CD166^{high} Uveal Melanoma Cells Represent a Subpopulation With Enhanced Migratory Capacity

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Purpose. Cancer stem cells (CSCs) are a subpopulation of cells with the capacity to drive tumor growth. While there is evidence of the existence of CSCs in uveal melanoma (UM), there is no consensus on their defining markers. In this study, we examined putative CSC markers in UM cell lines, primary UM (PUM), and normal choroidal melanocytes (NCM).

Methods. Nonadherent sphere assays were used to assess the tumorigenic potential of 15 PUMs, 8 high (M3) and 7 low (D3) metastatic risk. Flow cytometry was used to compare the expression of CSC markers between 10 PUMs and 4 NCMs, as well as in 8 UM cell lines grown under adherent and nonadherent conditions. Based on the data generated and from TCGA analyses, CD166 was investigated in detail, including its effect on cell migration using a tumor transendothelial migration assay.

Results. M3 PUM had a greater melanosphere-forming efficiency than D3 PUM. CD166 and Nestin expression was upregulated in PUM compared to NCM by flow cytometry. UM cell lines resistant to anoikis had increased levels of CD271, Nestin, and CD166 compared with adherent cells. TCGA analysis showed that patients with higher CD166 expression had a poorer prognosis: this was supported by a Mel270 CD166^{high} subpopulation that had enhanced migratory capabilities compared with CD166^{low} cells. IHC showed that CD166 is expressed in the cytoplasm and cell membrane of PUM cells.

Conclusions. UM contain a population of cells with characteristics of CSCs. In particular, CD166^{high} UM cells appear to represent a subpopulation with enhanced migratory capacity.

Keywords: uveal melanoma, cancer stem cells, CD166

Cancer stem cells (CSCs) are a subpopulation within a tumor with the capacity to self-renew, generate the bulk of the tumor and facilitate continued tumor propagation.[1] It is also believed that CSCs contribute to chemo- and radio-resistance in tumors that relapse, despite "successful" first-line treatment.[2] The enhanced survival mechanism(s) of CSCs enables them to survive in the circulation and form distant metastases.[3] Several cell surface markers have been described in breast, colon, and prostate carcinomas as well as other cancers, which putatively identify/enrich for the CSCs.

In skin melanoma, CSC markers include CD271,[4] CD133,[5] CD166,[6] Nestin,[7] ABCB5,[8] and CD20,[9] which identify cells able to form tumors when xenotransplanted in mice, to form melanospheres (MS) in nonadherent culture, and showed a stepwise increase in expression from nevi to metastatic lesions.

Although only a few studies exist in UM, there is some evidence for the existence of stem-like cells in UM cell lines,[10,11] and an association of high metastatic risk UM with a primitive neuroectodermal phenotype.[12] Putative CSC markers identified in eight UM cell lines included Nestin, CXCR4, CD44, and c-kit. In formalin-fixed paraffin embedded (FFPE) UM, CD133, Pax6, Musashi, Nestin, Sox2, and ABCB5 were observed predominantly at the "invading" tumor edge.[11] Doherty et al.[13] demonstrated expression of ALDH, CD44, and CD133 in UM cell lines and short-term cultures, although the cellular phenotype altered in response to environmental stimuli. They suggested this cellular plasticity may be related to the neural crest origin of intraocular melanocytes and all UM cells have the potential to drive tumor progression.

It is clear, therefore, there is lack a consensus regarding the presence of CSCs in UM and the markers that can be used to identify this subpopulation. In this study, we examined several properties associated with CSCs in UM cell lines and short-term cultures of primary UM (PUM) cells and normal choroidal melanocytes (NCM). These include, expression of melanocytic, neural crest and putative stem cell markers and resistance to anoikis.

Materials and Methods

Sample Collection

All PUM samples were obtained from the Ocular Oncology Biobank (REC ref. no. 16/NW/0380) and were used with patient consent and according to project-specific ethical approvals from the Health Research Authority (REC ref. no. 11/NW/0759 and 15/SS/0097). Human umbilical vein endothelial cells (HUVECs) were provided by Lugang Yu of the

University of Liverpool. The study was conducted in accordance with the tenets of the Declaration of Helsinki.

Four postmortem globes were obtained at the Centre for Eye Research, University of Oslo, Norway and used for cornea isolation and transplantation according to the ethically approved protocol of the Cornea Bank (REK ref. no. 2017/418). After isolation of the cornea, NCM were isolated and cultured according to a standard protocol.

Cell Culture

Isolation and culturing of NCM was performed as previously described.[14] Briefly, the enucleated eye was washed with PBS + 1% penicillin-streptomycin solution (Sigma Aldrich Corp., Dorset, UK). A circumferential incision was made in the sclera and the vitreous, sensory neuroretina, and retinal pigment epithelial cells were removed. The choroid was washed with the PBS-antibiotic mix and peeled from the sclera. It was mechanically minced with a blade and resuspended in a solution of 0.2 U Dispase (Sigma Aldrich Corp.). Following overnight incubation at 37°C, the digested choroid was collected, filtered, and spun down. The pelleted cells were plated into a six-well plate in melanocyte growth medium (Promocell, Heidelberg, Germany).

Culture of PUM cells was also performed as previously published.[15] The fresh tumor tissue was minced using a sterile blade. The tissues pieces were then resuspended in collagenase IV (Sigma Aldrich Corp.) and incubated at 37°C for 1 hour. Isolated cells were counted and used either directly in the nonadherent sphere assay or grown as adherent cultures until they reached ~60% confluence. In both assays, PUM cells were grown in 1:1 αMEM (Sigma): amnioselect (Metachem Diagnostics Ltd, Northampton, UK), 10% fetal calf serum (FCS) (Labtech International Ltd, Heathfield, UK), 2 mM L-glutamine (Sigma) and antibiotics.

UM cell lines, derived from both primary (92.1,[16] Mel270,[17] MP41,[18] MP46[18]) and metastatic UM (MUM) (OMM1,[19] MM66,[18] OMM2.3,[17] OMM2.5[17]), were maintained in RPMI medium (Life Technologies, Warrington, UK) with 10% FCS, 2 mM L-glutamine and antibiotics. Cells were passaged once a week and used when they reached ~60% confluence. Cell line details are included in Supplementary Table S1. All cell lines were mycoplasma free at the time of study and authenticated by STR profiling according to the guidelines recommended by the International Cell Line Authentication Committee (ICLAC).

HUVECs were cultured in a specialized medium, EGM (Lonza Ltd, Slough, UK), which contains growth factors, supplements, and antibiotics. The medium was changed every 3 days, and cells were used when they formed a confluent monolayer.

Chromosomal Copy Number Analysis

The multiplex ligation dependent probe amplification (MLPA) procedure for the assessment of chromosome 1, 3, 6, and 8 copy number alterations were performed as previously described.[20]

Nonadherent Sphere Assay

PUM cells were seeded in 20 mL PUM medium, at a density of 2000 cells/mL into a 75-cm^2 flask coated with poly 2-hydroxyethyl methacrylate (poly-HEMA; Sigma Aldrich Corp.) to prevent cell attachment. Every 7 days, 10 mL of medium was replaced with fresh PUM medium (10 mL). After 21 days, MS of at least 50 µm in diameter (size was determined using an eyepiece graticule with crossed scales) were counted.

Flow Cytometry

The NCM, PUM and cell lines were used for flow cytometry upon reaching ~60% confluence. Cell detachment was by collagenase IV and nonenzymatic dissociation solution (Life Technologies). After incubation for 5 minutes at 37°C, the blocking buffer (10%FCS, 0.02%EDTA in PBS) was added. Centrifugation was performed at 250*g* for 2 minutes and following cell counting, 200,000 cells were resuspended in 100 µl "flow cytometry" buffer (PBS containing 1% bovine serum albumin: BSA). A fluorescently-labeled antibody was added to the appropriate tubes for direct labeling of the surface antigens. The antibodies used were all from Biolegend (London, UK): PE-conjugated anti-CD166 (12.5 µg/ml), FITC-conjugated anti-CD146 (10 µg/ml) and PE-conjugated anti-CD133 (5 µg/ml). After 30 minutes, the samples were washed with PBS and centrifuged at 250*g* for 10 minutes. The pellet was suspended in 500 µl of buffer and analyzed in the FACS Canto II cytometer (BD Biosciences, Berkshire, UK).

Indirect labeling for the intracellular proteins was performed following cell fixation in 1% paraformaldehyde for 10 minutes. After washing with PBS, the cells were permeabilized with 0.5% Tween-20 solution for 10 minutes. Blocking was performed using 10% normal goat serum in 1% BSA+PBS for 10 minutes. The cells were washed with PBS and incubated with the primary antibody for 30 minutes. An AlexaFluor 488–conjugated fluorescent secondary antibody was then added for a further 30 minutes. After a final wash step, the sample was resuspended in 500 µl of flow cytometry buffer and analyzed on the cytometer. The mouse monoclonal antibodies labeled indirectly were Melan-A (DAKO, 1 µg/mL), Nestin (Abcam, 10 µg/mL) and CD271 (Abcam, 5 µg/mL).

Assessment of Anoikis Resistance

To assess anoikis resistance, UM cell lines were grown in adherent and nonadherent (using ultralow attachment [ULA] plates) conditions. Briefly, cell lines at ~60% confluence were detached with a nonenzymatic cell dissociation solution. After centrifugation at 1500*g* for 2 minutes, the cells were counted and 5×10^5 cells were added to either a 75 cm^2 tissue culture treated flask or a 75 cm^2 ULA flask (Sigma Aldrich Corp.) in RPMI +10% FCS. Cells were maintained in these conditions for 72 hours, then labeled for flow cytometry according to the standard protocol.

Immunohistochemistry (IHC)

IHC for CD166 (Abcam, 44 µg/mL) was performed on 4-µm FFPE sections using commercial equipment (Leica Bond RXm System; Leica Microsystems Ltd, Milton Keynes, UK) and a detection kit (Bond Polymer Refine Red Detection Kit; Leica Biosystems, Inc., Buffalo Grove, IL, USA). Slides were counterstained with hematoxylin and mounted using DPX mountant (Sigma Aldrich, Corp.). Normal pancreas served as the positive control; negative control was omission of the primary antibody. Slides were scanned using a slide scanner (Aperio CS2; Leica Biosystems, Inc.) and analyzed with imaging software (Aperio Image Scope version 11.2; Leica Biosystems, Inc.).

Fluorescence Activated Cell Sorting (FACS)

The Mel270 UM cell line was used for FACS because it contains two distinct subpopulations, $CD166^{high}$ and $CD166^{low}$. After dissociation, 7×10^6 cells were resuspended in FACS buffer (PBS, 1% BSA, 10% serum). They were then labeled with the PE-conjugated CD166 antibody according to the protocol

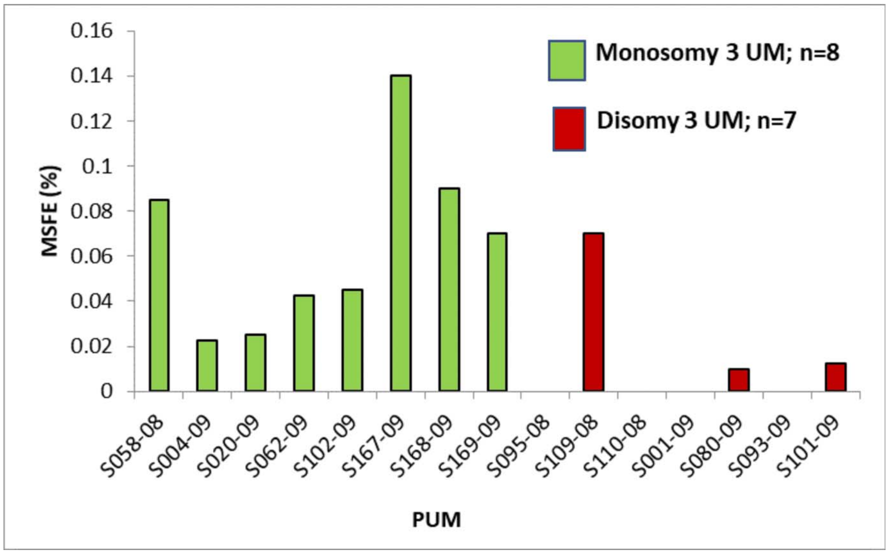

FIGURE 1. MSFE of PUM grown at low density in non-adherent culture conditions. A *bar graph* showing a greater MSFE for M3 UM (*green*) as compared with the D3 UM (*red*).

described above. After 30 minutes, samples were washed and resuspended in FACS buffer. Cell sorting was performed using the FACS Aria III (BD Biosciences). Following sorting, cells were collected in RPMI medium +10% FCS and plated into 25-cm^2 flasks.

Tumor Transendothelial Migration Assay

To mimic the metastatic process of extravasation, a trans-endothelial migration assay was performed as previously described.[21] First, HUVECs were harvested and counted: 30,000 cells were plated onto each 0.8-µm transwell insert in a 24-well plate and media changed daily for 3 days. When a confluent monolayer had formed, Mel270 cells were added onto the HUVEC cell layer at a density of 40,000 cells per well in RPMI containing 1% serum. The bottom of the chamber contained RPMI + 10% FCS. The plate was incubated at 37°C for 48 hours, after which the cell-dissociation and calcein-AM solution was added for 1 hour. Fluorescence was measured in a plate reader at a wavelength of 485 (excitation) and 520 nm (emission).

Statistical Analysis

Student's *t*-test or Mann–Whitney test was used to examine linear variables where data did or did not fit a normal distribution, respectively. When the Mann-Whitney test was used to compare the medians between two groups (PUM and NCM), a Bonferroni correction was applied and a value of $P \leq 0.008$ was considered statistically significant. In all other cases $P \leq 0.05$ was considered statistically significant. The difference in proportion for marker expression in the UM cell lines was assessed by *z*-statistics. Survival analysis was performed using the Kaplan–Meier test. All analyses were performed using statistical software (SPSS version 24.0; SPSS Science, Chicago, IL, USA).

RESULTS

Details of UM cell lines used in the study are given in Supplementary Table S1. Cells isolated from 10 PUM were used for flow cytometry and a further 15 PUM for MS assays. The details of the PUMs are provided in Supplementary Table S2. Demographics of the four human NCM donors and the cause of death are included in Supplementary Table S3.

Monosomy 3 UM Have Higher Colony Forming Efficiency Than Disomy 3 UM

The 15 UM samples used for MS assays were tested for chromosomal alterations by MLPA and classified as either being at a high or low risk of developing metastasis, according to chromosome 3 status, as previously described.[20] When tested for their MS-forming efficiency (MSFE), all M3 UM were able to form MS (median 0.05%, range: 0.02%–0.14%). In contrast, D3 UM were not able to form MS in 4/7 samples tested (median 0%, range 0%–0.07%; Fig. 1).

CD166 and Nestin Are Upregulated in PUMs Compared to NCMs

Short-term cultures of both NCMs and PUMs investigated by flow cytometry found stem cell markers CD166 and Nestin to be elevated in PUMs compared to NCMs. CD166 expression in the cultured PUM was 4-fold greater (mean 78%, median 78%, range: 54%–100%) than its expression in the NCM (mean 19%, median 16%, range 4%–41%; Supplementary Table S4). This difference was statistically significant ($P = 0.0003$; Mann-Whitney). The mean Nestin expression in PUM (mean 33%, median 19%, range: 0.04%–99%) was 1.6-fold higher when compared to the mean expression level in NCM (mean 20%, median 17%, range: 5%–42%). However, increased Nestin expression ($P = 0.12$) 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 significantly different between PUM and NCM, using a Mann-Whitney test (Fig. 2).

There were five PUMs with features of high metastatic risk (M3). When the expression of CSC markers was compared between M3 and D3 UM, the M3 UM had elevated expression of CD271 and Nestin when compared to D3 tumors; however, none of the CSC markers examined reached statistical significance (Mann-Whitney test; Supplementary Table S3).

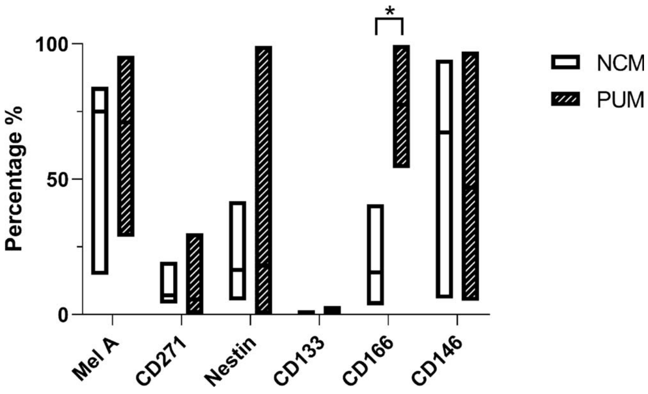

FIGURE 2. Expression of CSC markers in the NCM ($n = 4$) vs. PUM ($n = 10$) cells. The *bars* represent the min-max percentage expression, while the line is drawn at the median expression level of the markers. Statistically significant difference is shown with a star ($P = 0.0003$; Mann-Whitney).

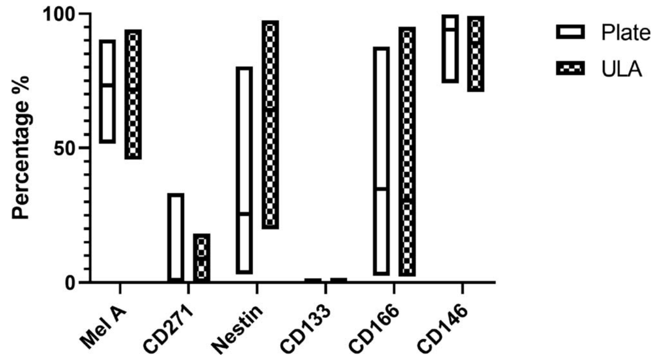

FIGURE 3. Expression of CSC markers across eight UM cell lines cultured in adherent (plate) and nonadherent (ULA) conditions. The *bars* represent the min-max percentage expression, while the line is drawn at the median expression level of the markers. The experiment was repeated three times ($n = 3$).

CSC Markers Are Upregulated in UM Cells During Anoikis Resistance

Eight UM cell lines were also examined for CSC markers by flow cytometry under adherent and nonadherent culture conditions. These were PUM derived (92.1, Mel 270, MP41, MP46), and MUM derived (OMM1, Omm2.3, Omm2.5, MM66). Melan-A was expressed in at least half of all the cells in both adherent and nonadherent culture (median 73% vs. 72%). The expression of CD271 was low (median 1%, range 0.03%–33%) in all cell lines grown as adherent cultures but increased expression (median 9%, range 0.04%–18%) was noted in the cells resistant to anoikis. These changes were statistically significant ($P < 0.01$, z-statistic) in MP46, OMM1, and MM66. The median expression of Nestin was 26% (range: 3%–80%) in the adherent cultures. This increased in the cells that survived anoikis (median 64%, range: 20%–98%; Fig. 3). Upregulation of Nestin expression was observed in 7/8 of the cell lines examined. This difference was statistically significant ($P < 0.01$, z-statistic) in the 92.1, MP41, MP46, OMM2.3, and OMM 2.5 cell lines (Supplementary Table S5).

CD133 was expressed in <1% of UM cells in both adherent (median 0.3%, range: 0.06%–0.6%) and nonadherent cultures (median 0.4%, range: 0.2%–0.8%). CD146 was expressed in >70% of the cells examined in both culture conditions. CD166 expression was variable in the cell lines examined both in adherent (median 35%, range: 3%–88%) and nonadherent culture (median 31%, range: 2%–95%; Fig. 3). Three MUM cell lines and one PUM, however, upregulated their mean expression of CD166 during anoikis resistance as compared with cells in adherent culture; OMM1 (88%–95%), OMM2.3 (4%–7%), OMM2.5 (18%–26%), and MP41 (3%–18%). Analyses by z-statistics showed that these changes were statistically significant only for MP41 cells ($P < 0.01$; Supplementary Table S5).

CD166, Nestin and CD271 Gene Expression in PUM Analyzed by TCGA

Data regarding mRNA expression of Nestin, CD271, and CD166 were downloaded from GDC The Cancer Genome Atlas (TCGA) database, which contains genetic information and clinical/survival data of 80 well characterized PUM patients followed-up for at least 5 years.[22] Data were analyzed in the Xena Browser and the results compared with chromosome 3 copy number variations, *BAP1* mutations, and patient outcome (Fig. 4).

Median expression levels of the genes (white color) were used as the cutoff points and upregulation was displayed in red while downregulation was displayed in blue in the heat maps. The median expression levels (unit log2[fpkm-uq+1]) of the genes were: *BAP1* (19.5), *ALCAM* (14.2), *Nestin* (19.0) and *CD271/NGRF* (13.2). UM with M3 and decreased *BAP1* gene expression compared to the median were associated with upregulation of *CD166/ALCAM* expression. Expression of *Nestin* and *CD271/NGRF* was more variable across the 80 samples.

Kaplan-Meier plots (Fig. 5) were created using the median expression levels as the cutoff and differences were analyzed by Log rank tests. Survival probability was calculated based on event (death from metastatic UM) and time to event (time in years) as parameters. Three patients were censored during this analysis; one who died from pancreatic cancer and two from unknown causes. UM expressing *CD166/ALCAM* above the median were associated with a worse prognosis than those with *CD166/ALCAM* expression below the median ($P = 0.03$, Log rank). Expression of *Nestin* ($P = 0.59$) and *CD271/NGRF* ($P = 0.91$) above and below the median had no statistically significant association with patient survival. Based on data from both the flow cytometry analyses and gene expression data, the functional role of CD166/ALCAM was investigated further.

CD166^{high} Subpopulation Has Higher Tumor Transendothelial Migration Potential Than CD166^{low}

The influence of CD166 to aid migration and metastasis was investigated using a transendothelial assay and CD166^{high} and CD166^{low} Mel270 cells isolated by FACS (Fig. 6A). These were plated separately on HUVEC cells under chemoattractant/serum gradient conditions. After 48 hours, cells with CD166^{high} expression migrated across the HUVEC monolayer significantly more than those with CD166^{low} expression ($P = 0.02$, Mann-Whitney; Figs. 6B).

CD166 Is Expressed in PUM Tissue by IHC Analysis

FFPE sections of nine enucleated PUM were examined for CD166 protein expression by IHC. These were the same tumor samples used flow cytometry analysis.

Normal pancreas (positive controls) expressed CD166 on the membrane of cells in the islets of Langerhans (Fig. 7A). In

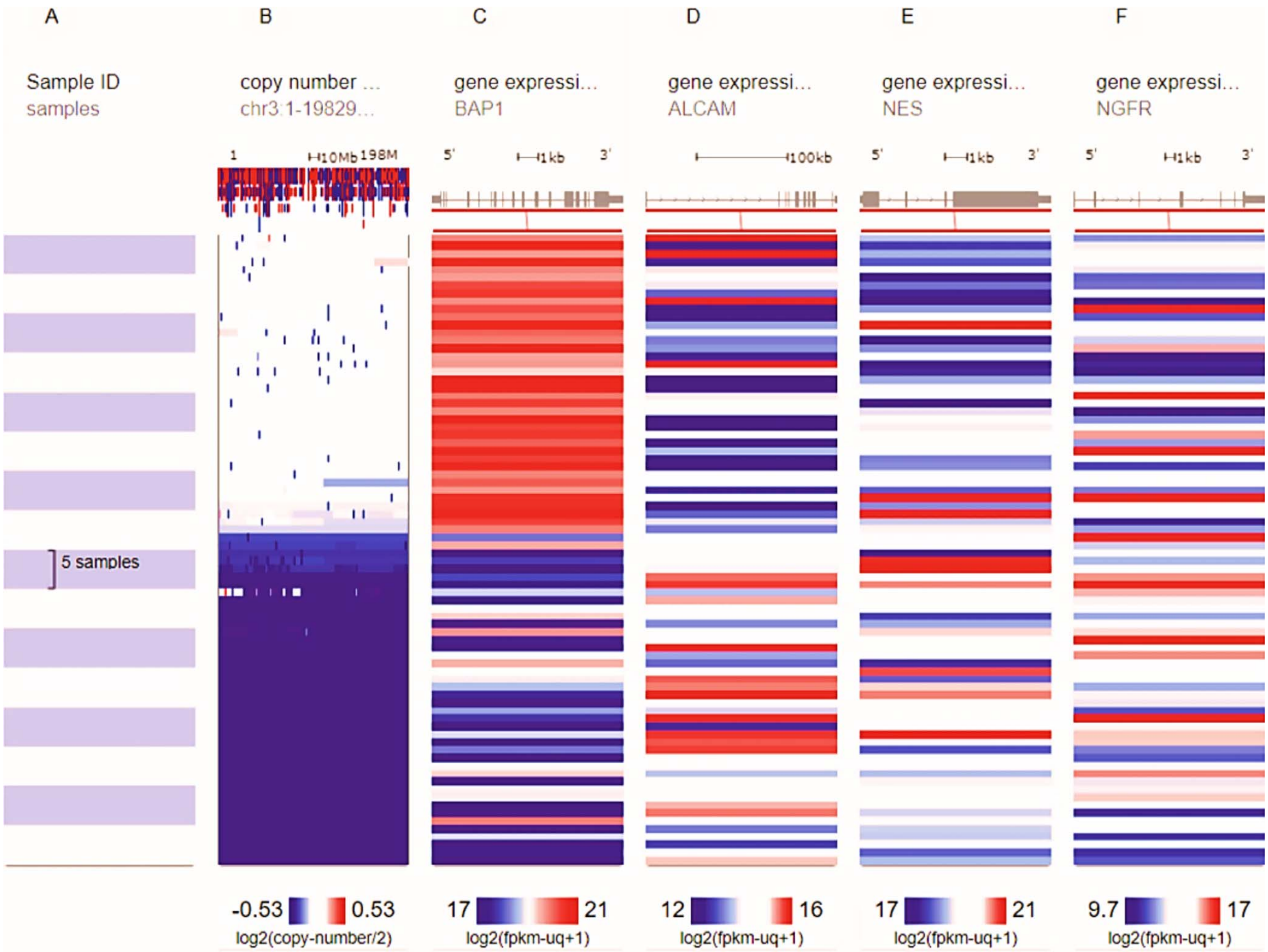

Figure 4. Heat map showing association between CD166/ALCAM, Nestin and CD271/NGFR expression to BAP1 and Chromosome 3 loss. The patient samples are arranged in rows under column (**A**), chromosome 3 status in column (**B**), BAP1 in column (**C**), CD166/ALCAM in column (**D**), Nestin in column (**E**) and CD271/NGFR in column (**F**). *Blue color* shows downregulation, *red* shows upregulation and *white* shows median expression of a gene on a log scale. Higher CD166/ALCAM expression was more abundant in M3 UM with BAP1 mutations while Nestin and CD271 expression was variable across the 80 PUM samples.

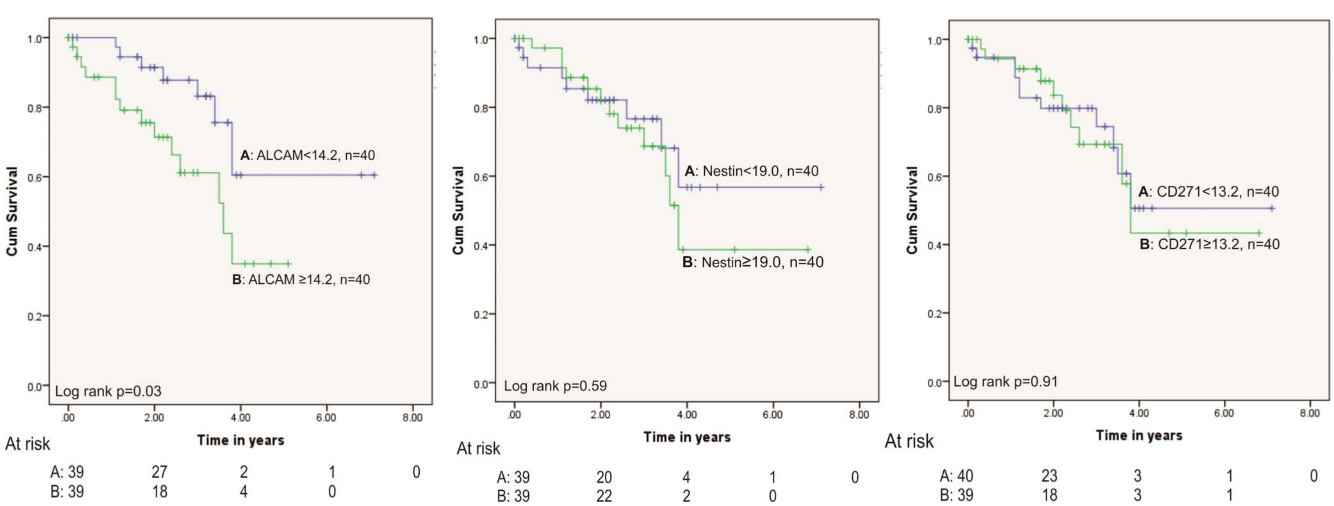

Figure 5. Kaplan-Meier survival plots showing that (*Left*) CD166/ALCAM expression is significantly associated with survival, ($P = 0.03$, Log rank). The gene expression levels of Nestin (*Middle*) and CD271/NGRF (*Right*) showed no significant association with patient survival.

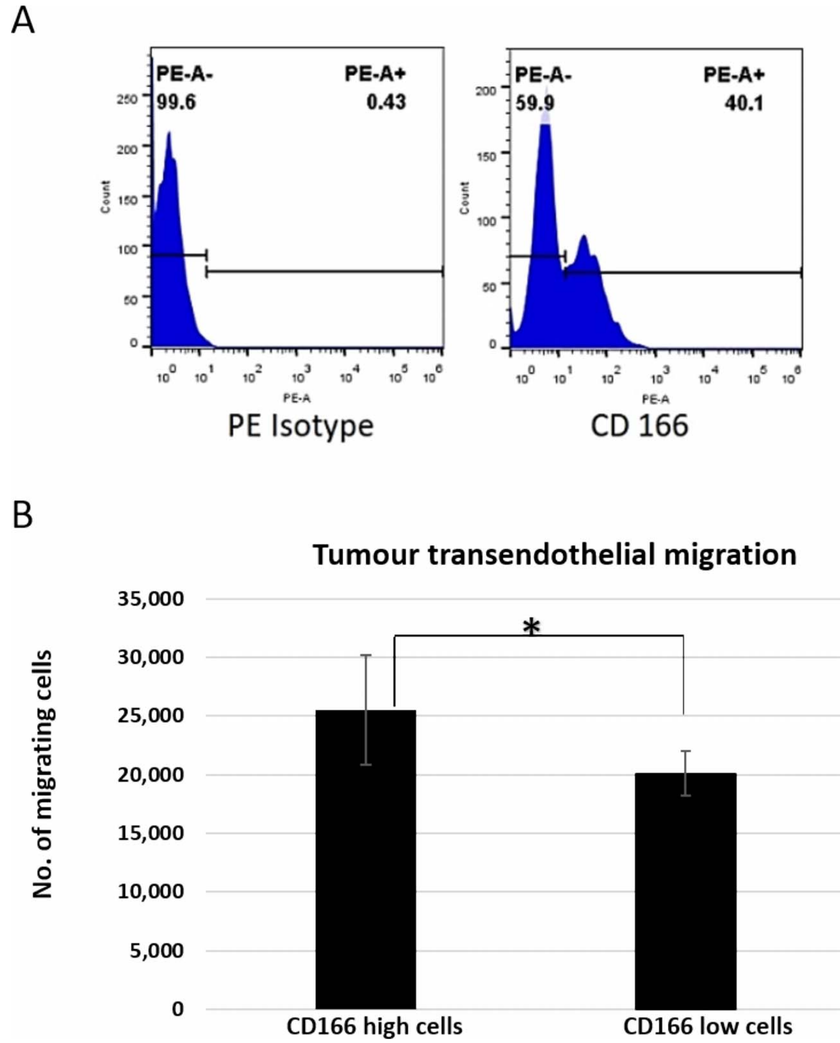

FIGURE 6. (**A**) Flow cytometry profile of Mel 270 cell line. The expression level of the markers (%) is shown on top of the histograms. Isotype controls were used to gate for the negative populations. The cells positive for CD166 (40.1%) are shown. (**B**) *Bar graph* representing the number of Mel270 tumor cells migrating across the HUVEC layer. Experiments were repeated twice and mean ± SD is shown. A Mann-Whitney test shows that extravasation was significantly ($^*P = 0.02$) higher in CD166^{high} compared to CD166^{low} cells.

the tumor sections, endothelial cells and tumor-associated macrophages (TAM) consistently expressed CD166 (Fig. 7B, Supplementary Fig. S1).

CD166 expression was seen in both the cytoplasm and the membrane of PUM cells. However, this was clear in only 2/9 samples examined. These two samples were from a D3, and an M3, PUM. The M3 tumor had few (<20%) CD166 expressing PUM cells (cytoplasmic and membranous) scattered throughout the sample. In the D3 sample, staining was only found in the anterior portion of the tumor (Fig. 7C). In this region, 70% of the cells stained positive for CD166 (Figs. 7D–F), while PUM cells in the rest of the tumor were negative. It was difficult to determine cytoplasmic or membranous staining in the heavily pigmented or macrophage dense tumor sections (4/9). The NCMs expressed CD166 on their cell membrane in 2/9 cases examined.

DISCUSSION

In this study, we have shown that poor prognosis (M3) UM are able to form more melanospheres from single cells than D3 UM, indirectly suggesting an increased presence of CSCs. Moreover, flow cytometry demonstrated significantly increased expression of CD166 in PUM compared with NCM, with a trend toward elevated expression for Nestin and CD271. It is of interest that none of the markers examined were significantly increased in M3 as compared with D3 UM, although elevated CD166 mRNA was associated with M3, decreased BAP1 mRNA and reduced survival time, suggesting that CD166 has an important role in the pathogenesis and progression of UM.

A higher overall expression of CD166 in PUM as compared to NCM is consistent with studies of skin melanoma where primary tumors expressed more CD166 than benign lesions by IHC.[6,23] Nestin expression was also higher in the PUM compared to the NCM, consistent with our previous findings.[24] Our data for CD146 support the findings of Lai et al.[25] who examined expression of CD146 in the uvea. They reported that CD146 is expressed in the NCM, FFPE tumor 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.

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.[26] The different causes of anoikis resistance in cancer cells include genetic instability,

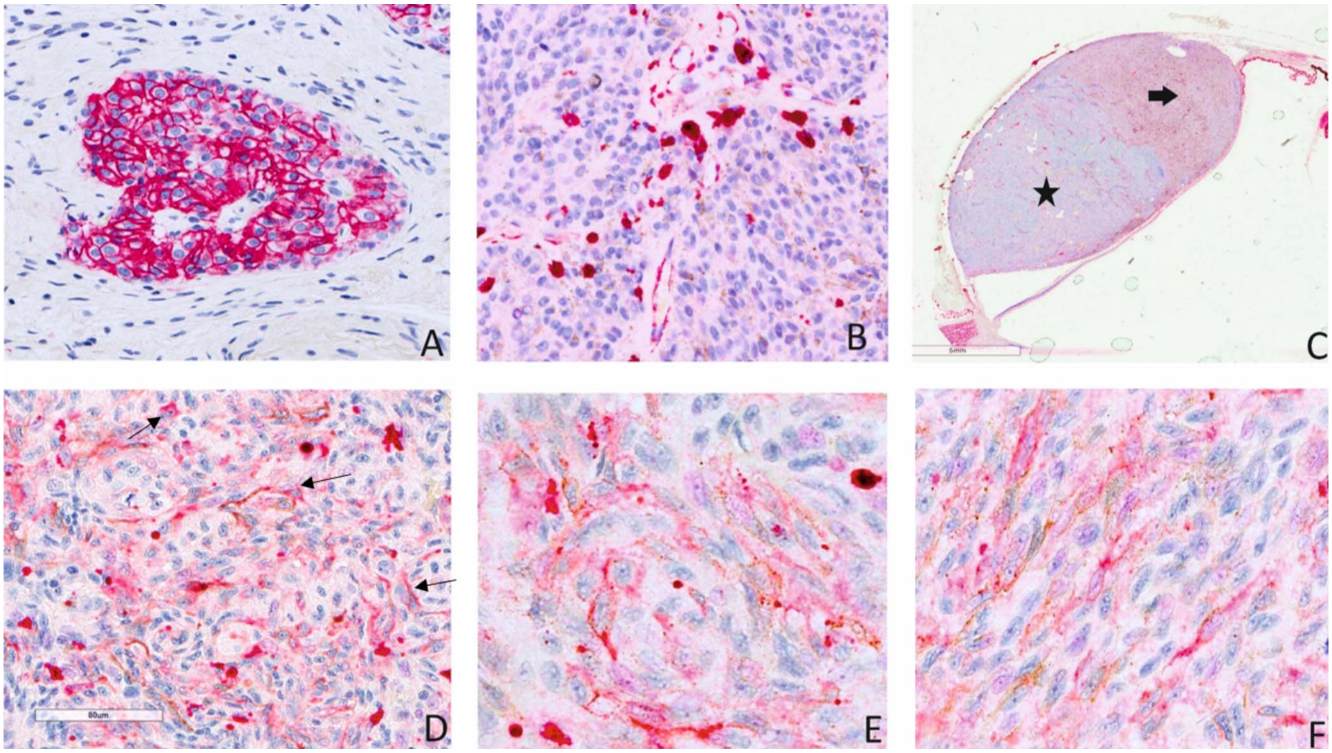

FIGURE 7. (**A**) Positive control for CD166 stain in the pancreas. (**B**) Internal controls in PUM tissue showing positively stained macrophages and endothelial cells. (**C**) PUM tissue from an enucleated eye with positive staining for CD166 in the anterior one-third (*bold black arrow*) and negative staining in the posterior two-thirds (*star*). (**D**) Low magnification of the anterior region (*bold black arrow*) showing membranous and cytoplasmic staining of PUM cells (*small black arrows*). (**E**, **F**) Higher magnification of CD166 expressing PUM cells.

epithelial-mesenchymal transition, and overexpression of stemness markers.[27] CSCs in several cancers including breast have been shown to be resistant to anoikis, form spheres in nonadherent culture and have enhanced tumor growth capacity.[28]

UM cells surviving anoikis showed an increased expression of several markers previously associated with neural crest development and stem cells: CD271, Nestin, and CD166. CD271 has been shown to mark the CSC population in skin melanoma. These cells were able to form tumors in xenograft models that resembled the parent tumor.[4,29] In UM, CD271 was also expressed in the cells that formed vasculogenic mimicry patterns, a poor prognostic feature likely to cause metastasis.[30] Increased Nestin expression in PUM is associated with poor prognostic parameters including M3 and chromosome 8q gain.[24] These results suggest that PUM cells surviving anoikis may be enriched by the CSC population, as evidenced by their increased expression of stemness markers.

CD166/ALCAM, a cell adhesion molecule has been reported to mark mesenchymal stem cells,[31] hematopoietic progenitor cells,[32] and CSCs in prostate[33] and colon cancer,[34] glioblastoma,[35] and skin melanoma.[6] Studies in skin melanoma have described its role in controlling the transition from local cell proliferation to tissue invasion.[36,37] This is consistent with increased migration observed in the CD166^{high} Mel270 cells as compared to CD166^{low} cells. A previous study has shown that silencing CD166 by shRNA knockdown in a high-ALCAM expressing UM cell line (MUM-2B), resulted in reduced motility in gap-closure assays and a reduction in invasiveness as measured by a transwell migration assay.[38] This suggests CD166 may play a role in UM tumor cell motility, migration, and invasion. Indeed, analysis of TCGA data demonstrated that increased *CD166/ALCAM* gene expression was significantly associated with metastasis and a reduced overall survival.

In PUM sections, CD166/ALCAM expression was abundant in the cytoplasm of TAMs. These results support studies which showed that CD166/ALCAM is expressed in macrophages of arthritic patients in response to cytokine release.[39] Tumor endothelial cells also expressed CD166/ALCAM, which has been reported to be involved in early embryonic hematopoiesis and vasculoangiogenesis.[32] CD166 expression has been documented in the retina, stromal cells, and melanocytes of mice eyes, where it is proposed to play a role in the development, structure, and function of these cells.[40] Expression of CD166/ALCAM in melanoma cells analyzed by IHC was not as abundant as demonstrated by flow cytometry. The presence of CD166 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 tumor cells.

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.

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