAQ/11 and is required for GNAQ/11-induced tumorigenicity in UM. The YAP-TEAD cascade seems to be implemented in CM cells that have lost MITF and engages in an invasive gene expression program (Verfaillie et al. 2015). These observations suggest that these signaling molecules (MITF, BAP1 and YAP) may represent suitable pharmacological intervention strategies in both tumor types.

Role of UV

It has long been suspected that UVR exposure was the main environmental risk factor for melanoma. However, the link with melanoma development was not fully understood until recently. Large-scale genomic studies have revealed a higher rate of somatic mutations in CM tumors than in any other tumor types (Lawrence et al. 2013) with a median of 16.8 mutations per megabase (/Mb) (Berger et al. 2012; Hodis et al. 2012; Krauthammer et al. 2012; Cancer Genome Atlas 2015). The highest average mutation rate was observed in chronically sun damaged skin melanomas (21/Mb), whereas acral and mucosal melanomas displayed a very lower mutational load (Cancer Genome Atlas 2015). These studies have unequivocally provided genomic evidence for a direct mutagenic role of UV light in melanoma pathogenesis. The mutations are predominantly C to T or tandem CC to TT transitions at specific dipyrimidine sequences, which is the mutational signature of UVB light (Harris 2013) or G to T substitutions that might reflect a transversion following oxidative DNA damage (Cheng et al. 1992). Specifically, it was found that 46% and 9% of melanoma driver mutations can be attributed to C>T or G>T mutations, respectively (Hodis et al. 2012). BRAF^{V600} variants, particularly BRAF^{V600E}, which is the main driver gene in melanoma, do not bear the traditional UVB signature mutations. However, sunlight UV is also composed of UVA, which is thought to promote mutagenic lesions through oxidative damage (Besaratina et al. 2004). Accordingly, DNA lesions induced by UVA exposure resemble the BRAF^{V600E} variant mutation (Thomas 2006; Besaratinia and Pfeifer 2008).

From the genomic analyses, it appears that UM tumors have a low mutation burden. Johansson et al. identified a mean of 10.6 protein changing mutations per sample (range 0 to 53) (Johansson et al. 2016), which is consistent with other studies

reporting 17 variants per tumor on average (Royer-Bertrand et al. 2016). The mean mutation rate across the UM patient genomes range between 0.24-0.50/Mb, which is lower than that of metastatic CM, and the mutation spectrum is not consistent with an ultraviolet radiation signature (Johansson et al. 2016; Royer-Bertrand et al. 2016), strengthening the lack of UV involvement in UM etiology. However, a C to T transition has been described in few cases of UM with a GNAQ/11 R183 mutation, suggesting a possible role of UV in UM.

Comparative biology of uveal and cutaneous melanoma

Why are driver mutations different in UM and CM?

As mentioned above, despite the fact that both CM and UM are derived from melanocytes that originate from neural crest cells, the driver mutation landscape is completely different in these two neoplasms. This difference might be ascribed to the lack of UVR involvement in UM etiology, while UVR is a proven risk factor for CM.

However, the risk of intraocular melanoma is much higher in Caucasians than in African Americans and in people with light colored eyes (Seddon et al. 1990; Vajdic et al. 2002; Tsai et al. 2005), suggesting a possible link to sunlight exposure. Further studies have also proposed a role for short-wavelength blue light exposure in the etiology of UM (de Lange et al. 2015). Blue light, which is part of the visible light spectrum, reaches deeper into the eye and causes damage to the back of the eye. Although only a part of blue light is harmful, our exposure to it is increasing due to the use of digital devices and modern lighting, which emit blue light. Furthermore, as has been shown for CM, the pheomelanin pigment pathway might contribute to uveal melanomagenesis by an ultraviolet-radiation-independent carcinogenic mechanism

that depends on oxidative damage (Mitra et al. 2012). In agreement with this notion, both cutaneous and ocular melanomas are more common in Caucasians than in African-Americans (Tsai et al. 2005), *i.e.*, in individuals with pale skin and eyes with light-colored iris in comparison to those with dark brown skin and eyes.

Of note, the main driver mutations, *GNAQ/11*^{Q209L} (c.626A>T), *CYSLTR2*^{L129Q} (c.386T>A) in UM and *BRAF*^{V600E} (c.1799T>A) in CM, display homologous base substitutions, which is not related to UVR, suggesting a converging mutational mechanism.

In addition to understanding the cause of the mutations, it is also important to consider why causative driver mutations are not identical in UM and CM. It should be noted that *BRAF* and *NRAS* mutations dominate in lesions arising in an epithelial context such as conjunctival melanoma and CM, excluding blue nevi and blue-nevi-like melanoma. *GNAQ/11* mutations are found in melanocytic lesions with an extra-epithelial location.

Indeed, *GNAQ/11* mutations are not solely restricted to UM but are also found in melanocytic lesions located in leptomeninges (diffuse melanocytosis and meningeal melanomatosis) and dermis (nevus of Ota, blue nevi and blue-nevi-like melanomas) (Van Raamsdonk et al. 2009; Van Raamsdonk et al. 2010; Murali et al. 2012). Most of these lesions arise in the craniofacial region, albeit blue nevi can be found anywhere on the body. A plausible explanation for *GNAQ/11* mutations predominance in non-epithelial melanocytes involves subtle geographic variances in the embryonic origin of epithelial and non-epithelial melanocytes. Along the embryonic axis, several distinct neural crest populations differ both in their migratory pathways and range of derivatives (Bronner-Fraser 1994). In this context, it has been suggested that non-epithelial melanocytes derive from cranial rather than truncal

neural crest (Francis et al. 2016), which indicates that although the melanocytes have the same embryonic origin, *i.e.*, the neural crest, they might behave differently as a function of their relative anterior-posterior position (Mayor and Theveneau 2013).

However, blue nevi located on the legs for instance are unlikely to derive from cranial neural crest.

Further, it should be noted that epidermal melanocytes interact with epithelial cells, whereas non-cutaneous melanocytes interact with mesodermal stroma. The growth and differentiation of epidermal melanocytes appear to be dependent on KIT signaling, whereas non-cutaneous melanocytes seem more dependent on the endothelin and HGF signaling pathways (Wilson et al. 2004; Aoki et al. 2009). Therefore, it could be argued that CM and UM acquire the same mutations, but direct cell contact or the paracrine signal produced by the tissue-specific environment might only allow proliferation of cells with specific mutational events and therefore favor selection of specific driver mutations.

Finally, Adameyko et al. showed that Schwann cells, which also originate from the neural crest and differentiate to form myelin sheaths that surround the mature nerve, constitute another source of melanocytes (Adameyko et al. 2009). A mutation in GNA11 has been reported in a melanotic Schwannoma, a soft tissue benign neoplasm of Schwann cells, which shares histologic features with melanocytic tumors and Schwannomas (Tatsi et al. 2016). Because the mutational spectrum of these lesions overlaps, it has been previously hypothesized that both cell types could derive from the same developmental mechanism (Van Raamsdonk et al. 2010; Bastian 2014).

Why are gene expression signatures different in UM and CM?

In both CM and UM, extensive efforts have been undertaken to identify genes or sets of genes that can predict the clinical outcome of the patients, including metastasis development and survival. As mentioned earlier, the study of numerous primary UM patients has led to a well-defined molecular classification (Table 2). Class 2 UM has the poorest survival prognosis and is characterized by an increase in epithelial markers such as E-cadherin (*CDH1*), and by a loss of differentiation markers such as tyrosinase (*TYR*) or dopachrome tautomerase (*DCT*). For CM, the gene expression signature has been obtained by analysis of metastatic melanoma cell lines or short-term cultures and defines a high-MITF/differentiated group endowed with fast proliferation capacity and a low-MITF/poorly differentiated group with highly invasive behavior. The low-MITF CM generally lost epithelial markers such as E-cadherin and gained a mesenchymal phenotype. This apparent discrepancy can be explained by the differences in the UM tumor tissue versus cultured metastatic CM cells of the analyzed samples. Of note, analysis of CM tumor tissue also identified a “keratin subclass” of CM with high expression levels of keratin and epithelial markers (*CDH1*) indicating a worse prognosis (Cancer Genome Atlas 2015). However, this subclass also displays a high level of differentiation markers, in contrast to reports for primary UM samples. The nature of analyzed UM and CM samples are not similar. Primary lesions are predominant in UM cohorts, while CM tissues are mainly from metastatic samples (80%), of which approximately 50% are lymph node metastases (Cancer Genome Atlas 2015).

The natural history of metastatic tumor development requires, first, an *in situ* proliferation phase followed by an invasion phase, allowing melanoma cells to reach

metastatic sites. Once at the metastatic site, cells with invasive properties can migrate and colonize other organs, or can proliferate to promote an increased tumor size, functional failure of the affected organ and ultimately patient death. Therefore, survival will be affected by the ability of tumor cells to implement both the invasive and subsequent proliferative programs. In primary skin melanoma lesions, cells must enable the invasive program, while metastatic cells require only the proliferative program. Accordingly, the gene expression signature associated with poor prognosis might be different if it stems from primary UM lesions, or from CM metastatic samples.

Despite the invaluable basic information provided by gene expression analyses of melanoma lesions, their translation into clinical advances are currently minimal for CM. Furthermore, primary CM shows a very high level of contamination by keratinocytes that may hinder accurate gene expression profiling. For UM, gene expression profiling has a real clinical impact, perhaps because the analyzed samples are more homogenous.

Why does UM display prominent liver tropism?

Metastasizing UM displays a tropism to the liver in 90% of cases, while CM does not demonstrate such preferential tropism. Indeed, CM cells disseminate to skin or lymph nodes, and almost equivalently to lungs, liver, brain and bones (Balch et al. 2003). Several factors might be important for favoring UM liver metastasis progression, such as the invasion route used by tumoral cells. It is accepted that CM cells prefer the lymphatic system, while UM cells spread almost exclusively hematogenously. The hematogenous routes might favor dissemination of tumor cells from the eye to the liver rather than to others organs. It is also possible that the specific loose structure

of the liver endothelial vasculature presenting large fenestrae (Wisse et al. 2008), might favor liver colonization by UM cells. Another mechanism could be related to the metabolic status, which has been shown in breast cancer cells to dictate the site of metastasis (Dupuy et al. 2015).

Finally, recent data demonstrated that UM-derived exosomes, expressing integrin alpha V/integrin beta5, are taken up by liver-specific cells to prepare the pre-metastatic niches and to steer the liver tropism of UM cells (Hoshino et al. 2015). In addition to favoring the nesting of UM cells, the liver might also provide a favorable environment for sustaining the growth of UM. This phenomenon could be achieved through liver production of hepatocyte growth factor (HGF), which stimulates the proliferation and survival of UM cells expressing the surface receptor c-Met (Bakalian et al. 2008). A complete identification of the mechanisms mediating this tropism will undoubtedly help to improve patient surveillance and outcome.

From Bench-to-Bedside: the therapeutic options

The identification of specific biological, molecular and genetic tumor features have led to the development of “personalized therapy,” *i.e.*, therapies tailored to patients-specific molecular aberrations. More precisely, the discovery of the BRAF^{V600E} mutation led to the development of BRAF (vemurafenib and dabrafenib) and MEK (trametinib and cobimetinib) inhibitors (Sullivan and Flaherty 2015b). Bi-therapy, combining both BRAF and MEK inhibitors, is the reference treatment for patients with metastatic CM harboring a mutation at codon 600 of BRAF, yet in some centers it is superseded by immunotherapy (described below). The targeted therapies have allowed, for the first time, a more than 12 month-increase in the median overall survival of the patients (Long et al. 2014; Ugurel et al. 2016). However,

despite the success of these treatments, most patients eventually develop secondary resistance and relapse.

The genetic studies suggest that conjunctival melanoma behaves more similar to cutaneous than uveal melanoma. Although there is no standard recommendation for the treatment of patients with conjunctival melanoma, there is ample evidence to test patients for mutations at codon 600 of BRAF and to assess the efficacy of the BRAF and MEK inhibitors.

Currently, there is no systemic treatment for UM once it has spread. UM metastases are remarkably refractory to conventional chemotherapy and non-sensitive to external radiotherapy. Mortality rates have not changed in the last decades. In patients with UM metastases, BRAF and/or MEK inhibitors are generally ineffective due to the absence of the BRAF mutation (for review (Oliva et al. 2016)). However, because a phase 2 trial showed promising results with the MEK-inhibitor selumetinib (Carvajal et al. 2014), there are several ongoing trials for UM patients assessing the efficacy of selumetinib in combination with chemotherapies or with PKC and AKT inhibitors (Chattopadhyay et al. 2016).

Nevertheless, the discovery of *GNAQ*, *GNA11*, *CYSLTR2* and *PLCB4* mutations has paved the road towards specific targeted therapies for UM. In this context, FR900359, a selective inhibitor of GNAQ/11/14, has been recently identified and shown to blunt the signaling downstream of GNAQ^{R183C} and GNAQ^{Q209L} (Schrage et al. 2015). Moreover, compounds with anti-CYSLTR2 activity are currently being tested in phase 2 clinical trials (Wunder et al. 2010). As CYSLTR2 functions upstream of GNAQ/11, CYSLTR2 inhibitors are expected to be efficient only in UM without GNAQ/11 or PLCB4 mutations.

Inhibitors of TRIO (Blangy et al. 2006; Schmidt and Debant 2013) or ARF6 (Yoo et al.

2016) have been described and would be of wider use because they will block oncogenic signaling downstream of mutated GNAQ/11 and CYSLTR2, overall representing approximately 95% of the UM cases. These inhibitors might not be efficient in UM with PLCB4 mutations.

Finally, CM and UM might be sensitive to the same inhibitors. For example, UM with mutations in EIF1AX might benefit inhibitors of the formation of the EIF4F complex, which is required downstream of EIF1AX for the regulation of cap-dependent translation. Such inhibitors have been described and have shown some efficacy in a preclinical model of CM (Boussemart et al. 2014).

The potential targets and drugs are summarized in Table 3.

Additionally, studies have elucidated how CTLA4 and PD-1 decrease activation of the immune system, thereby leading to the development of monoclonal antibodies against CTLA4 (ipilimumab) and PD-1 (nivolumab and pembrolizumab). These antibodies have demonstrated clear clinical benefits in patients with metastatic CM. Indeed, objective response rates are obtained in 15-30% and demonstrate durable responses, reaching an 18-month increase in median survival (Hodi et al. 2010; Topalian et al. 2014; Carlino and Long 2016). The combination of ipilimumab and nivolumab showed even greater improvements in patient overall survival compared with anti-CTLA4 or anti-PD1 monotherapy (Kaufman et al. 2013; Sullivan and Flaherty 2015a). Clinical trials investigating antibodies against PD-L1, a PD1 ligand, have demonstrated promising activity in advanced CM, albeit with generally lower response rates than PD-1 antibodies (Tsai et al. 2014; Mahoney et al. 2015).

Until now, immunotherapeutic approaches targeting immune checkpoints have shown limited efficacy in metastatic UM (Danielli et al. 2012; Algazi et al. 2016; van der Kooij et al. 2017). Ipilimumab has failed to demonstrate a clear objective clinical

response (reviewed in (Oliva et al. 2016)). Similarly, anti-PD1 (either pembrolizumab or nivolumab) or anti-PDL1 (Algazi et al. 2016) has shown poor clinical activity (Algazi et al. 2016; van der Kooij et al. 2017).

The weak response of metastatic UM to immunotherapies might be ascribed to the maintenance of ocular immune privilege, which has been involved in the suppression of both adaptive and innate immune effector mechanisms (McKenna and Chen 2010). Moreover, the CM response to immunotherapies seems to be correlated to the mutational burden, which is thought to generate neo-antigens (Johansson et al. 2016; Royer-Bertrand et al. 2016). The low mutational burden of UM might also explain the moderate response to immunotherapies.

Clinical trials evaluating the efficacy of pembrolizumab in monotherapy, which nevertheless has shown a more favorable response in one study (Kottschade et al. 2016), and the combination of ipilimumab and nivolumab are currently under way (Chattopadhyay et al. 2016). In the absence of rational treatment options for metastatic UM, immunotherapy should be considered, and additional clinical trials should be scheduled. In support of this idea, combination therapies of checkpoint inhibitors with local, targeted and immuno-therapy for metastatic UM must be explored to determine whether they could improve patient prognosis.

Moreover, strategies using genetically modified T-cell-based adoptive immunotherapy approaches, including chimeric antigen receptor (CAR) T-cell therapy and engineered T-cell receptor (TCR) T-cell therapy, have yielded encouraging clinical responses by overcoming immune evasion and by redirecting the specificity of cytotoxic T lymphocyte to tumor cells (Sharpe and Mount 2015). Due to advances in sequencing technology, somatically mutated UM antigens, or neoantigens, have been identified and have become compelling targets for

immunotherapy. Adoptive immunotherapies could therefore represent therapeutic options for low mutation burden cancers such as UM.

Furthermore, PRAME is associated with class 1b metastatic UM (Field et al. 2016a) and is immunogenic, increasing the attractiveness of the development of novel immune therapies for PRAME. Phase 1 clinical trials are currently evaluating the safety and immunogenicity of a PRAME vaccine (Gutzmer et al. 2016). This new strategy might soon be offered to patients with UM and improve their outcome.

Conclusion

Cutaneous and uveal melanocytes share the same embryonic origin and the same cellular function; however, they are subjected to different oncogenic transformation processes. In recent years, immense progress has been achieved in the cellular and molecular characterization of uveal and skin melanomas. Large-scale genomic studies have demonstrated the direct mutagenic role of UVR in CM pathogenesis, whereas there is no conclusive proof linking UV exposure to UM etiology. The majority of CM (80%) carries a mutation in BRAF, NRAS or NF1, leading to the deregulation of the ERK pathway. In UM, activating mutations in GNAQ/11 dominate (83%) and engage specific signaling pathways including ARF6/TRIO/RHO/RAC/YAP and PLC β PKC/ERK cascades. Moreover, recurrent genetic alterations in BAP1 that function in the cell cycle, cell identity and genome integrity are found in UM (40%) and are associated with the development of metastasis. UM metastases display very strong liver tropism, while CM metastases involve, almost equivalently, the lungs, liver, bones and brain. Because of these cellular and molecular differences, the recently developed therapies (targeted and immunotherapies) that show clinical activity for metastatic CM are still ineffective in patients with metastatic UM.

Nevertheless, recent discoveries have delineated the contours of UM disease and have identified targets for rational therapies. The concerted effort of talented researchers and clinicians working in the field will undoubtedly replicate in UM the extraordinary clinical advances recently achieved in CM.

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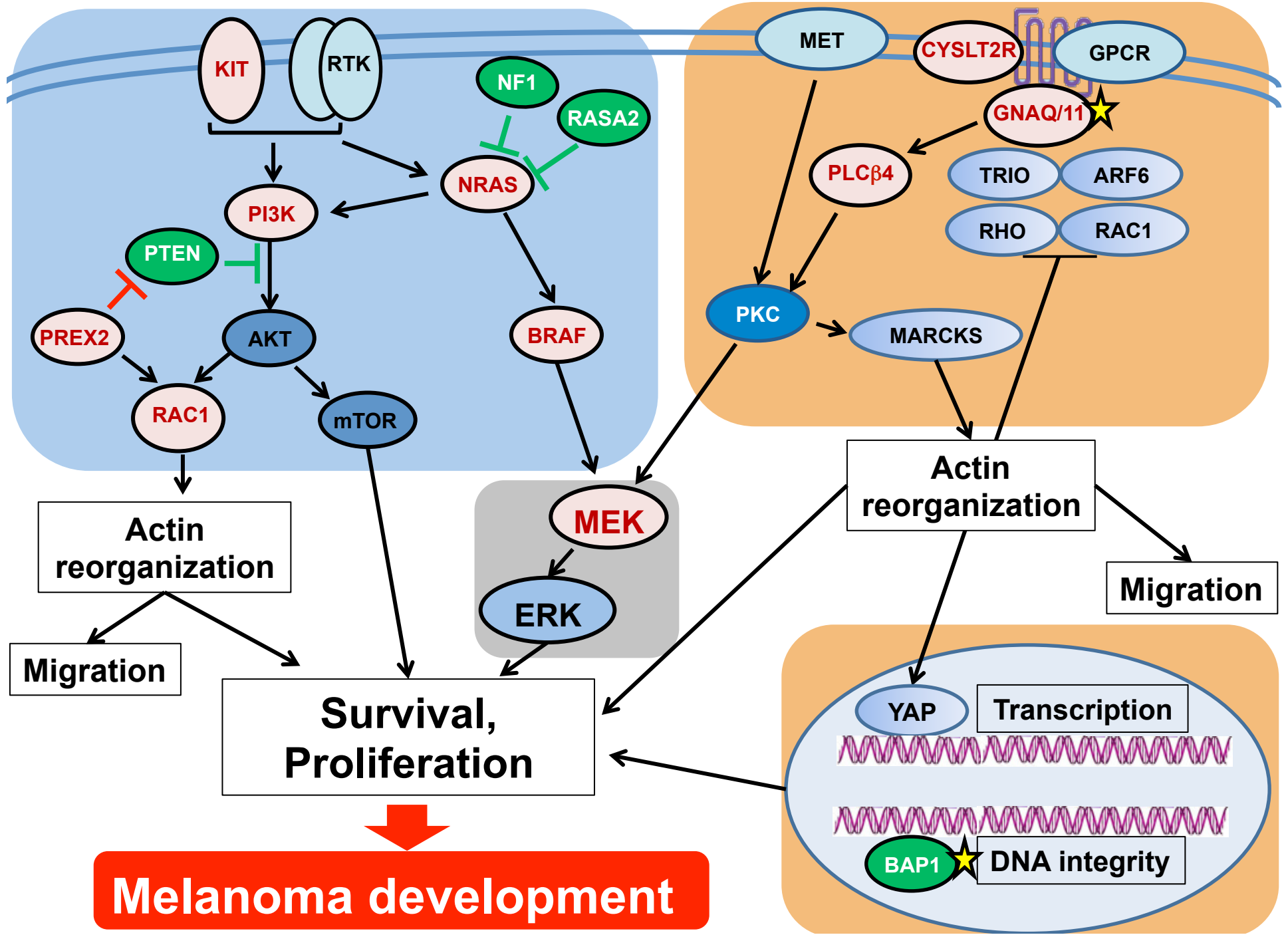
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Table 1: Comparison of cutaneous and uveal melanoma features.

Table 2: Gene expression signature in uveal melanoma.

List of genes that distinguish low metastatic risk (class 1 signature) and high metastatic risk (class 2 signature) primary uveal melanomas (Onken et al. 2004; Onken et al. 2010b). This list contains the high stringency genes that discriminate class 1 and class 2 genes (¶) (fold change >5 and FDR <0.001) (Onken et al. 2006) and the set of 12 genes (*) that have been proposed using a clinically practical PCR-based prognosis assay to identify high-risk patients (Onken et al. 2010b). The direction of the gene expression change in class 2 versus class 1 primary uveal melanomas is shown (column 4). Kaplan-Meier survival analysis for TCGA cutaneous melanoma data between the lower and upper quartiles was computed (column 6). A positive correlation indicates that high gene expression is associated with a good prognosis (p-value is given). No correlation is indicated as “no”. Thus, some genes may have important functions in both uveal and cutaneous melanomas.

Table 3: Clinically relevant targets in uveal melanoma. Novel potential treatment strategies (column 1) against essential genetic and pathway alterations (column 2) in uveal melanoma. Clinical indications (column 3), clinical trials that are underway (column 4) and the stages of drug development (column 5) are indicated.



Pandiani_Table 1

Column1	OCULAR MELANOMA		CUTANEOUS MELANOMA
	Uveal melanoma2	Conjunctival melanoma	SSM, NM, AM, MM, LM
Incidence worldwide	8 000	800	230 000
Median Survival rate	2-8 months	8 months	6-10 months
Hereditary cases	1-2%		10%
Main susceptibility alleles	OCA2-HERC2	BAP1	Cell cycle (CDKN2A, CDK4, RB1) DNA damage (BRCA1), Telomere maintenance (TERT, POT1, ACD, TERF2IP) Pigmentation (MC1R, MITF, TYR, TYRP1, OCA2)
UV radiation involvement	No	Possible	Yes
Mutation burden	Low		High
Somatic genetic alterations			
Gain of function	GNAQ/GNA11	BRAF	GNAQ/GNA11
	SF3B1	NRAS	NRAS
	EIF1AX	TERT	MEK1/2
	CYTLR2		PIK3CA, PIK3CG
	PLCβ4		AKT1/AKT3
			RAC1
			KIT
Loss of function	BAP1		CDKN2A
			PTEN
			NF1
			RASA2
Signaling pathways activation	ARF6-TRIO-RHO-RAC-YAP PLCβ/PKC		ERK PI3K/AKT
Chromosome anomalies	"Simple"		"Complex"
	loss of 1p, 3, and 6q and gain of 6p and 8q		
Dissemination	Hematogenous	Lymphogenous and hematogenous	Lymphogenous and hematogenous
Common first metastatic sites	Liver	Regional Lymph nodes	Regional Lymph nodes
Targeted therapies	No	Anti-BRAF, anti-MEK to be tested	Anti-BRAF, anti-MEK
Immunotherapies	Inefficient	No	Anti-CTLA4, anti-PD1, anti-PDL1

Pandiani_Table 2

Gene Symbol	Gene Name	Genomic location	Direction in class 2 primary uveal melanoma	Function	Correlation with survival in cutaneous melanoma; p-value
CDH1*	E-cadherin	16q22.1	Up	Cell-cell adhesion, mobility and proliferation of epithelial cells	no
ECM1*	Extracellular matrix protein 1	1q21.2	Up	Cell adhesion, cell-to-cell communication and differentiation	no
RAB31*	RAB31, member RAS oncogene family	18p11.22	Up	Regulator of vesicle trafficking	no
HTR2B*	5-hydroxytryptamine (serotonin) receptor 2B	2q37.1	Up	Receptor	Positive (p=0.044)
ID2*¶	Inhibitor of DNA binding 2	2p25.1	Down	Transcriptional regulator	Positive (p=0.008)
EIF1B*	Eukaryotic translation initiation factor 1B	3p22.1	Down	Poly(A) RNA binding and translation initiation factor activity	no
FXR1*	Fragile X mental retardation autosomal homolog 1	3q26.33	Down	Nucleic acid binding and RNA binding	no
LMCD1*	LIM and cysteine-rich domains 1	3p25.3	Down	Protein-protein interactions, Transcriptional regulator	no
ROBO1*¶	Roundabout, axon guidance receptor 1	3p12.3	Down	Receptor	no
SATB1*	SATB homeobox 1	3p24.3	Down	Transcriptional regulator	Positive (p=0.046)
MTUS1*¶	Microtubule-associated tumor suppressor 1	8p22	Down	Signaling	Positive (p=0.019)
LTA4H*	Leukotriene A4 hydrolase	12q23.1	Down	Poly(A) RNA binding and peptidase activity	no
AZGP1¶	Alpha-2-glycoprotein 1, zinc	7q22.1	Down	Metabolism	no
ENPP2¶	Ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)	8q24.12	Down	Metabolism	no
EDNRB¶	Endothelin receptor type B	13q22.3	Down	Receptor	Positive (p=0.004)
GPR37¶	G protein-coupled receptor 37 (endothelin receptor type B-like)	7q31.33	Down	Receptor	no
IL12RB2¶	Interleukin 12 receptor, beta 2	1p31.3	Down	Receptor	no
SPP1¶	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	4q22.1	Down	Cell matrix interaction	no
VAMP8¶	Vesicle-associated membrane protein 8 (endobrevin)	2p11.2	Down	Regulator of vesicle trafficking	Positive (p=0.006)

Pandiani_Table 3

Drugs	Targets	Indication	Clinical Trials in uveal melanoma	Comments
ONO-6950	CYSLTR2	Asthma		Asthma: NCT01551147
HAMI3379	CYSLTR2	Asthma		Pre-clinical dev
FR900359	GNAQ/11	Asthma		Pre-clinical dev
NAV-2729	ARF6	Inflammatory conditions		Pre-clinical dev
ITX3	TRIO			Pre-clinical dev
TRIP	TRIO			Pre-clinical dev
FL3	EIF1AX			Pre-clinical dev
Crizotinib	MET	Lung cancer	NCT02223819	FDA approved
Cabozantinib	MET	Kidney cancer	NCT01835145	FDA approved
Verteporfin	YAP	Retinopathy		FDA approved
Sotrastaurin	PKC	DLBCL, Uveal melanoma	NCT01801358 NCT01430416 NCT02273219	Trials in combination with MEK of PI3K inhibitors
LXS-196	PKC	Uveal melanoma	NCT02601378	
Trametinib	MEK	Cutaneous melanoma	NCT01979523	FDA approved in combination with BRAF inhibitor
Selumetinib (Adjuvant)	MEK	Thyroid cancer	NCT02768766 NCT01143402	