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Biology of Advanced Uveal Melanoma and Next Steps for Clinical Therapeutics

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Summary

Uveal melanoma is the most common intraocular malignancy though it is a rare subset of all melanomas. Uveal melanoma has distinct biology relative to cutaneous melanoma, with widely divergent patient outcomes. Patients diagnosed with a primary uveal melanoma can be stratified for risk of metastasis by cytogenetics or gene expression profiling, with approximately half of patients developing metastatic disease, predominately hepatic in location, over a 15 year period. Historically, no systemic therapy has been associated with a clear clinical benefit for patients with advanced disease and median survival remains poor. Here, as a joint effort between CURE OM and the National Cancer Institute, the current understanding of the molecular and immunobiology of uveal melanoma is reviewed, and on-going laboratory research into the disease is highlighted. Finally, recent investigations relevant to clinical management via targeted and immunotherpies are reviewed and next steps in the development of clinical therapeutics are discussed.

Keywords

Ocular; Uveal; Melanoma; MEK; GNAQ; GNA11; metastasis; cancer

There has been a recent increased interest in uveal melanoma (UM), largely due to advances in the understanding of the molecular underpinnings of the disease, and the report of a clinical trial showing an improvement in the progression-free survival of patients with advanced disease by MEK inhibition. As a joint effort between the CURE OM foundation and the National Cancer Institute (NCI), this manuscript reviews the current understanding of the molecular and immunobiology of UM, on-going laboratory research into the disease, as well as investigations and challenges relevant to the development of new treatments for patients with UM.

PATHOGENESIS OF UM

Molecular Biology of UM

Uveal melanoma belongs to a clade of melanocytic neoplasms that are thought to arise from melanocytes not associated with epithelial structures (Bastian, 2014), and are genetically characterized by frequent, mutually exclusive mutations in *guanine nucleotide-binding protein G(q) subunit alpha* (*GNAQ)* and g*uanine nucleotide-binding protein subunit alpha-11 (GNA11)*, two closely related large GTPases of the Gαq family (Van Raamsdonk et al., 2009; Van Raamsdonk et al., 2010). These mutations render the heterotrimeric G protein α subunits $G\alpha_q$ and $G\alpha_{11}$ GTPase defective, and hence constitutively active (O'Hayre et al., 2013). The oncogenic activity of *GNAQ* was initially revealed as part of a systematic analysis of the transforming potential of G proteins and their coupled receptors in the early 90's (Kalinec et al., 1992). The best-known downstream signaling event initiated by Gαq involves its ability to activate phospholipase C (PLC) β and the consequent increase in inositol 1,4,5-trisphosphate (IP_3), and diacylglycerol (DAG) (Hubbard and Hepler, 2006). IP_3 induces the rapid increase in cytoplasmic Ca^{2+} levels, thereby controlling a variety of calcium-regulated pathways, and together with DAG, stimulates the classical isoforms of protein kinase C (PKC) (Griner and Kazanietz, 2007). Of direct relevance to UM, GNAQ utilizes PLCβ to stimulate the mitogen activated protein kinase (MAPK). This is similar to

the consequence of mutations in the *B-RAF* or *N-RAS* oncogenes in cutaneous melanomas (Davies et al., 2002).

BRCA1 associated protein 1 (BAP1) is a gene that is mutated in approximately 47% of primary UM lesions (Harbour et al., 2010). These mutations are thought to arise after the activating mutations of *GNAQ* or *GNA11*. The presence of a *BAP1* mutation in UM is associated with a high likelihood of metastasis. Potential functions of BAP1 include cell cycle regulation and maintenance of cell identity and genomic integrity (Ladanyi et al., 2012). The *BAP1* gene maps to chromosome 3p21 and *BAP1* mutations in UMs are accompanied by primarily somatic complete or partial loss of chromosome 3 (Harbour et al., 2010). This is consistent with a two hit model for loss of activity of a tumor suppressor gene. Approximately 1–3% of patients with UM are likely to harbor a predisposing germline mutation in *BAP1* (Harbour et al., 2010), although tumor development will also depend on loss of wild type *BAP1*. *BAP1* germline alterations, while rare, are also associated with predisposition to a variety of other cancers including mesothelioma, cutaneous melanoma and renal cell cancer (termed a Tumor Predisposition Syndrome) (Abdel-Rahman et al., 2011; Testa et al., 2011; Wiesner et al., 2011).

Most *BAP1* alterations are likely to lead to loss of the BAP1 peptide in tumors. However, some tumors harbor missense alterations that affect BAP1 function. Critical domains of BAP1 that are altered in such tumors are the ubiquitin carboxy-terminal hydrolase (UCH) domains, suggesting that loss of UCH activity in UM predisposes to metastasis. Targets of the BAP1 UCH activity in UM are not well defined but include histone H2A, host cell factor-1 (HCF1) and O-linked N-acetylglucosamine transferase (OGT) (Dey et al., 2012; Sowa et al., 2009). When BAP1 is depleted, UM cells exhibit stem-cell like characteristics (Matatall et al., 2013). These include a loss of morphological differentiation and down-regulation of the melanocyte transcriptional program as revealed by down-regulation of Microphthalmia-associated transcription factor (MITF), transient receptor potential cation channel subfamily M member 1, tyrosinase and Dopachrome tautomerase genes and up-regulation of genes enriched in stem cells and developmental processes. Cells where BAP1 has been knocked down also have fewer dendritic aborizations and less differentiated spindle morphology. Depletion of BAP1 does not lead to increased proliferation, migration, invasion or tumorigenicity. These observations are consistent with a role for BAP1 in melanocyte differentiation and the maintenance of cell identity (Matatall et al., 2013).

Two additional genes that are recurrently mutated in UM include *Splicing factor 3b, subunit 1* (*SF3B1*) and *eukaryotic translation initiation factor 1A* (*EIF1AX*) (Harbour et al., 2013; Martin et al., 2013). Mutations in *SF3B1* and *EIF1AX* have been associated with low-grade UM and a good prognosis. These mutations rarely co-exist with *BAP1* mutations and seem to confer a phenotype associated with a lower risk of systemic recurrence. Expression of mutant SF3B1 has been associated with alternative RNA splicing in multiple tumor types including UM. Specifically, differential alternative splicing has been observed, via RNA sequencing of tumor samples from patients with UM, in genes such as *ABCC5* and *UQCC* (Furney et al., 2013). However, the precise role of SF3B1 alterations in UM tumorigenesis is not yet defined. The role of *EIF1AX*, has also yet to be clarified.

IMMUNOBIOLOGY OF UM

Uveal melanoma is characterized by tumor dormancy, with many patients experiencing metastatic recurrence more than five years after treatment of the primary lesion and no evidence for local recurrence. The molecular events involved in maintaining this dormancy are not known. However, several lines of evidence implicate immune surveillance. In RET.AAD mice, which spontaneously develop UM, tumor dormancy is observed and is mediated in part by cytostatic $CD8^+$ T cells (Eyles et al., 2010). In other intraocular melanoma mouse models, natural killer (NK) cells have been shown to regulate outgrowth of liver micrometastases (Dithmar et al., 2000; Ly et al., 2010; Yang et al., 2011). Clinical observations also suggest immune responses are operational in maintenance of UM tumor dormancy. For example, decreased tumor expression of MHC Class I, a ligand for NK inhibitory receptors is associated with longer metastasis-free survival (Maat et al., 2009) whereas tumor loss of NK activating receptors (MICA and MICB) is associated with tumor progression (Vetter et al., 2004).

The eye is characterized as a site of “immune privilege” where immune responses to antigens (including tumor antigens) are modulated to protect non-regenerating ocular tissues that, if damaged by inflammation, would compromise vision. Hence, immune suppressive mechanisms that maintain ocular “immune privilege” may be utilized by uveal melanomas to limit immune surveillance and promote emergence from dormancy. It is clear that stringent control of ocular immune responses limits immune-surveillance as primary UMs that are heavily infiltrated by CD8+ T cells and macrophages are larger tumors with a genetic profile indicating increased risk for liver metastases (Maat et al., 2008). In addition, immunogenic tumor cell lines that are normally rejected by host immune responses when transplanted in the skin of mice grow progressively when transplanted in the anterior chamber (McKenna and Chen, 2010) or vitreous cavity of the eye (Jiang and Streilein, 1991). Progressive ocular tumor growth occurs despite systemic priming of tumor-specific CD8+ T cells that infiltrate ocular tumors (Ksander et al., 1991; McKenna and Kapp, 2006), suggesting regional immune suppression.

Ocular immune privilege is maintained by anatomical and biochemical barriers to immune responses along with the generation of systemic tolerance to ocular antigens (reviewed in (Forrester and Xu, 2012; McKenna and Previte, 2012; Niederkorn, 2012)). Anatomical constraints include the absence of afferent lymphatics within the eye, and blood-ocular barriers that raise the threshold for priming immune cells in secondary lymphoid organs and limit their traffic to the eye respectively. Immune cells that do enter the eye encounter significant biochemical barriers including soluble immunosuppressive factors such as transforming growth factor beta (TGF-β), α-melanocyte stimulating hormone, cytotoxic T-lymphocyte antigen (CTLA)-2α, retinoic acid, and indoleamine dioxygenase (IDO). These factors limit T cell proliferation and effector function, and can convert T effectors into immunosuppressive T regulatory (T_{reg}) cells. In addition, intraocular tumor growth in mice has been shown to induce systemic T cell tolerance to tumor antigens which is mediated by the generation of immunosuppressive CD8+ T_{reg} cells that inhibit type IV hypersensitivity reactions (Streilein and Niederkorn, 1985).

Several immunosuppressive mechanisms, such as Programmed Death Ligand-1 (Yang et al., 2008) and IDO (Chen et al., 2007), that normally preserve ocular immune privilege are utilized by UMs to escape immune surveillance and as a result may transfer "immune privilege" to the metastatic site (Niederkorn, 2012). Primary UM cell lines are also rendered resistant to CD8 T cell cytolytic activity (Hallermalm et al., 2008) by IFN-γ. This promotes the expression of soluble FasL (Hallermalm et al., 2004) to provide protection from FasL induced apoptosis and limit inflammation. In addition, primary and metastatic UM are resistant to NK cell responses via increased expression of migration inhibitory factor (Repp et al., 2000) and TGF-β2 (Esser et al., 2001).

Recent studies have identified CD4+, forkhead box P3 (FoxP3)+ T_{reg} cells within primary UMs whose frequencies correlated with metastatic spread (Lagouros et al., 2009; Mougiakakos et al., 2010). In patients with primary UM followed from diagnosis, at which time there was no clinical or radiographic evidence of metastasis, until metastasis manifested, circulating anti-tumor $CD3^{-}CD56^{dim}$ NK cells and $CD8^{+}$ and double-negative $CD3^{+}CD56^{+}$ NKT cells decreased while pro-tumor $ICOS^{+}CD4^{+}FoxP3^{+}$ T_{reg} cells increased (Achberger et al., 2014) which further supports a role for T_{reg} in tumor progression. Whether UM growth induces the generation of CD8+ T_{reg} is not known. However, the peculiar association of larger tumor size and increased risk of metastases in primary UMs infiltrated by CD8+ T cells (de la Cruz et al., 1990; Durie et al., 1990) could suggest that these are T_{reg}. Elimination of CD8 T_{reg} in a mouse intraocular tumor model caused spontaneous tumor rejection suggesting a potential therapeutic target (Streilein and Niederkorn, 1985; Streilein and Niederkorn, 1981). Patients with primary UMs and liver metastases also have elevated frequencies of CD11b+CD15+ cells in blood (Achberger et al., 2014; McKenna et al., 2009) which may act as immunosuppressive myeloid derived suppressor cells (MDSC). Immune suppression in liver metastases is not well characterized due to limited animal models. However, B16LS9 melanomas have been shown to metastasize to the liver after transplantation in the vitreous cavity of mice (Dithmar et al., 2000), and recently Yang and coworkers (Yang et al., 2011) identified that liver NK T cells inhibited tumoricidal NK cell responses by their production of IL-10.

The molecular mechanisms by which immune responses are regulated over time to account for the emergence from dormancy have not been elucidated. Very little is known regarding the relative contributions of intra-tumoral changes and changes inherent to the host immune response. Changes within the tumor may be epigenetic as well as genetic. Although immune responses are predominantly controlled at the transcriptional level, epigenetic mechanisms are also increasingly being recognized, including those mediated by specific microRNAs (miRs). There is emerging evidence that tumor burden in multiple cancer histologies can influence miR expression in immune cells. The specific effects vary substantially between different cancers and the expression of individual miRs. Plasma levels of miRs are implicated in immune regulation and miR-20a, 125b, 146a, 155, 181a, and 223, have been found to be higher in patients with UM at diagnosis compared to healthy controls (Achberger et al., 2014). Plasma levels of miR-20a, 125b, 146a, 155, and 223 increase, and miR181a decrease in patients with UM followed from diagnosis to the development of metastasis. Alterations in immune regulatory miRs are also observed in $CD3^{+}$, $CD15^{+}$, and $CD56^{+}$ cell populations. Thus, there is evidence that the development of metastasis in UM is

associated with changes in immune effector and regulatory cells consistent with lessening tumor immune surveillance. Epigenetic mechanisms may be involved, as these changes are associated with changes in plasma and cellular levels of immune regulatory miRs. Improved understanding of the mechanisms controlling immune surveillance of UM may help the development of more effective biomarkers of metastatic risk as well as therapeutic immunotherapies for patients with UM.

ONGOING LABORATORY RESEARCH IN UM

Novel and Potentially Druggable G Protein Signaling Targets

Knock down of $G\alpha_q$ in cell lines derived from primary or metastatic UM results in decreased output from the MAPK pathway at ERK and reduced deoxyribonucleic acid synthesis (Van Raamsdonk et al., 2009; Vaque et al., 2013). However, recent data has suggested that a small molecule inhibitor that abolished the activation of phosphoinositide phospholipase C-β (PLCβ) exerts a less than expected impact on the proliferative capacity of UM cells (Vaque et al., 2013). These findings are aligned with the recent report of MEK inhibition conferring a progression-free survival (PFS) benefit in patients with metastatic UM (2013). These clinical and experimental results raise the possibility that *GNAQ* oncogenes may activate signaling events in addition to the ERK pathway eventually limiting the clinical benefit of MEK inhibitors.

Consistent with this possibility, a recent report has described Gαq as binding and activating Trio, a guanine nucleotide exchange factor (GEF) for the small GTPases RhoA and Rac1, thereby providing a direct biochemical link between the *GNAQ* oncogene and the sustained activation of these Rho GTPases (Vaque et al., 2013). This is in contrast to the many biological responses elicited by Gαq that are mediated by PLC activation. The *GNAQ*-induced activation of RhoA and Rac1 initiate the nuclear expression of growth promoting genes by inducing rapid cytoskeletal changes and the activation multiple MAPKs, including JNK and p38 (Vaque et al., 2013). These signaling pathways act in parallel to ERK activation.

Also aligned with the possibility that *GNAQ* oncogenes may activate non-MAPK signaling events, it was shown that preventing Trio activation diminished UM tumor formation without affecting ERK (Vaque et al., 2013). In turn, RhoA and Rac1 activation may enable *GNAQ* to stimulate UM cell migration and growth even in the presence of a MEK inhibitor, thus providing a potential treatment resistance mechanism that could be considered for co-targeting in conjunction with MEK inhibition.

In this context, a search is on-going for druggable transcriptional events that are initiated by *GNAQ* through these Rho GTPases. Emerging evidence indicates that *GNAQ* stimulates the transcriptional co-activator YAP, a Hippo signaling pathway component involved in organ size control during embryogenesis, through a Trio-Rho/Rac signaling circuitry (Feng et al., 2014). The activation of YAP by *GNAQ* is independent of the canonical Hippo pathway, but instead involves a novel mechanism initiated by the polymerization of the actin cytoskeleton. YAP was shown to be essential for UM cell proliferation, thereby representing a novel therapeutic target for UM treatment (Feng et al., 2014). In this regard, a recent

small-molecule library screen identified verteporfin as a potent inhibitor of the YAP transcriptional activity *in vitro* (Liu-Chittenden et al., 2012). Treatment of mice bearing human UM xenografts with verteporfin was quite potent in diminishing tumor growth (Feng et al., 2014). As verteporfin is already in clinical use as a photosensitizer for photodynamic therapy in wet age-related macular degeneration (Michels and Schmidt-Erfurth, 2001), these findings raise the possibility of repurposing verteporfin in future clinical trials for UM treatment.

Hypoxia as a Therapeutic Target and Arylsulfonamide KCN1 as an Inhibitor of UM *in vivo*

Hypoxia inducible factors (HIF) are key transcription factors that orchestrate a range of molecular responses, allowing cancer cells to survive in a hypoxic environment, including the direct or indirect activation of gene products that control anaerobic metabolism, stimulate angiogenesis, cell motility and metastasis (Burroughs et al., 2013). This includes vascular endothelial growth factor (VEGF), C-X-C chemokine receptor type 4, hepatocyte-growth factor and MET, signal transducer and activator of transcription 3 (STAT3) and matrix metalloproteinases 2 and 9. Metastatic cancers commonly overexpress HIF-1 (Zhong et al., 1999), and the reduction of HIF transcription factor levels or activity in cancer cells can significantly antagonize the growth of a variety of tumors *in vivo* (Burroughs et al., 2013). Hypoxia inducible factors are heterodimeric transcription factors and consist of one of three oxygen-regulated α subunits (1α, 2α and 3α) and the constitutively expressed HIF-1β. Under normoxic conditions, α subunits are hydroxylated and ubiquitylated, which leads to rapid degradation by the proteasome. Under hypoxic conditions, α subunits are stabilized, translocate into the nucleus where they interact with the HIF-1β subunit, recruit co-activators p300/CBP, and transcriptionally activate over 100 target genes via binding to specific DNAs sequences termed hypoxia-response elements (HRE). While the differential function of HIF-1 and HIF-2 is still under investigation, both are associated with cancer stem cells and most studies suggest that one or both isoforms need targeting, depending on tumor and cancer type (Keith et al., 2012). HIF-3 has not been extensively studied, but may function as a dominant negative isoform as it lacks the C-terminal transactivation domain (Hara et al., 2001).

Targeting of hypoxia-mediated pathways, which regulate the multistep process of metastasis in several cancers (Zhong et al., 1999) has surprisingly received little attention in UM (el Filali et al., 2010; Victor et al., 2006). To target HIF for cancer therapy and potentially UM, a combinatorial library of natural product-like compounds were screened. This screen was based upon a 2-dimethylbenzopyrane scaffold, which is found in more than 4,000 natural products using a cell-based reporter assay for HIF activity, and revealed a new class of chemicals (arylsulfonamides) with potent HIF pathway inhibitory activity (Mun et al., 2012a; Mun et al., 2012b; Reid Mooring S, 2011; Shi et al., 2012; Tan et al., 2011). Initial studies have revealed that these molecules selectively reduce the transcriptional activity of HIF-1 in the mid to high nanomolar range in the absence of appreciable cytotoxic effects. The mechanisms of HIF transcription blockade continues to be under study.

One of the lead compounds in this pipeline, KCN1 (3,4-dimethoxy-N-[(2,2-dimethyl-2H-chromen-6-yl) methyl]-N-phenylbenzenesulfonamide) has been observed to have anti-tumor

activity in multiple cancer models. In glioma cell lines, KCN1 has been observed to disrupt the interaction between the HIF-1α subunit and transcription co-factors p300/CBP, possibly due to binding to the CH1 domain of the p300/CBP (Shi et al., 2012; Yin et al., 2012). KCN1 has also been found to exhibit HIF-1α-independent cytostatic activities in pancreatic cancer cell lines (Wang et al., 2012) and anti-tumor effects have been observed in *in vivo* models of malignant glioma (Yin et al., 2012), pancreatic cancer (Wang et al., 2012), and Ewing sarcoma (communication from Erwin G. Van Meir, Ph.D). KCN1 was also noted to be well tolerated in mice. The precise mechanism(s) of the anti-tumor action of KCN1 are still under investigation and it is currently unknown whether they are solely driven by HIF inhibition.

In UM, *in vitro* cytotoxicity assays have shown that KCN1 inhibits the growth of human and mouse UM cells only at higher concentrations (IC_{50} ~30–80 μM), while sparing normal melanocytes (communication from Erwin G. Van Meir, Ph.D.). Treatment of an orthotopic, syngeneic UM mouse model with KCN1 reduced the size of intraocular tumors while significantly reducing metastatic potential. Kaplan-Meier survival curves of murine treatment studies revealed that KCN1 extended survival; immunostaining demonstrated a reduction in phosphorylation of MET, MAPK and STAT3 as well as Ki67, and VEGF. Microvascular density was also reduced. These findings suggest that KCN1 and analogous arylsulfonamide compounds may hold promise as new therapeutic agents for the treatment of UM.

BAP1 in UM and the Therapeutic Potential of Histone Deacetylase Inhibitors

Uveal melanoma can be subdivided into groups with low metastatic risk (class 1 tumors) and high metastatic risk (class 2 tumors) based on a gene expression signature (Onken et al., 2004). The likelihood of metastasis is also associated with loss of one copy of chromosome 3 (Onken et al., 2007). Placing tumors into these two classes is now possible with a routine clinical test with fine needle biopsy where 15 genes are profiled on a microfluidics platform (Harbour and Chen, 2013). As described earlier the presence of *BAP1* mutations is also strongly correlated with likelihood of metastasis. BAP1 mutations are usually sporadic and are found in approximately 85% of class 2 (metastatic) tumors and less than 5% of class 1 tumors. Loss of BAP1 in uveal melanocytes recapitulates the class 2 phenotype. In turn, transcriptome analysis of class 2 UMs reveals a profile similar to primitive ectodermal and neural stem cells. Transition of an early stage uveal melanoma to a class 2 gene expression profile may be responsible for metastasis in these highly aggressive tumors (Chang et al., 2008). Inferior outcomes in melanomas with neural crest like features have been identified for both uveal and cutaneous melanoma (Thies et al., 2004).

The polycomb repressive deubiquitinase (PR-DUB) complex of which BAP1 is a component catalyzes the removal of monoubiquitin moieties from histone H2A in opposition to the ubiquitinating activity of the PRC1 complex. Loss of BAP1 in mammalian cells results in abnormal ubiquitination of histone H2A and that this can be reversed with histone deacetylase (HDAC) inhibitors (Landreville et al., 2012). Further, HDAC inhibitors revert primary class 2 UM cells to a differentiated class 1 phenotype, and restore to normal levels the expression of melanocyte differentiation genes that are down-regulated by BAP1

depletion. Inhibitors of HDACs also induce morphological changes that are consistent with melanocyte differentiation. Given the important role of BAP1 in tumor progression and metastasis, there may be a role for HDAC inhibitors in preventing the progression of micro-metastatic disease or in combination with other therapies for advanced disease (Landreville et al., 2012).

The Cancer Genome Atlas (TCGA) in UM Research

An exciting development in the UM research arena that has the potential to significantly enhance our understanding of UM biology is the inclusion of UM in the NIH/NCI TCGA Rare Tumor Project. The TCGA was designed as a collaborative effort to create a comprehensive collection of maps that chart genomic changes that occur in each type of cancer, with a specific plan to comprehensively molecularly characterize up to 500 melanomas. The TCGA program was further expanded in March 2012 with rollout and promulgation of the TCGA Rare Tumor Project, the goal of which is to characterize at least 50 qualifying cases for each of ten or so uncommon malignancies into its well-established overall TCGA effort (i.e., inclusion of approximately 1/10 the number of cases allocated to the principal TCGA tumor types). A UM TCGA Disease Working Group (DWG) was configured in January 2013 and receipt of all UM specimens was complete by December 31, 2013.

The TCGA UM Rare Tumor project specifically solicited fresh frozen, previously untreated (i.e., no radiotherapy to tumor or systemic therapy prior to tumor acquisition) primary UM biospecimens of sufficient quality (e.g., ≥60% tumor nuclei, ≤20% necrosis, RIN ≥7, etc.) and quantity for which matching blood and sufficient clinical annotation were also available. Some clinical data was collected in conjunction with the specimen including but not limited to tumor morphology, anatomic site, chromosomal alterations, gene expression profile, PET/CT SUV, mitotic count, presence of extravascular matrix patterns, TIL, TIM, tumor basal diameter, tumor thickness, extrascleral extension, and follow-up. TCGA platforms currently proposed for molecular interrogation of these cases include whole exome sequencing, SNP and copy number, DNA methylation, mRNAseq, and miRNAseq; possible additional platforms include low-pass whole genome sequencing and reverse-phase protein array. Analysis of these specimens is on-going and it is particularly exciting to learn that among these a high percentage (65%) qualified initial review, had analytes prepared, passed Biospecimen Core Resource (BCR) quality control, and are currently awaiting shipment to TCGA platforms for molecular profiling (https://tcga-data.nci.nih.gov/datareports/BCRPipelineReport.htm). It is anticipated that data from this international collaboration will be available to the TCGA UM rare tumor team for initial analysis sometime in 2014.

CLINICAL EXPERIENCE IN UM AND NEXT STEPS

Ipilimumab for Advanced UM and Combination Strategies for Investigation

It is hypothesized that UM may be a more immunogenic tumor relative to others given that it arises in the eye, an immunologically privileged site. Uveal melanoma has high expression of multiple immunogenic cancer antigens such as gp100, MAGE, MART-1, tyrosinase and TRP-1 (de Vries et al., 1998; Luyten et al., 1998). Ipilimumab, the fully human monoclonal

antibody against CTLA-4, has recently been approved for melanoma though no prospective trials have yet documented the activity of this agent for metastatic UM.

Multiple groups have investigated the activity of ipilimumab in UM in retrospective series (Kelderman et al., 2013; Khattak et al., 2013; Luke et al., 2013; Maio et al., 2013), demonstrating response rates of approximately 5% and three month disease control rates of approximately 36%, based on the immune-related response criteria described for ipilimumab (Wolchok et al., 2009). Within each of these series however, patients were identified who had long term stabilization of disease and tumor responses. In one series, in addition to Eastern Cooperative Group performance status of zero and lactate dehydrogenase within normal institutional limits, stratification of OS by absolute lymphocyte count (ALC) of ≥ 1000 cells/μL (vs ≤1000 cells/μL) showed median OS of 13.4 months (95% CI, 9.6 months to ∞) vs 4.8 months (95% CI, 3.6–7.0 months), respectively (Luke et al., 2013). The seven patients who responded or had stable disease at last follow up in this analysis had a median rise in ALC from baseline to week 7 of 600 cells/μL. These results suggest that patients most likely to benefit from ipilimumab treatment are those treated early in their disease course before clinical decline and those who maintain an intact immune system. It also suggests that a rise in ALC at week 7 of ipilimumab treatment may be a useful biomarker related to ipilimumab clinical benefit for further investigation in the future.

To improve these results, several avenues of research appear potentially promising. There is emerging interest in MEK inhibition in this disease (Carvajal et al.), and combinations with ipilimumab would be of interest. Such interest may be tempered somewhat however based on the described preclinical effects of MEK inhibitors in dampening T cell activation (Luke and Ott, 2013; Ott et al., 2013). A second approach may be consideration of combination immunotherapy with other immune-checkpoint agents such as anti-Programmed Death 1 antibodies. This combination has shown an impressive response rate in pre-treated patients with cutaneous melanoma (Wolchok et al., 2013). A third approach may be consideration of combining ipilimumab with anti-angiogenesis agents. Metastatic UM is a highly vascular disease that produces high levels of VEGF and demonstrates elevated levels of phosphorylated VEGF receptor (VEGFR) (Logan et al., 2013). The feasibility of combining ipilimumab with the anti-VEGF antibody bevacizumab has previously been documented in patients with melanoma (Hodi et al.). Finally, strong consideration should be given to combination trials of ipilimumab with liver directed therapies. As UM predominately demonstrates a pattern of hepatic metastasis, liver directed therapies have been a standard of care for many years (Leyvraz et al., 1997). More recently, radioembolization of hepatic lesions entered clinical practice and a growing body of literature supports the premise that radiation-induced macrophage phenotype switching may improve T cell trafficking into tumors (Klug et al., 2013). This suggests a potential synergy between liver directed radioembolization and immunotherapies.

Targeted Therapy in UM: Clinical Experience

The median survival of patients with metastatic UM has been described as approximately 12 months (Rietschel et al., 2005), and no systemic chemotherapy has been associated with an overall survival benefit (Salmon et al., 1998). Trials of various chemotherapy regimens,

including biochemotherapy have been performed, with most utilizing single-arm designs and all demonstrating limited clinical activity (Bedikian et al., 2003; Homsi et al., 2010; Kivela et al., 2003; O'Neill et al., 2006; Schmidt-Hieber et al., 2004; Schmittel et al., 2005; Schmittel et al., 2006). Considering this, there is consensus among expert UM clinicians that chemotherapy and high-dose interleukin-2 are of minimal value in this disease and patients should not be treated with them unless no other treatment option is available. With the development of targeted therapies toward BRAF in melanoma, there is growing interest in targeted approaches in UM.

High cell surface membrane expression of the receptor tyrosine kinase c-KIT has been documented in UM leading to interest in the evaluation of KIT-inhibitors. In a pilot study of thirteen patients, the kinase inhibitor imatinib was evaluated (Penel et al., 2008). Minimal activity was observed, with no clinical responses and one patient experiencing stable disease. In a phase II study of 20 patients, sunitinib was administered at 37.5 mg daily continuously in 4-week cycles (Mahipal et al., 2012). One patient who had a partial response and 12 patients with stable disease were reported, though the median survival of 8.2 months did not appear to vary from historical controls. Given these results, surface expression of c-KIT has been abandoned as a target for kinase inhibitor therapy in UM.

A second target of interest has been the VEGF/R axis. UM cell lines have been associated with high levels of VEGF, suggesting that anti-VEGF/R therapies could be relevance (Logan et al., 2013). Three VEGF/R axis directed treatments have been investigated in patients with advanced UM including sunitinib, sorafenib (in combination with chemotherapy) and VEGF-trap. As above, treatment with sunitinib showed limited activity. Similarly, in a phase II study of sorafenib in combination with carboplatin and paclitaxel chemotherapy there were no objective responses observed in 24 evaluable patients with only 29% of patients having stable disease at six months (Bhatia et al., 2012). The VEGF-trap, known as aflibercept, has also been evaluated in ten patients with advanced UM (Tarhini et al., 2011); of the ten patients, five were noted to be progression free at four months though no objective responses were observed (Tarhini et al., 2011).

Other potential molecular targets of interest include the insulin-like growth factor 1 receptor (IGF1R) and c-MET. Targeting of IGF1R has been proposed as a putative target due to the finding of high IGF1R expression in primary specimens from patients who developed metastatic disease as compared to those who did not (All-Ericsson et al., 2002; Economou et al., 2008). A clinical trial evaluating the anti-IGF1R monoclonal antibody IMC-A12 as a monotherapy has been accrued, though data have not yet been disclosed (NCT01413191). Finally, c-MET, which is over-expressed in up to 80% of UM (Mallikarjuna et al., 2007), has also been of interest as blockade of MET has limited progression of UM in preclinical models (Surriga et al., 2013; Wu et al., 2012b). In a randomized discontinuation study of cabozantinib, a MET and VEGFR2 inhibitor, six-month PFS was described in nine of 23 (39%) patients treated, with a median PFS of 4.8 months (Gordon et al., 2011).

Rationale and Review of On-Going and Proposed Clinical Trials in Advanced UM

Based upon the finding that UM is characterized by functionally active mutations in *GNAQ* or *GNA11* (Ivey et al., 2003; Onken et al., 2008; Raamsdonk et al., 2008), as well as

preclinical findings demonstrating antitumor effects of MEK inhibition in UM in a genotype-specific fashion (Ambrosini et al., 2012), a randomized trial of selumetinib, a selective, orally-available, non-ATP competitive small molecule inhibitor of MEK 1/2, versus temozolomide in patients with metastatic UM was performed (Table 1). This study demonstrated a progression-free survival double that of chemotherapy, with a hazard ratio of 0.46 in favor of selumetinib, and represents the first trial to demonstrate clinical activity of any systemic therapy in advanced UM in a randomized fashion (Carvajal et al., 2013).

There are several MEK inhibitors in development at this time and it is not clear which is likely to be the most efficacious or least toxic. Thus further trials evaluating other MEK inhibitors in UM are needed as well as combination approaches relying on MEK inhibition as a backbone. Based upon preclinical data demonstrating enhancement of MEK inhibitor-induced antitumor effects by concurrent inhibition of AKT or PI3K (Ambrosini et al., 2013; Khalili et al., 2012), a phase II trial of trametinib in combination with GSK2141795, an oral AKT inhibitor, was recently initiated. Furthermore, building upon preclinical data demonstrating the enhanced susceptibility of tumor cells to chemotherapy with concurrent MEK inhibition (Holt et al., 2012), as well as the greater PFS observed with the combination in a study of cutaneous melanoma (Holt et al., 2012; Robert et al., 2013), a phase II trial of DTIC with or without selumetinib has also begun accrual (NCT01974752).

Beyond MEK alone, it is clear that other signaling mediators downstream of GNAQ/11 are also important. Using gain and loss of function mutants in human and mouse melanocytes, as well as human UM cell lines, it has been demonstrated that the MAPK pathway activation requires signaling through Protein Kinase C isoforms, and that the oncogenic effects of GNAQ or GNA11 can be partially blocked by PKC inhibition (Xu Chen and Boris Bastian, unpublished data, Figure 1). Inhibitors of PKC, including sotrastaurin (AEB071) and related compounds, selectively block the proliferation of melanoma cell lines with mutations in *GNAQ* or *GNA11*, without any effects in cell lines with mutations in other oncogenes (Wu et al., 2012a; Wu et al., 2012c). In *in vivo* studies with an allograft model of melanocytes stably transduced with mutant *GNAQ*, sotrastaurin slows tumor growth, but fails to induce tumor shrinkage. Similar results are observed with human melanoma cell lines with *GNAQ* or *GNA11* mutations. In these cell lines, PKC inhibition with multiple inhibitors results in MAPK pathway inhibition but not in cell death. Prolonged exposure to PKC inhibitors however, leads to a rebound of MAPK signaling, which becomes visible after 48 hours of treatment. Correspondingly, analysis of tumor lysates under therapy with sotrastaurin shows suppression of PKC activity but re-activation of MAPK signaling. While MEK inhibitors suppress the growth of UM cell lines, they show no selectivity compared to melanoma cell lines with mutations in other oncogenes. By contrast, in UM with *GNAQ* or *GNA11* mutations, a combination of PKC inhibition with sotrastaurin and MEK inhibitors leads to a highly synergistic effect, resulting in sustained MAPK pathway extinction and apoptosis *in vitro*. In melanoma cell lines with mutations in other oncogenes such as *BRAF* or *NRAS*, this combination does not have a synergistic effect. The combination of sotrastaurin with a MEK inhibitor, but neither compound as monotherapy, led to tumor shrinkage in a xenograft model of *GNAQ* mutant UM. These results suggest that combined inhibition of PKC and MEK represents a rational combination to be evaluated in humans.

Based upon these data, a phase I study of AEB071, an inhibitor of both conventional and novel PKC isoforms, was launched and is nearing completion of accrual. Data demonstrating promising preclinical activity of combined vertical pathway inhibition at the levels of MEK and PKC (Chen et al., 2013) has led to the recent initiation of phase Ib/II trial of MEK162 and AEB071. Antitumor synergy from the combination of PKC and PI3K-α inhibition has also been demonstrated (Musi et al., 2014), and a phase I trial of AEB071 in combination with BYL719, an α-specific PI3K inhibitor is in development. The potential utility of these combination approaches will need to be closely considered in what is likely to be increased toxicity to combination regimens.

A number of other molecularly informed therapeutic strategies are being pursued based upon promising preclinical and clinical activity, including assessment of the efficacy of combined c-MET and VEGFR2 inhibition using cabozantinib, the efficacy of HDAC inhibition using vorinostat, the efficacy of mTOR inhibition with a somatostatin analogue using everolimus and SOM230, the efficacy of heat shock protein 90 inhibition using ganetespib, and others. Regarding immunotherapy, studies of ipilimumab, tremelimumab, and adoptive T cell therapy have either been recently been completed or are ongoing. Finally, given the hepatotropic nature of this disease, studies assessing various liver targeted therapies, either alone or in combination with systemic therapy, are being pursued, including a phase 0 study of radioembolization in combination with ipilimumab, a phase II trial of SIR-Spheres 90Y Microspheres, and a randomized phase III trial of isolated hepatic perfusion versus best alternative care.

In the adjuvant setting, there are several ongoing clinical trials including a phase Ib/II trial of a dendritic cell vaccine, and a phase II trial of sunitinib, tamoxifen and cisplatin. Several additional trials are planned including a phase II trial of crizotinib and a phase II trial of sunitinib or valproic acid.

The Future of Therapeutic Clinical Trials in UM

The incidence of UM in the United States is approximately seven cases per million or 2200 cases annually. Of those, half develop advanced disease within 15 years (Singh and Topham, 2003). In considering this relatively small patient population, clinical trial design becomes especially important. Optimal clinical trial endpoints and designs differ when developing trials for common versus rare diseases such as UM. Endpoints including OS, PFS and response rate each have some merit however consideration must be given to differential preferences of regulatory bodies as well as the pragmatic aspects of completing accrual to a clinical trial.

While an improvement in OS would indicate a therapy with an indisputable clinical benefit, assessment of OS would require a large randomized trial and may not be feasible. Given the lack of standard of care therapeutics, the control arm must be thoughtfully considered as patients may be unwilling to be randomized to chemotherapy or placebo. Meta-analysis has recently suggested PFS as a robust surrogate for OS in advanced melanoma, thus cross-over designs may be of interest (Flaherty et al., 2014). Adaptive study designs or multi-arm trials in which multiple treatments could be evaluated simultaneously are also of particular interest. With the growing number of therapeutic strategies being developed, larger

collaborations will be necessary to efficiently complete clinical trials. The International Rare Cancer Initiative includes a working group for UM and this venue may be a useful framework for engagement by the National Cancer Institute, the European Organisation for Research and Treatment of Cancer and industry in coordinating clinical trials that can engage patients internationally.

Despite the logistical challenges inherent in improving the clinical management of patients with UM, there is nonetheless an increasing awareness of the need for research and drug development for UM in the patient and medical community. To further advance the field, patients with UM should be referred to centers with expertise and enrolled in clinical trials for adjuvant, first-line metastatic and subsequent settings of disease. Larger collaborations should also be developed with cooperative groups creating access to trials for more patients with UM. Such efforts will facilitate continued dialogue, increased research funding, and enhanced collaboration such that known challenges associated with a rare disease may be overcome.

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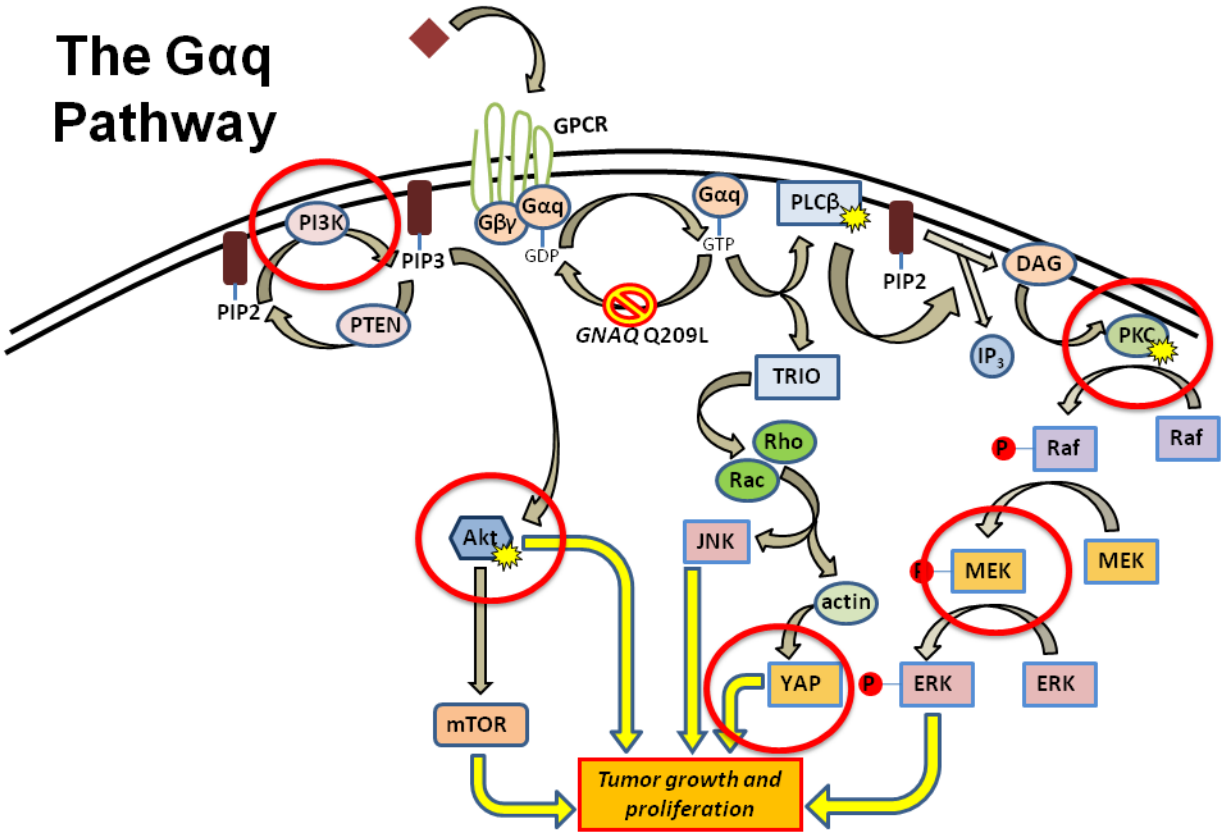

Figure 1. G-α Signaling Pathway

Multiple signaling pathways are important in uveal melanoma, but Protein Kinase C and MEK have been identified as points of therapeutic intervention in GNAQ/11 mutant disease. The Phosphoinositide 3-kinase/AKT pathway and Rho/Rac/Yap pathway have also been identified as a potential targets for therapeutic development as well.

Table 1

On-going or Planned Clinical Trials for Ocular and Uveal Melanoma

Phase	Name of Drugs	Molecular Target or Mechanism	ClinicalTrials.gov Number	Sponsor
Adjuvant	Ipilimumab	CTLA-4	NCT01585194	MD Anderson Cancer Center
Adjuvant	Tumor Antigen mRNA Transfected Dendritic Cells	Dendritic Cell Vaccine	NCT00929019	Rotterdam Eye Hospital, The Netherlands
Adjuvant	Sunitinib, Taxmoxifen, Cisplatin	VEGFR2, Estrogen, Chemotherapy	NCT00489944	San Diego Pacific Oncology & Hematology Associates
Adjuvant Adjuvant	Crizotinib Sunitinib, Valproic Acid	MET VEGFR2, HDAC	Pending Pending	NCI (MSKCC) Thomas Jefferson University
I	Sotrastaurin	PKC	NCT01430416	Novartis
I	Sotrastaurin, BYL719	PKC, PI3K-α	Pending	Novartis
I	Ipilimumab, Radioembolization	CTLA-4, Yttrium 90 glass microspheres	NCT01730157	Case Comprehensive Cancer Center
Ib/II	MEK162, Sotrastaurin	MEK +/− PKC	NCT01801358	Novartis
II	Selumetinib, Temozolomide	MEK, Chemotherapy	NCT01143402	NCI (MSKCC)
II	Trametinib, GSK2141795	MEK +/− AKT	NCT01979523	NCI (MSKCC)
II	Selumetinib, Dacarbazine	MEK, Chemotherapy	NCT01974752	AstraZeneca
II	Cabozantinib, Temozolomide	MET, VEGFR2, Chemotherapy	NCT01835145	Alliance for Clinical Trials in Oncology
II	Vorinostat	HDAC	NCT01587352	NCI (MSKCC)
II	Everolimus, Pasireotide	mTOR, Somatostatin Receptor	NCT01252251	MSKCC
II	Ganetespib	Hsp90	NCT01200238	Dana-Farber Cancer Institute
II	Ipilimumab	CTLA-4	NCT01355120	University Hospital, Essen, Germany
II	Tremelimumab	CTLA-4	NCT01034787	Alberta Health Services, Canada
II	Tumor Infiltrating Lymphocytes	Adoptive Cell Transfer	NCT01814046	NCI
II	Radioembolization	Yttrium 90 glass microspheres	NCT01473004	Thomas Jefferson University
III	Hepatic Perfusion, Palliative Care	Chemotherapy	NCT01785316	Sahlgrenska University Hospital, Sweden

Legend: Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), Heat Shock Protein 90 (Hsp90), Histone deacetylase (HDAC), National Cancer Institute (NCI), Memorial Sloan Kettering Cancer Center (MSKCC), Phosphoinositide 3-kinase (PI3K), Protein Kinase C (PKC), Vascular endothelial growth factor 2 (VEGFR2)