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PHENOTYPIC PROFILE AND ROLE OF CANCER STEM CELLS IN UVEAL MELANOMA

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Phenotypic profile and role of cancer stem cells in uveal melanoma

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Introduction

Uveal melanoma (UM) is a malignant tumour thought to arise from an uncontrolled division and proliferation of melanocytes within the uveal tract, comprising the iris, ciliary body and choroid. The uveal tract is the vascularised middle layer of the eye between the sclera and retina(1). Although a rare tumour, UM is the most common primary intraocular tumour in adults.

UM occurs in about 6-8 individuals per million each year (2). It accounts for about 5% of all melanomas in humans (3). Fair-skinned populations are more affected than others, especially those with blue or grey eyes and blonde or red hair with cutaneous freckles or nevi (4). UM is seen more commonly in older people with only 1% being reported in patients of 18 years and under (5). The incidence of UM rises with increasing age, with more than 20 cases per million per year being reported in individuals aged 70 years and above(2). Males and females are affected equally(3).

Anatomical and clinical features of UM

Choroidal Melanoma

Ninety percent of UM arise from the choroid. Choroidal melanomas may remain restricted by the Bruch's membrane and thereby have a "dome shaped" pattern resulting in small retinal detachment and deposition of lipofuscin in the subretinal space. If the tumour breaks through Bruch's membrane, it may appear as a "collar- stud" or "mushroom" shape and leads to subretinal exudation, and typically to a large retinal detachment.

Ciliary Body Melanoma

Five percent of UM develop in the ciliary body. Ciliary body melanomas tend to be diagnosed later than choroidal ones because they present with visual symptoms later and may be overlooked if dilation of the pupil is not undertaken during routine ophthalmological examinations. Presenting symptoms may include unilateral cataract, elevated IOP, and dilated episcleral vessels in the corresponding eye. Visual field defects do not occur until the tumour is large enough to cause obstruction of the pupil.

Iris melanoma

Iris melanomas are often small nodular lesions that are deeply pigmented. The anatomic location allows for earlier detection. Diffuse iris melanoma, which is a rare, more aggressive variant of the tumour may also occur. It typically causes glaucoma by obstructing the aqueous outflow pathway, including the trabecular meshwork and Schlemm's canal. Such iris melanomas have an increased risk of metastasis.

Diagnosis and Treatment

Signs, symptoms and diagnosis

Blurred vision, flashing lights or shadows, opaqueness of the lens, and glaucoma may be the first symptoms that patients experience. Upon visiting an optometrist or ophthalmologist, the diagnosis of UM would be made after a slit lamp, direct ophthalmoscope examination, ultrasonography or optical coherence tomography (OCT). For many patients, the diagnosis is made during routine eye examination as the tumour may not present any symptoms. Pain is experienced in <1% of cases and may be due to increased IOP from iris neovascularization or closed angle glaucoma.

Characterization of the tumour size is often performed by ultrasonography. Traditionally, this was based on the largest basal diameter (LBD). This classification has been updated to the 8th

Tumour, Node and Metastasis (TNM) staging system, devised by the American Joint Committee on Cancer (AJCC), which is an anatomical staging system applied in all cancers. It enables the UM to be staged based not only by its LBD and thickness, but also by its extraocular extension (EOE) and/or ciliary body involvement (CBI).

Treatment

After the diagnosis of UM, management options are offered to the patient according to the tumour size and its location. Associated ocular co-morbidities and patient preference are other factors considered when determining UM treatment. Currently available treatment options include forms of radiotherapy, surgical excision, enucleation or phototherapy.

Posterior UM are most commonly treated by radiotherapy. Brachytherapy using a radioactive plaque of Ruthenium-106 is most widely used in Europe, while the Iodine-125 isotope is the most popular in the USA. Tumour control is rated at 90% within 5 years using this treatment.

Proton beam radiotherapy (PBR) is offered to patients in some specialised centres. It involves focusing a beam of radiation from an external source onto the tumour while sparing the surrounding tissue. It is modified to suit the tumour based on its size (between 2.5 and 10 mm in height and <16 mm basal diameter) and location and may be used for any UM, including iris melanomas.

The two surgical techniques used to remove UM are called endoresection and 'exo' local resection. The former involves a pars plana vitrectomy after phacoemulsification with intraocular lens implantation. A 25-gauge cutter is used to cause posterior vitreous detachment and the choroidal tumour is 'hoovered' through the retina. Exoresection or local resection involves a partial lamellar sclera-uvectomy technique. To improve local control, adjuvant brachytherapy is commonly done after primary tumour resections. Transpupillary thermotherapy and photodynamic therapy are two of the phototherapeutic treatment modalities available in UM management.

Dissemination, detection and treatment of metastatic UM

Unfortunately, about 50% of UM patients develop metastasis despite successful treatment of the primary tumour. Metastatic spread is via the blood stream since the eye has no lymphatic drainage. Local dissemination of the tumour rarely occurs especially if the conjunctiva is intact and has not been infiltrated transclerally.

The liver is the main site for the metastatic dissemination of UM occurring in around 90% of cases. Other sites such as the lung, bone, skin occur occasionally but typically after liver metastasis. Occult micrometastases may be present in UM patients at the time of ocular treatment. Detectable metastatic tumours are present at the time of ocular diagnosis in only 1-2% of patients. Typically, liver metastases are detected 1-3 years after ocular treatment. Sometimes the metastasis appears 10, 20 even 40 years after primary tumour treatment. The reason for such latency is yet to be understood. Theories suggest that a subset of stem-like cancer cells remain dormant and are reactivated after many years. They then proliferate and give rise to the bulk of the tumour leading to a clinically detectable metastasis. Once liver metastasis has developed, median survival time ranges from 2-12 months with 1-year survival being only 10-15%. Patients typically die from parenchymal invasion of the metastasis or from toxicity of the chemotherapeutic drugs.

Stem cells in normal tissue and cancer.

The development of the embryo relies heavily on a group of cells with self-renewal and multi-lineage differentiation potential. These cells can undergo asymmetric cell division generating one daughter cell that retains the self-renewal and multi-lineage differentiation capacity while the other can become committed to a specific differentiation pathway. Stem cells in adult tissue also produce transient amplifying progenitor cells with the ability to undergo a limited number of cell divisions before finally differentiating terminally. The first evidence of stem cells was from the hematopoietic stem cell (HSC). A single HSC was able to regenerate the blood system of a mouse. The transient amplifying progenitor cell, however, did not have self-renewal properties. When transplanted into an irradiated mouse, it could only produce cells for a short period of time (73).

There are striking similarities between normal organogenesis and carcinogenesis. Many of the oncogenes are known to promote self-renewal in normal stem cells and some tumour suppressor genes also inhibit self-renewal in non-tumour tissue. Theories have therefore been proposed that cancer originates from deregulated stem cells or mature differentiated cells that have dedifferentiated and gained self-renewal properties. The consensus on which of these theories is correct is yet to be reached. However, compelling evidence for the existence of stem cells in cancer has been provided. The American Association for Cancer Research (AACR) held a workshop to review this evidence, providing guidelines and defining several terms in this rapidly growing field. They gave the definition that cancer stem cells (CSCs) are cells with the capacity to divide asymmetrically to produce another CSC and a daughter cell that differentiates giving rise to the bulk of the tumour. CSCs may also be referred to as tumour initiating cells (TICs), describing their more functional role i.e. the capacity to form tumours when xenotransplanted.

In addition to the properties of self-renewal and differentiation, CSC/TIC also possess certain features that enable them to generate, maintain, enhance tumour growth and resist conventional therapy. These include expression of putative stem cell markers, activation of embryonic signalling pathways, anoikis resistance/anchorage independent growth, dye/drug efflux, EMT and the ability to change their metabolic signature among others. Several of these properties have been used to identify and isolate CSCs in different cancers.

Aims of this study

1. To investigate the presence of CSC in UM by examination of several properties including the expression of putative stem cell markers, reactivation of developmental pathways and anoikis resistance.

2. To examine the expression of CSC- and adhesion markers in NCM, UM cell lines and in primary UM cells grown in adherent and non-adherent culture conditions.

3. To examine if CSC markers influence patient outcome using the TCGA database.

4. To investigate the functional role of CSCs in UM cell lines.

5. To examine whether the developmental/stem cell markers that control cell proliferation and migration are expressed in UM tissue.

6. To investigate if the developmental/stem cell markers have an influence on UM prognostication and patient outcome by examining PUM and MUM tissue.

Materials and Methods

Sample Collection

The globes of human cadavers were enucleated with consent at the Department of Pathology, University of Szeged and the Center for Eye Research, University of Oslo, Norway. These eyes were used for cornea isolation and transplantation according to the ethically approved protocol of the Hungarian National Medical Research Council (14387/2013/EKU-182/2013) and the Cornea and Tissue Bank in Oslo (REK ref.nr.:2017/418). After isolation of the cornea, normal choroidal melanocytes (NCM) were isolated and cultured, according to standard protocol that is described below under 'Cell culture'.

Primary UM (PUM) samples were obtained from the Ocular Oncology Biobank (REC Ref 16/NW/0380) and Metastatic UM (MUM) samples were obtained from the Liverpool Bio-innovation Hub Biobank. They were used with patient consent and according to project specific ethical approvals from the Health Research Authority (REC Refs 11/NW/0759 and 15/SS/0097).

Human Umbilical Vein Endothelial Cells (HUVECs) were kindly donated by Professor Lu-Gang Yu of the University of Liverpool. This study was approved by the Health Research Authority and conducted in accordance with the Declaration of Helsinki.

Cell Culture

The isolation of the NCM was done according to standard protocol, as previously reported. Briefly, after enucleation, the eye was put in a petri-dish and washed with PBS+1% penicillin-streptomycin solution. A circumferential incision was made in the sclera, behind the limbus and the anterior segment, then the vitreous and sensory neuroretina were removed. Trypsin was then added inside the eye bulb and it was incubated at 37◦C for 1hr to remove the retinal pigment epithelial (RPE) cells. The choroid was washed with the PBS-antibiotic mix, peeled from the sclera and placed in a petri dish. It was mechanically minced with a blade and re-suspended in a solution of 0.2U of Dispase. This was incubated for 18 hours at 4°C with mild shaking, then at 37°C for 1hr. A wash medium was then added, the supernatant collected, centrifuged and the pellet was plated onto a 6-well plate in melanocyte growth media.

Culture of PUM was performed as previously published. The piece of tumour tissue obtained from surgery was placed in a petri dish and cut to small pieces using a sterile blade. These tissue pieces were then re-suspended in collagenase I and incubated at 37°C for 1hr. Enzyme activity was stopped by addition of media containing 1:1 αMEM: amnioselect, 10% FCS, 2 mM L-glutamine and antibiotics. The solution was centrifuged at 1500rpm for 2 mins. The supernatant was discarded and the cell pellet was re-suspended in fresh media and plated into a 25cm² flask. The media was changed every three days.

The UM cell lines, both PUM and metastatic UM (MUM), were maintained in RPMI media with 10% FCS, 2mM L-glutamine and penicillin-streptomycin. The cells were passaged once a week, and then used when they reached ~60% confluence. At the time of experimental analyses, the cell lines were mycoplasma free and had STR profiles consistent with previously published data. These cell lines have been authenticated according to the guidelines recommended by the International Cell Line Authentication Committee (ICLAC).

HUVEC cells were cultured in a specialised media called EGM which contains growth factors and supplements such as bovine pituitary extract, hydrocortisone, epidermal growth

factor and antibiotics. The media was changed every three days, and when the cells formed a confluent monolayer, they were used for the experiments.

Flow cytometry

The NCM and the PUM cells were used for flow cytometry upon reaching ~60% confluency. They were detached using collagenase IV and a non-enzymatic dissociation solution, respectively. Following this, the blocking buffer was added. The cells were centrifuged at 1500rpm for 2 mins and the supernatant removed. A flow cytometry buffer was added to the pellet and the cells were counted. 200,000 cells were then placed into each FACS tube in 100µl of flow cytometry buffer. The fluorescent antibody of interest was then added for the direct labelling of surface antigens. After 30 mins, the sample was washed with PBS and centrifuged. The remaining pellet was re-suspended in 500µl of flow cytometry buffer and run on the FACS Canto II cytometer. Indirect labelling for the intracellular proteins involved cell fixation in 1% PFA for 10 mins. After washing with PBS, the cells were permeabilized with 0.5% Tween-20 solution for 10 mins. Blocking was performed using 10% normal goat serum in a 1% BSA and PBS solution. 10 mins later, the cells were washed with PBS and the primary antibody was added for 30 mins. A wash was subsequently performed followed by a further 30 mins incubation at room temperature with a fluorescently conjugated secondary antibody. After a final wash, the sample was re-suspended in 500µl of flow cytometry buffer and analysed on the FACS Canto II cytometer.

Adherent and non-adherent cultures for assessment of anoikis resistance

For the purpose of assessing possible changes in putative CSC marker expression during anoikis resistance in the UM cell lines, adherent and non-adherent cultures were employed. Briefly, cell lines that had been cultured to ~60% confluence were detached using the non-enzymatic dissociation solution. The cells were counted and $5x10^5$ cells were added to either a 75cm² tissue culture treated flask or a 75cm² ULA flask. An equal volume of media was added in both flasks. The cells were maintained in these conditions for 72 hrs, and subsequently labelled for flow cytometry according to the protocol detailed above.

Fluorescence activated cell sorting (FACS)

The Mel270 cell line was used for FACS sorting because it had two distinct subpopulations of $CD166^{high}$ and $CD166^{low}$ cells. The cell line was cultured in a 175cm² tissue culture flask until it reached ~60% confluence. The cells were dissociated using a non-enzymatic solution and counted. A total of 7 million cells were re-suspended in a FACS tube using FACS buffer. Cells were labelled with the PE-conjugated CD166 antibody according to the protocol described under 'flow cytometry'. After 30 mins, the sample was washed with a PBS-based wash buffer and re-suspended in FACS buffer. Cell sorting was then performed after establishing the negative and positive gates using a PE-isotype control. The post-sorted cells were collected in media and plated into 25cm² flasks for 24hrs.

Tumour transendothelial migration assay

To mimic the process of extravasation across an endothelial cell layer into the blood stream a tumour transendothelial migration assay was performed as previously described. For this a 24-well plate with trans-well inserts having membranes with 0.8µm pores was used. Firstly, the HUVEC cells were harvested and counted. 30,000 cells were plated into each well insert and media changed daily for 3 days. When a confluent monolayer had been formed, the Mel270 cells were added. 40,000 cells of the Mel270 cell line were plated onto the HUVEC

cell layer with media containing 1% serum on the top while the in the bottom of the chamber the media contained 10% serum. This plate was incubated at 37°C for 48 hours. Afterwards, the cell-dissociation and calcein-AM cocktail was added and after a 1hr incubation, the fluorescence was measured at 485nm excitation and 520nm emission wavelengths in a GeNios Tecan plate reader (Tecan UK Ltd, Reading, UK).

Western Blotting

The specificity of the CD166 and Nestin antibodies used for immunohistochemistry was examined by Western blotting. Briefly, UM cell lines were grown to ~60% confluence in an adherent plate. Media was removed and the flask washed with cold PBS. RIPA buffer together with a protease cocktail inhibitor were added to the flask and a scraper was used to detach the cells. The suspension was then incubated on ice for 30 mins. Sonication was performed for 3 mins followed by heating at 95°C for 10 mins. After centrifugation at 14,000rpm, the supernatant was transferred to a new Eppendorf tube and stored at -20°C. The protein concentration was determined by a BCA assay (Biorad) according to manufacturer's instructions. 20µg of protein was loaded onto an SDS-PAGE gel and run for 2hrs, followed by transfer onto a nitrocellulose membrane of 0.45µm pore size. The membranes were blocked for 1 hr at room temperature in a solution of 5% non-fat milk (NFM) powder in 0.05% TBS-Tween20 (TBST). Overnight incubation in primary antibody diluted in 5% NFM powder was performed at 4°C. This was followed by three wash steps in TBST and a 1hr room temperature incubation with anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody. After washing, the membranes were developed with an enhanced chemiluminescence kit according to manufacturer's guidelines. Imaging was undertaken on a GeneGnome XRQ Image capture machine.

Immunohistochemistry (IHC)

IHC for CD166 in the enucleated eye sections was performed on the automated Bond RXm System. The samples were prepared as 4 µm thick sections cut from FFPE tissue blocks. The kit used was the Bond™ Polymer Refine Red Detection, which applied a Fast-Red substrate chromogen. The slides were counterstained with haematoxylin and mounted using DPX mountant. Normal pancreas tissue sections were used as controls and positive staining in these demonstrated a valid IHC run. Slides were scanned using the Aperio CS2 Slide scanner from Leica and analysed with the Aperio Image Scope software version 11.2.

IHC staining for Nestin was performed on 4-µm-thick sections cut from FFPE tissue blocks. A Dako Pre-treatment module was used for dewaxing and heat-induced epitope retrieval. Slides were incubated in a high pH bath containing EnVision^{TM} FLEX target retrieval solution (Tris/EDTA buffer pH9.0) at 96°C for 20 min, then stained with a primary antibody on an autostainer using the FLEX EnVision^{TM} kit reagents.

IHC staining of UM cell lines grown on chamber slides was performed as follows: 0.2% Triton-X in PBS was added to each chamber for 5 mins to permeabilize the cells. After being washed with FLEX wash buffer, the samples were blocked with an EnVision^{TM} FLEX peroxidase block for 5 mins and incubated with primary monoclonal antibody for 30 mins.

After being washed again, both the chamber slides and Nestin slides were incubated with a FLEX linker for 15 min. Bound antibody was then detected with HRP for 20 min and visualized with an AEC Substrate Kit or DAB for 30 min. Sections were counterstained with Mayer's haematoxylin, blued with Scott's tap water and mounted with either Aquatex^{TM} aqueous mountant (AEC) or DPX mountant (DAB). Normal colon tissue sections listed in were used as controls and positive staining in these demonstrated a valid IHC run for Nestin.

Omission of primary antibody was used as a negative control. Pictures were taken with the Nikon Eclipse Ci (Nikon UK Ltd, Surrey, UK).

IHC Scoring

IHC stained slides for all antibodies were scored by three independent observers. The percentage of positively-stained tumour cells was determined for all antibodies and this was used to grade the staining on a scale of 0-100%. In the Nestin stained slides, positive expression in the intratumoural capillary endothelium was also assessed, as either being present or absent.

Statistical analysis

Statistical analyses were undertaken for all the samples and markers. Categorical variables were tested using the Pearson's Chi-squared test and Fisher's exact test. A student t-test or Mann–Whitney test was used for to examine linear variables where the data did or did not fit a normal distribution respectively. To determine the threshold for Nestin positivity, a receiver operating curve (ROC) analysis was used. Difference in proportion for marker expression in the UM cell lines was assessed by z-statistics. Survival correlation was performed using the Kaplan– Meier test. A p-value <0.05 was considered to be statistically significant and Bonferroni corrections were made to the comparison between NCM and PUM($p \leq 0.008$). All analyses were performed using SPSS software (ver.24.0; SPSS Science, Chicago, IL, USA).

Results

Cell culture of primary tissue

The NCM were cultured until they reached 80% confluence and formed mature cells producing pigment. The cells were of spindle morphology with many melanosomes present in the cytoplasm. PUM cells showed both spindle and epithelioid morphology**.** There were varying amounts of melanin production in these cells consistent with histological sections of the primary tumour.

CD166 and Nestin are upregulated in cultured PUM compared to NCM

When short term cultures (STC) of both NCM and PUM were investigated by flow cytometry, the stem cell markers CD166 and Nestin were found to be upregulated in PUM compared to NCM.

CD166 expression was 4-fold greater in the cultured PUM, (mean 78.3%, median 77.6%, range 54.1-99.6%) compared to its expression in the NCM, (mean 18.8%, median 15.5%, range 3.5-40.8%). This 4-fold increase in expression was found to be statistically significant **(p=0.0003**; Mann-Whitney). When correlated with chromosomal aberrations and histological prognostic factors, CD166 expression did not correlate with any known predictors of survival or disease outcome. Nestin expression was 1.6-fold higher in PUM (mean 33.1%, median 18.5%, range 0.04-99.3%) compared to NCM (mean 19.9%, median 16.5%, range 5.2-41.9%). However, this increased Nestin (p=0.12) together with the expression of Melan A(p=0.12), CD271 (p=0.14) CD146 (p=0.12) and CD133 (p=0.01) were not statistically significant when a Mann-Whitney U test was performed.

Of the ten PUM samples, five had features associated with a high metastatic risk (M3), four of these also having a loss of nBAP1 expression. When prognostic factors were correlated with the PUM results, they showed that M3 patients had a higher expression of CD271 (median 6.8%, range 0.5-30%) compared to D3 tumours (median 4.5%, range 0.2-22.2%). M3 patients also had a higher Nestin expression (median 19.8%, range 9.8-98.3%) than D3 patients (median 16.5%, range 0.04-99.3%). These changes were however not statistically significant when a Mann-Whitney test was performed CD271 (p=0.42) and Nestin (p=1).

CSC markers are upregulated in PUM cells during anoikis resistance

Culturing cells in non-adherent/ultra-low attachment (ULA) conditions for 72 hrs ensured that only UM cells that were able to survive anoikis were viable. Comparison of the expression of CSC markers was made between the cells surviving anoikis/anchorage independent conditions and those grown in adherent monolayers. The UM cell lines used were derived from either PUM (92.1, Mel 270, MP41, MP46) or MUM (OMM1, OMM2.3, OMM2.5, MM66) samples. All the experiments were repeated at least 3 times and an average ± standard deviation (SD) was calculated.

Melan A, the marker of differentiated melanocytes was expressed in at least 50% of all the cells in both adherent and non-adherent conditions (median 73.3% vs 71.8%).

CD271, the neural crest stem cell marker was expressed at low levels (median 1.0%, range 0.03-33.3%) in the cells grown as adherent cultures. The cells surviving anoikis, however, had a higher expression of this marker (median 8.9%, range 0.04-18.2%). These changes were statistically significant (**p=0.00**, z-statistic) in MP46, OMM1, and MM66.

Nestin, a neural stem cell marker of prognostic significance in both skin and uveal melanoma was upregulated in all cell lines after anchorage independent growth. During adherent culture, the median expression of Nestin was 25.5% (range 3.0-80.4%). After non-adherent culture, the cells had a median expression of 64.1% (range 19.8-97.5). Although this upregulation of Nestin expression was observed in 7/8 of the cell lines examined, these changes were statistically significant (**p=0.00**, z-statistic) only in 92.1, MP41, MP46, OMM2.3 and OMM 2.5.

CD133 is a stem cell marker in hepatocellular carcinoma, colorectal cancer and glioblastoma. Its expression was unchanged in both culture conditions, adherent (median 0.3%, range 0.1-0.6%) and non-adherent (median 0.4%, range 0.2-0.8%).

CD146 (MUC18 or MCAM) is a protein of the immunoglobulin family. It strongly expressed in metastatic skin melanoma cells for the purpose of interacting with vascular tissue during haematogenous spread. The expression of CD146 was present in >70% of the UM cells and showed little change in either of the culture conditions.

CD166 is also a member of the immunoglobulin superfamily. It functions as a cell surface sensor for cell density, controlling the transition between local cell proliferation and tissue invasion in melanoma progression. Its expression was variable in the UM cell lines examined in adherent culture (median 34.8%, range 2.5-87.7%) and in non-adherent culture (median 30.5%, range 2.2-95.1%). Three of the four MUM cell lines [OMM1 (87.7-95.1%), OMM2.3 (3.7-6.9%), OMM2.5 (17.7-26.4%)] and one PUM; MP41 (3.1-18.0%) however, upregulated their expression of CD166 during anoikis resistance as compared with cells in adherent culture. Analyses by z-statistics showed that these changes were statistically significant MP41 (**p=0.00**) but not OMM1 (p=0.07), OMM2.3(p=0.35), or OMM2.5 (p=0.17).

Nestin, CD271 and CD166 gene expression in 80 PUM analysed by TCGA

The role of *Nestin*, *CD271* and *CD166/ALCAM* gene expression in UM and their correlation to other prognostic factors were investigated. This was undertaken by analysing the recently-published and publicly available data from The Cancer Genome Atlas (TCGA) for UM. It contains the genetic data of 80 well characterised PUM patients followed up for 5 years. mRNA expression data was obtained from the database and compared with chromosome 3 CNVs, BAP1 gene expression and patient outcome.

Median expression levels of the genes were used as the cut off points and upregulation or downregulation was displayed in the heat maps. The median expression levels (unit log2(fpkm-uq+1)) of the genes were: *BAP1*(19.50), *ALCAM* (14.22), *Nestin* (19.02) and *CD271/NGRF* (13.2). Results showed that UM with M3 (i.e. associated with a poor prognosis) and decreased *BAP1* gene expression (compared to the median) also had upregulations in *CD166/ALCAM* expression. The expression of *Nestin* and *CD271/NGRF* was more variable across the 80 samples with upregulations and downregulations occurring in the M3 as well as the D3 group in no distinct pattern.

The Kaplan-Meier plots were created using the median expression levels as the cut off and statistics analysed by Chi-square test ($\chi 2$). The survival probability was calculated based on event (metastatic death) and time to event (number of days) as parameters. They show that UM expressing *CD166/ALCAM* above the median were associated with a poorer prognosis than those with expression below the median (**p=0.00321**). The expression of Nestin (p=0.31) and CD271/NGRF (p=0.68) had no statistically significant association with patient survival. *CD166/ALCAM* association with survival was not as a result of its covariation with tumour stage. Based on data from both the flow cytometry analyses and gene expression data, the functional role of CD166/ALCAM was investigated further.

Western blotting

In order to perform functional studies in UM cell lines, we purchased a CD166 antibody (Abcam: ab109215) that was not fluorescently-conjugated. The antibody was tested for antigen specificity by Western blot. The cell lysate of UM cell line Mel270 was run on an SDS-PAGE gel to determine CD166 protein expression. The cell line expressed CD166, visible as a band at the predicted molecular weight (100-105kDa)**.** The antibody was specific for only this protein.

CD166 protein is expressed in the cytoplasm of PUM cell lines by IHC.

The expression pattern and cellular location of CD166 protein was examined in UM by IHC. Three cell lines (92.1, Mel270, OMM1) were cultured in chamber slides and stained by IHC for CD166. The UM cells 92.1 expressed CD166 in the cytoplasm of ~60% of the cells**.** The Mel270 cell line also showed cytoplasmic CD166 expression in approximately 40% of the cells. OMM1 cells expressed CD166 only on the membrane. This expression was in at least 80% of the cells.

$CD166^{high}$ Mel270 cells have a higher tumour transendothelial migration potential than $CD166^{low}$ Mel270 cells

After obtaining distinct populations of $CD166^{high}$ and $CD166^{low}$ Mel270 cells by FACS the cells were plated on a layer of HUVEC cells cultured on transwell inserts with a 0.8µm pore size.

The HUVECs had been in culture for three days and had formed a confluent monolayer with tight junctions between the cells. Serum was used as a chemoattractant in the media, with a gradient of 1% serum on top and 10% serum at the bottom. The cells with $CD166^{high}$ expression (mean 25,531, median 25,474, range:17,514-3363) migrated across the HUVEC layer more than those with $CD166^{low}$ expression (mean 20,100, median 20,031, range: 17,617-22,474). A t-test showed that the differences were statistically significant, **p=0.041**.

CD166 is expressed in the cornea, ciliary body and optic nerve

IHC staining undertaken in eyes enucleated due to the presence of UM, revealed several normal ocular structures that expressed CD166. These served as an internal positive control when examining the tumour cells. They include the corneal epithelium, ciliary processes and ciliary muscle, trabecular meshwork and the meningeal layer of the optic nerve.

IHC analysis shows that CD166 is expressed in the PUM tissue

IHC staining was performed in FFPE sections obtained from enucleated eyes of PUM patients. Melan A-stained slides of these tissue sections were obtained from the pathology archives and used to identify the UM cells. The identified PUM cells were then examined for CD166 expression.

PUM cells stained for CD166 in both the cytoplasm and the membrane. This was clearly observed in only two of the nine samples examined (eight were from enucleations and one was from an endoresection). In one of the samples, the staining was weak and involved only the anterior one-third of the tumour. In this region, 70% of the UM cells stained positive for CD166 while tumour cells in the posterior two-thirds of the tumour were negative. It was difficult to determine cytoplasmic or membranous staining in the heavily pigmented or macrophage dense UM sections (4/9).

In addition to positive staining for CD166 in the tumour cells it was also detected in the cytoplasm of the tumour associated macrophages. Normal pancreas sections stained positively for CD166 in the islets of Langerhans. The NCM expressed membranous and cytoplasmic CD166. Endothelial cells in some tumour sections also stained positive for CD166.

Neural crest markers are expressed in PUM

Evidence from skin melanoma shows that similar pathways and features are employed during malignant transformation and metastasis as those found in the embryonic NC cells. These include EMT, migration through different microenvironments and expression of NC regulatory factors/markers. Our hypothesis was that UM cells reactivate developmental pathways that control cell proliferation and migration. One of the aims of this study was to investigate if the developmental/neural crest markers are expressed in UM tissue. We focused on MITF, SOX10, PAX3, Notch 1 and Nestin, all which play a role in melanocyte development.

IHC analysis showed that MITF, SOX10 and PAX3 are expressed in the nuclei of >80% of PUM cells in all six samples. The Notch1 antibody used detects activated Notch1 and is thus used to mark nuclear Notch 1 expression. Activated Notch1 was also expressed in the nuclei of >80% of PUM cells in all six samples. There was no difference in the expression pattern of these markers when high-risk M3 tumours were compared to the low-risk D3 tumours. Poor prognostic features such as the presence of loops and epithelioid cells were seen in some of the M3 tumours, but this did not influence the expression of these markers.

C-KIT was expressed on the cell membrane of PUM in both the M3 and D3 tumours. It was expressed in >70% of cells in the M3 tumours and <50% of cells in the D3 tumours. Nestin was expressed only in the endothelium of the blood vessels of the D3 samples, with no expression in the tumour cells. In the M3 UM, Nestin expression was evident in the membrane and cytoplasm of the tumour cells. Of the markers examined, C-Kit and Nestin showed variation in expression between the high and low metastatic risk tumours.

Overexpression of C-KIT in UM has been previously described and a therapeutic agent (imatinib mesylate) acting against this tyrosine kinase inhibitor has been tested in a phase II multicentre clinical trial involving patients with unresectable MUM. The trial showed no effect of the drug and the clinical trial did not proceed to the next stage. Although C-KIT expression showed variation between M3 and D3 tumours in this study, it was not examined further. An investigation of Nestin, which had showed a distinct difference between M3 and D3 tumours was undertaken in a larger cohort of PUM samples, MUM and UM cell lines.

Nestin expression *in vitro*

A western blot was performed using two UM cell lines to determine the specificity of the Nestin antibody used. Nestin was expressed in the Mel270 and MM66 UM cell lines. A preliminary IHC staining of six UM cell lines was also undertaken. Nestin was expressed in the cytoplasm as well as the membrane of these cell lines**.** Following this a large cohort of PUM samples (144) were stained for Nestin.

Nestin expression in PUM

144 PUM samples underwent IHC staining for Nestin. The results were scored on a 0-100% scale for positively stained tumour cells. Nestin was positive in several non-tumour areas of the globes and these were used as positive internal controls to validate the IHC stain alongside the normal control colon sections. These areas include the cells of the neuroretina, the inner and outer nuclear layer, the ganglion cells and optic nerve fibre layer. The blood vessels were also Nestin positive. The retinal pigment epithelium and the NCM did not express Nestin

Melan A-stained slides of the tissue sections obtained from the pathology archives were used to identify the UM cells across the tissue sections, and to determine the quality of immunostaining of the samples following formalin fixation. The identified UM cells were then examined for their Nestin expression. The UM cells expressed Nestin in their membrane and cytoplasm. The endothelium of the intratumoural blood vessels were also positive for Nestin in some tissue sections**.** To determine the highest levels of sensitivity and specificity for Nestin expression, a ROC analysis was performed. The data identified that a range of expression between 8.5% (0.86/0.58; sensitivity/specificity) and 12.5% (0.68/0.48; sensitivity/specificity) would give the highest sensitivity and specificity respectively at 95% confidence interval (CI). A 10% threshold was therefore used as the cut-off point. Previous studies examining Nestin had also used this threshold. UM samples with <10% Nestin expression were thus considered negative for this marker.

Fifty-two of 141 (36%) of the examined PUM were scored as negative for Nestin expression. Sixty-three PUM (44%) showed varying intensity of Nestin expression ranging from 10-50% positivity in the tumour cells**.** Nestin was expressed in 50– 100% of UM cells in the remaining 26 PUM samples (18%). In these samples, Nestin was expressed both in the cytoplasm and membrane of cells**.** Positive staining in the endothelium of the intratumoural blood vessels was observed in a total of 36 PUM (25%).

Survival analysis

When correlated with known poor prognostic factors in PUM, Nestin positivity (≥10%) was significantly associated several predictors of metastasis. These include epithelioid morphology (Pearson's Chi-square p<0.0001), high mitotic count (Mann–Whitney p<0.0001), closed connective tissue loops (Pearson's Chi-square p=0.001) monosomy 3 (Pearson's Chi square p=0.007) and chromosome 8q gain (Fisher's exact test p<0.0001). Increasing Nestin expression was significantly correlated with the absence of nBAP1 protein expression (p=0.015, Mann–Whitney U). Nestin expression in the capillary endothelium showed no significant correlation with any prognostic factor. Patients with PUM classified as negative for Nestin expression (<10%) had a better prognosis than those patients with positive Nestin expression in the tumour cells as shown by the Kaplan–Meier analysis (L**og-rank p = 0.002**).

Nestin expression in MUM

Melan A-stained slides of MUM were obtained from the Pathology archives and assessed, similarly to the PUM described above. They were used as a reference to show the antigenicity of the tissue and to identify the tumour cells. The hepatocytes and hepatic stellate cells did not express Nestin while the MUM cells and associated blood vessels were positive for Nestin. nBAP1 data were available for 19 of the 26 MUM 17 MUM were negative for nBAP1 expression and two were positive for nBAP1.

50-100% of Nestin positive tumour cells was were seen in ten of the 26 MUM (38%). Nine of the 26 samples (34%) had 10-50% of Nestin expressing cells. In 17 (65%) of the samples, Nestin was expressed in the UM cells located within closed connective tissue loops. Six (23%) of the 26 MUM had <10% Nestin-expressing tumour cells, and in one other, Nestin expression was completely negative. Nestin expression in the intratumoral capillary endothelium was visible in nine MUM samples which also showed a high proportion (50-90%) of Nestin-positive melanoma cells in the tissue.

Nestin expression in matched PUM-MUM

Scattered UM cells that were located away from the bulk of the tumour in the MUM sections also stained positive for Nestin. Eleven of the MUM samples had matched PUM sections available. When examined, Nestin was expressed in the cytoplasm of the UM cells in all the PUM tissue sections. In one (9%) PUM, there were <10% of Nestin positive cells in the tumour section. Eight (72%) of the eleven PUM had 10-50% of Nestin expressing cells while in the remaining two samples (18%), Nestin was expressed in 50-90% of the PUM cells. In five of these PUM samples, staining was also seen in the intratumoural capillary endothelium. Nine of these matched PUM were negative for nBAP1 expression, one was positive and for one, data were not available.

A varied expression of Nestin was seen in the MUM: four MUM (36%) expressed Nestin in >50% of the tumour cells, three (27%) expressed Nestin in 10–50% of UM cells. In two (18%) other samples, only scattered large ovoid MUM cells stained positive for Nestin. Only the intratumoural capillary endothelium stained positive for Nestin in the remaining two samples (18%). Seven of these MUM were negative for nBAP1 expression, two were positive while the BAP1 expression data were unavailable for the remaining two samples.

Interestingly, three of the four MUM samples with >50% of cytoplasmic Nestin-expressing cells had shorter metastasis free and overall survival than the MUM in which Nestin expression was <50%. One of the patients died 2 weeks after the diagnosis of metastatic

melanoma while the two others died within one month of diagnosis. The fourth patient is still alive with features of good prognosis such as nuclear BAP1 expression in the PUM and a normal (disomy) chromosome 3 status. The MUM cells in the liver resection are also nBAP1 positive for this patient. The three-remaining matched M3 PUM and MUM were negative for nBAP1 expression.

Discussion

This study has shown the expression of CSC markers in UM. Flow cytometry results have demonstrated increased expression of the CSC markers, Nestin, CD271 and CD166 in high metastatic risk PUM compared with low metastatic risk PUM and NCM. The ability of a subpopulation of UM cell lines to escape anoikis by upregulating stemness markers such as CD166, CD271 and Nestin provides further evidence for the existence of CSCs in UM. A tumour transendothelial migration assay has shown that the CD166 high subpopulation of UM cell line had higher migratory capacity compared to CD166 low population. This study has also shown that protein markers associated with NC developmental pathways leading to melanocyte specification are expressed in UM, suggesting that these primitive pathways may be reactivated in this tumour. These markers, including MITF, SOX10, PAX3, Notch 1, C-KIT and Nestin, have been shown to play a role in the tumourigenesis and metastasis of several cancers, particularly skin melanoma. They may function in a similar manner in UM.

Studies in skin melanoma have shown a higher overall expression of CD166 in primary tumours than benign lesions by IHC. Consistent with these studies, flow cytometry analysis showed that PUM expressed more CD166 as compared to NCM. Nestin expression was also higher in the PUM compared to the NCM, consistent with previous reports in literature. The findings of Lai et al. who examined expression of CD146/MCAM/MUC18 in the uvea support the CD146 results in this study. They reported that CD146 is expressed in the NCM, FFPE tumour sections and UM cell lines. However, the high levels of expression of this marker in both UM and NCM suggest that it lacks specificity as a CSC marker.

Anoikis is an apoptotic cell death process induced in cells after their detachment from the extracellular matrix (ECM). It is a physiological process that prevents attachment and seeding of displaced cells at inappropriate sites. Resistance to anoikis is a hallmark of tumorigenesis and metastasis, as it enables cancer cells to survive and spread in the blood or lymphatic system. The different causes of anoikis resistance in cancer cells, include genetic instability, intra-tumoral hypoxia, epithelial-mesenchymal transition and overexpression of stemness markers. Overexpression of stemness markers activates processes including cell proliferation, survival, motility, migration, apoptosis and anoikis resistance. CSCs in several cancers including breast have been shown to be resistant to anoikis, form spheres in non-adherent culture and have enhanced tumour growth capacity.

In this study, UM cell lines surviving anoikis showed an increased expression of several markers previously associated with neural crest development and stem cells; CD271, Nestin and CD166. The CSC population in skin melanoma has been shown to express CD271. Samples having >5% of CD271/SOX10 positive cells correlated with poor tumour specific survival. $CD271^{+}$ cells were able to form tumours in xenograft models that resembled the parent tumour. In UM, CD271 was also expressed in the cells that formed vasculogenic mimicry patterns, a poor prognostic feature likely to cause metastasis.

Nestin was identified as a marker of skin melanoma stem cells along with CD133 and CD166. It was expressed at moderate to strong intensity in >15% of cells of the primary and

metastatic melanomas but not nevi. Nestin protein and mRNA expression were also described in UM cell lines and primary tissue in the study investigating putative CSCs in UM. The $CD133^{+}/Nestin^{+}$ cells ranged from 3.1-17.6% in Mel270, OMM2.3 and OMM2.5 UM cell lines by FACS analysis. Although the $CD133^{+}/Nestin^{+}$ double positive cells were not examined in this study, the presence of Nestin in the UM cell lines supports the findings of the study by Thill et al. These results suggest that PUM cells surviving anoikis may be enriched by the CSC population, as evidenced by their increased expression of stemness markers.

In the PUM tissue sections stained by IHC, CD166/ALCAM expression was abundant in the cytoplasm of the tumour associated macrophages. Variable CD166 staining was also observed in macrophages in the study by van Kempen et al. examining ALCAM expression in skin melanoma. Tumour endothelial cells also expressed CD166/ALCAM, which has been reported to be involved in early embryonic haematopoiesis and vasculogenesis. In this study, expression of CD166/ALCAM in the PUM cells analysed by IHC was not as abundant as demonstrated by flow cytometry. The presence of CD166 positive macrophages and endothelial cells may account for this. However, its location was similar to that in skin melanoma, being positive both in the membrane and the cytoplasm of the tumour cells.

CD166/ALCAM was expressed in several normal structures of the eye including the normal choroidal melanocytes. This had only been previously reported in the choroid of mice although its role is yet to be established. The ciliary muscle and ciliary processes also strongly expressed ALCAM in their membrane. Additionally, the trabecular meshwork also had ALCAM positivity. Expression of ALCAM in the corneal endothelium has previously been reported, both *in vivo* and *in vitro*. Similarly, ALCAM is important in neurogenesis and in supporting neurite extension. It is expressed in spinal cord motor neurons and those of the peripheral nervous system. Its expression in the body and meninges of the optic nerve was therefore anticipated. Further investigations are necessary to ascertain if these data may be of clinical relevance. Our findings also present new insights into the expression of CD166 in the other structures of the eye, which have not been reported previously.

In this study a detailed examination of Nestin expression in PUM and MUM was also performed. The results show that Nestin expression is associated with a reduced survival time in UM. Nestin expression also significantly correlates with known poor prognostic factors in PUM, such as epithelioid cell morphology, high mitotic count, the presence of closed connective loops, monosomy 3 and polysomy 8q.

Gene expression profiling has shown that these poor prognostic parameters are also associated with so-called high metastatic risk ‘class 2’ UM. These class 2 PUM express genes similar to those seen primitive neuroectodermal and neural crest cells. The authors suggest that class 2 PUM have cells with primitive stem-like cell phenotype. Nestin is expressed in the migrating and proliferating neuroectodermal cells during embryogenesis. In adult tissues, it may identify primitive multipotent cells with regenerative capacity that can be re-activated during injury.

The absence of Nestin positivity in NCM as compared to its presence in UM cells may suggest reversion to a more primitive phenotype during tumourigenesis. This theory may be supported by evidence from recent studies in several tumours, including cutaneous melanoma, squamous cell carcinoma, basal cell carcinoma, osteosarcomas and gliomas that have associated increased Nestin expression with an immature and invasive cell phenotype. Data from this thesis also supports these findings. Nestin expression was higher in the PUM

compared to NCM as well as in M3 tumours (higher metastatic risk) compared to D3. The Nestin expressing UM cells may represent a subpopulation with stem cell-like characteristics.

In support of this, data generated by The Cancer Genome Atlas (TCGA)-UM study and from the analysis of gene expression data previously generated by Laurent et al., showed that Nestin mRNA expression was identified in a panel of genes associated with reduced time to metastasis after diagnosis of the PUM. However, this association was not found to be significant when examining the hazard ratio and 95% CI. This may be due to the relatively small cohort examined by the TCGA (n = 80), as compared to the 141 PUM examined in the current study.

The presence of putative CSC with self-renewal capacity that were resistant to chemotherapy was previously reported in UM cell lines. Nestin expression has been shown in the tissue of melanoma patients as well as in their circulating tumour cells (CTC). An analysis of the blood obtained from both cutaneous and UM patients revealed Nestin-expressing cells, which were absent in healthy volunteers. 17 skin melanoma patients with stage IV disease had Nestin mRNA expression in their blood samples compared to 4 stage III/II patients. Nestin mRNA expression was also significantly ($p=0.041$) higher in the blood samples of patients with high versus low tumour burden. This strongly proposes the possible use of Nestin as a biomarker for early detection of metastatic disease in high-risk UM patients. Its sensitivity may be more than that of previously proposed serum biomarkers, such as osteopontin, S100B and melanoma inhibitory activity (MIA). Nestin may be added to the panel of sensitive serum biomarkers of MUM along with the proposed cytokeratin 18 and GDF-15.

The molecular function of Nestin has also been investigated in several cancers. Suppression of Nestin expression by shRNA in cutaneous melanoma cell lines has been shown to lead to reduced cell growth, migration and invasion into Matrigel. These cells also have less spheroid formation ability than the control. When these cells were injected into mice, they formed smaller tumours, which did not metastasize to the liver. In gliomas, similar results were observed following Nestin knockdown. In pancreatic ductal carcinoma, Nestin downregulation inhibited liver metastasis *in vivo*. In UM, further functional studies are necessary to help us to delineate the role of Nestin. In this regard, Nestin IHC has been undertaken in a panel of six UM cell lines to determine baseline expression of this protein. Several UM cell lines with high levels of Nestin expression (e.g. 92.1, Mel270 and MP41) have been identified. These may then be used to create isogenic cell lines for *in vitro* functional assays. These assays have been included in our future plans.

In conclusion, we have shown that UM contains a population of cells with characteristics of CSCs *in vitro*. In particular, $CD166^{high}$ UM cells may represent a subpopulation with enhanced migratory capacity. Our future plans include using *in vivo* models to investigate if these findings can be recapitulated in living organisms. Additionally, this study has suggested that developmental pathways may be activated in UM cells, evidenced by the expression of NC and melanocytic lineage markers in PUM samples. PUM samples with >10% of Nestin-expressing cells correlate with poor survival. Nestin is also expressed in MUM, which together with previous studies showing Nestin expression in CTC, suggests that Nestin may be used as a biomarker in high-risk UM patients for early detection of disseminated disease. It is possible therefore that UM CSCs may be identified using several markers including CD166 and Nestin.

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