Nestin expression in primary and metastatic uveal melanoma – possible biomarker for high-risk uveal melanoma

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Abstract

Purpose: Nestin, a member of the intermediate filament protein family, has been described as a putative cancer stem cell marker (CSC) in uveal melanoma and poor prognostic factor in a variety of tumours, including cutaneous melanoma. In this study, we examined the expression of nestin in primary (PUM) and metastatic uveal melanoma (MUM) samples, and correlated the findings with histological, clinical and survival data.

Methods: Nestin expression was assessed by immunohistochemistry in 141 PUM and 26 MUM samples; 11 PUM cases were matched with their corresponding metastases. The percentage of tumour cells expressing nestin was scored by three independent observers. Statistical analysis of all data was performed with SPSS.

Results: Nestin expression was identified in both the cytoplasm and membrane of UM cells. Increased expression of nestin in PUM samples was associated with known poor prognostic parameters, including epithelioid cell morphology ($p<0.001$), closed loops ($p=0.001$), higher mitotic count ($p<0.001$), monosomy 3 ($p=0.007$) and chromosome 8q gain ($p<0.001$). PUM with nestin expression levels above a cut-off value of 10% (as determined by ROC analysis) were associated with a significantly reduced survival time (Log rank, $p=0.002$). In MUM, a higher percentage of nestin positive tumour cells combined with poor prognostic markers in the PUM led to a shorter survival time following the development of metastases.

Conclusion: In conclusion, increased nestin expression in PUM is a predictor of a tumour phenotype associated with metastatic progression and reduced survival time at onset of metastasis.

Key words: Nestin, uveal melanoma, cancer stem cell, metastatic uveal melanoma

Introduction

Cancer stem cells (CSC) are a small subpopulation of tumour cells with the capacity to self-renew and produce the cells that comprise the bulk of the tumour (Al-Hajj et al. 2003; Kalirai et al. 2011). It is thought that these cells are also responsible for tumour progression (i.e. invasion, metastasis, colonization) and resistance to therapy (Schatton et al. 2009). Studies have attempted to isolate and characterise these cells based on putative stem cell markers, and according to their *in vitro* and *in vivo* characteristics. Putative stem cell markers include CD15, CD24, CD44, CD133, CXCR4, NCAM, and ABC transporters (Sancho et al. 2016). Cytoplasmic proteins, like nestin, Musashi-1, and aldehyde dehydrogenase, or nuclear proteins Sox-2, Oct3/4, and Nanog, are also used to identify CSC (Neradil & Veselska 2015).

Nestin, a member of the intermediate filament (IF) class VI protein family, was first discovered in the developing nervous system of mice (Lendahl et al. 1990). It was observed to be a marker for neural stem and progenitor cells, thus it was named nestin: **ne**ural **st**em cell prote**in**. Its functions include promoting the disassembly of phosphorylated vimentin IF during mitosis, and the trafficking and distribution of IF proteins and other cellular factors to daughter cells during progenitor cell division. Nestin was recently classified as a CSC marker found in various tumours of neuroectodermal or mesenchymal origin arising in the brain, oropharynx, pancreas, kidney and muscle (Krupkova et al. 2010). Furthermore, it has been proposed that nestin expression correlates with a poor prognosis in malignancies, such as glioblastoma, lung and renal carcinoma, sarcoma and skin melanoma (Piras et al. 2010; Cros et al. 2016; Guadagno et al. 2016; Li et al. 2016; Zambo et al. 2016). In the latter, nestin expression was associated with more advanced tumour stages and predicted poor survival (Brychtova et al. 2007; Piras et al. 2010).

Uveal melanoma (UM), the most common intraocular tumour in adults, arises from the transformation of neural crest derived choroidal melanocytes. To date, there are only limited studies examining the expression of nestin in UM. Thill and colleagues used the putative CSC marker CD133 to isolate a subpopulation of CD133 positive/nestin positive cells in a panel of UM cell lines (Thill et al. 2011). In the peripheral blood of patients with metastatic UM (MUM), nestin positive circulating tumour cells (CTCs) were identified that also correlated with nestin positive tumour cells in the corresponding MUM tissue (Fusi et al. 2011)

In this study we examined nestin expression by immunohistochemistry in a large well-phenotyped cohort of primary (PUM) and MUM, correlating the results with their clinical, histomorphological and genetic features, and ultimate patient outcome.

Materials and methods

Patient samples

One hundred and forty-one PUM and 26 secondary hepatic metastases (MUM) were obtained from the Liverpool Ocular Oncology Biobank and the Liverpool Bioinnovation Hub Biobank, respectively. The study was approved by the Health Research Authority (REC Ref 11/NW/0759), and conducted in accordance with the Declaration of Helsinki. Full clinical, histopathological and follow-up data were available for all samples.

Immunohistochemistry (IHC)

Immunohistochemical staining was performed on 4 µm thick sections cut from formalin-fixed, paraffin embedded (FFPE) tissue blocks. A PT-link machine (Dako, Glostrup, Denmark) was used for dewaxing and heat-induced epitope retrieval; slides were incubated in a high-pH bath containing EnVision™ FLEX target retrieval solution (Tris/EDTA buffer pH9.0) (Dako) at 96◦C for 20 mins, then stained with a primary mouse anti-Nestin (ab18102) monoclonal antibody on an autostainer (Dako UK Ltd, Ely, Cambridgeshire, UK) as detailed below using the FLEX EnVision™ kit (Dako). After being washed with FLEX wash buffer, samples were blocked with an EnVision™ FLEX peroxidase block for 5 mins and incubated with a 1:200 dilution of primary monoclonal antibody (5 µg/µl) for 30 mins. Before being washed again, samples were incubated with a mouse linker (Dako) for 15 mins. Bound antibody was then detected with horseradish peroxidase (HRP) for 20 mins and visualised with a Vector AEC Substrate Kit (Vector Laboratories Ltd, Peterborough, UK) for 30 mins. Sections were counterstained with Mayer's haematoxylin (VWR International Ltd, Lutterworth, Leicestershire, UK), blued with Scott's tap water (Leica Microsystems, Linford Wood, Milton Keynes, UK) and mounted with Aquatex™ aqueous mountant (Merck Chemicals Ltd, Nottingham, UK). Normal colon sections were used as controls and positive

staining in these demonstrated a valid IHC run. Omission of primary antibody was used as a negative control. Pictures were taken using an Olympus BX51 microscope.

IHC Scoring

The slides were scored according to the percentage of positively stained tumour cells by 3 independent observers (SC, HK, LD). The percentage of positive tumour cells was used to grade the staining. Scores were given to each sample on a scale of 0-100%. The presence of positively stained capillary endothelium in the tissue sections was also assessed: for this, the sample was graded 'Yes' or 'No' accordingly.

Statistics

Statistical evaluation was undertaken for all the samples. The values shown are the mean ± SD. Survival correlation was done using the Kaplan-Meier test. The Pearson's χ^2 square test and Fisher's exact test were used for categorical variables. A student T test or Mann Whitney test were used for linear variables. A Receiver Operating curve (ROC) analysis was used to determine the threshold for nestin positivity. Two sided $p<0.05$ was considered to be statistically significant. All analyses were performed using SPSS software (ver.24.0; SPSS Science, Chicago, IL, USA).

Results

Clinicopathological characterisation

In this study, a total of 141 PUM were included: 82 were from male and 59 from female patients with a median age at diagnosis of 63 years (range, 20-87) (Table 1). The median follow-up was 33.2 months (range 5-384). Primary treatment was enucleation (n=111), local tumour resection (n=15), proton beam irradiation (n=10), and ruthenium plaque (n=5). At the end of our study (March 6, 2017), 105/141 (74%) of these patients were alive, 28 (20%) had died of metastatic melanoma, and 8 (6%) died of other causes. The median largest basal diameter (LBD) of the samples was 15.2mm (range 3.3-23.6), and the median tumour height as measured by ultrasound (UH) was 8.3mm (range 1.4-16.7). Ciliary body involvement was present in 51 UM (36%), and epithelioid cell morphology was recorded in 87 tumours (62%); 16 cases had extraocular extension. 89 cases were classified as monosomy 3, while a gain of chromosome 8q was seen in 81 cases. (Table 1)

The MUM included 14 liver resections and 12 fine needle biopsies. These were from 16 male and 10 female patients. Their median age at diagnosis was 63 years (range 39-78). Data from the primary tumour had classified 22 of these as monosomy 3, two as disomy 3, whilst for a further two cases the status of chromosome 3 was indeterminate. In 11 MUM the predominant cell type was spindle while the remaining 15 were predominantly epithelioid. The median time from primary tumour diagnosis to the development of metastasis was two years (range 1-8 years). By the end of the study, four patients with MUM were alive while 21 had died of their disease, and one patient was lost to follow up. The median survival time following detection of metastatic disease was 12 months (range 0.5-48).

11 MUM had matched PUM samples from enucleation. These PUM were from 7 male and 4 female patients with a median age of 59 years (range 47-75). The median LBD was 17.9mm (range 11.7-21.1) and median UH 9.0mm (range 1.8-14.7). The median mitotic count was 4

per 40 high-power field (40x objective) (range 2-38). Seven patients had epithelioid cell morphology in both the PUM and MUM, 10 had closed connective tissue loops, six had ciliary body involvement and only one had an extra ocular extension of the PUM. Ten PUM were monosomy 3 and of these eight also had an 8q gain. The eleventh patient had a normal chromosome 3 status and a gain in 8q.At the end of the study, 9/11 patients had died of MUM while two patients were still alive. These two patients had spindle cell morphology in their MUM and had been diagnosed of the PUM just a year prior to the onset of MUM; hence, the follow up time is short.

Nestin expression in PUM

In the non-tumorous areas of the enucleated eyes, nestin positivity was observed in the cells of the neuroretina; the inner and outer nuclear layer, the ganglion cells and optic nerve fibre layer (Fig. 1A). The blood vessels of the choroid and retina also expressed nestin. These structures acted as internal positive controls for the nestin IHC. The retinal pigment epithelium and the normal choroidal melanocytes were negative for nestin expression (Fig. 1B).

For the interpretation of nestin expression in the PUM, Melan A stained slides of the tissue sections were retrieved from the pathology archives, to identify the UM cells across the tissue sections. Whilst staining for Melan A was homogenous throughout the cohort, nestin staining was variable. Nestin expression was localised to the membrane and cytoplasm of the UM cells. In some tissue sections nestin expression was observed in the endothelium of the intratumoural blood vessels (Fig.1C). A ROC analysis of the nestin expression data demonstrated that the highest levels of sensitivity and specificity occurred between 8.5% and 12.5% (0.857/0.584 and 0.679/0.478 respectively). A 10% threshold was therefore used such that UM samples with <10% nestin expression (Fig. 1D) were considered negative for this

marker. This threshold is in accordance to previous studies examining nestin (Piras et al. 2010). Of the PUM examined 52/141 (36%) were evaluated as negative for nestin expression.

63 PUM (44%) showed nestin expression of varying intensity, in 10-50% of the tumour cells (Fig. 2A, B). The remaining 26 PUM (18%) demonstrated nestin expression in 50-100% of UM cells. In these tumours, there was moderate to strong expression of nestin, both in the cytoplasm and membrane of cells (Fig. 2C, D). A total of 36 PUM (25%) also showed positive staining in the endothelium of the intratumoural blood vessels.

Nestin positivity (>10%) significantly correlated with known poor prognostic factors in PUM, such as epithelioid morphology (Pearson's Chi square p-), high mitotic count (Mann Whitney p-), 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-). There was no significant correlation between nestin expression in the capillary endothelium and any prognostic factor.

Kaplan-Meier analysis showed that patients with PUM classified as negative for nestin expression had a better prognosis than those patients with nestin expression (Fig. 3; Log rank p=0.002).

Nestin expression in MUM

Similar to the protocol described for PUM, Melan A-stained slides of the MUM were used as a reference to identify the tumour cells and show the antigenicity of the tissue. The MUM showed positive nestin staining in the MUM cells and associated blood vessels while the adjacent hepatocytes and stellate cells were consistently negative. Twenty-one of the 26 MUM (80%) showed a high proportion of nestin positive tumour cells (Fig.4A). In particular, 17 MUM (65%) expressed nestin in the UM cells located within closed connective tissue

loops (Fig.4B). Two (7%) of the 26 MUM had very few nestin expressing tumour cells (Fig. 4C), and three others (11%) were completely negative for nestin staining. Nine MUM samples showed a high proportion of nestin positive melanoma cells in the tissue as well as nestin expression in the intratumoral capillary endothelium (Fig.4D). Nestin also stained scattered melanoma cells that were away from the bulk of the tumour.

Of the 11 matched samples, all had nestin expression in the cytoplasm of the melanoma cells in the PUM tissue sections (Fig 5A). Five of these PUM samples also showed nestin staining in the intratumoural capillary endothelium. The MUM showed a varied expression of nestin: four MUM (36%) had a high proportion of nestin expressing tumour cells (>50%) (Fig.5A), three (27%) had a medium proportion of nestin positive UM cells (10-50%), in two (18%) other samples, only scattered large ovoid MUM cells stained positive for nestin (Fig. 5B). In the remaining two sample (18%), only the intratumoural capillary endothelium stained positive for nestin.

Of the four MUM samples with a high proportion of cytoplasmic nestin expressing cells, one patient died two weeks after the diagnosis of metastatic melanoma while two others within one month of diagnosis. The fourth patient is still alive; this case showed nuclear BAP1 expression in the PUM, a normal chromosome 3 status and chromosome 8q gain.

Discussion.

In this study, we have examined in detail the putative CSC marker nestin in PUM and MUM. Our results show that high levels of nestin expression are associated with a reduced survival time in UM, significantly correlating 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.

These poor prognostic parameters are also associated with so-called high metastatic risk "class 2" UM, as determined using gene expression profiling (Onken et al. 2004). Chang and colleagues reported that class 2 UM are strongly associated with genes upregulated in primitive neuroectodermal cells (Chang et al. 2008). Nestin, a type VI intermediate filament protein, is associated with migrating and proliferating neuroectodermal cells during embryogenesis, and in adult tissues may identify primitive multipotent cells with regenerative capacity that can be re-activated during injury (Krupkova et al. 2010). Of interest in our current study was the absence of nestin positivity in normal choroidal melanocytes as compared with its presence in UM cells, which may suggest reversion to a more primitive phenotype following UM cell transformation. Indeed, recent studies in several tumours including cutaneous melanoma, squamous cell carcinoma, basal cell carcinoma, osteosarcomas and gliomas, (Singh et al. 2003; Veselska et al. 2008; Sabet et al. 2014) have associated increased nestin expression with an immature and invasive cell phenotype that may represent tumour cells with stem cell-like characteristics. In support of this, nestin mRNA expression was identified in a panel of genes associated with time to metastasis both from the data generated by The Cancer Genome Atlas and also from their analysis of gene expression data previously generated by Laurent et al. ; although this association was not found to be significant when examining the Hazard Ratio and 95% CI. Furthermore, unpublished gene expression data generated by our group comparing eight monosomy 3 UM

patients who died from metastatic disease with eight disomy 3 UM patients who are still alive without metastatic disease, identified a 2.8 fold increase in mRNA expression levels of nestin in M3 UM as compared with D3 UM.

The presence of putative CSC with self-renewal capacity and that were resistant to chemotherapy was previously reported in UM cell lines (Kalirai et al. 2011). This was followed by work demonstrating the presence of CD133/nestin positive cells in eight UM cell lines by Thill and colleagues (Thill et al. 2011). CD133 has been used to identify CSC in other tumours including those of the skin, colon, brain, and lung (Saigusa et al. 2010; Tamura et al. 2010; Sabet et al. 2014).

Nestin is not only expressed in the tissue of melanoma patients, but also in their CTC. An analysis of the blood obtained from both cutaneous and UM patients revealed nestin expressing cells, which were not present in healthy volunteers. Nestin was higher in cutaneous melanoma patients with stage IV disease compared to stage III/II patients (Fusi et al. 2010). This strongly suggests that nestin could be used as a possible biomarker for early detection of metastatic disease in high-risk UM patients, and that it could be more sensitive than 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 TPS cytokeratin 18 (Barak et al. 2007) and GDF-15 (Suesskind et al. 2012).

The molecular function of nestin has also been investigated in several cancers. Akiyama and colleagues showed that suppression of nestin expression by shRNA in cutaneous melanoma cell lines leads to reduced cell growth, migration and invasion into Matrigel (Akiyama et al. 2013). The spheroid formation ability of these cells was also less than the control. When these cells were injected into mice, they formed smaller tumours, which did not metastasize to the liver (Akiyama et al. 2013). Similar findings were observed in gliomas following nestin

knockdown (Ishiwata et al. 2011) and in pancreatic ductal carcinoma downregulation of nestin inhibited liver metastasis *in vivo* (Matsuda et al. 2012). Further functional studies using UM cell lines are necessary to help us to delineate the role of the putative CSC marker, nestin. In this regard we have undertaken nestin IHC in a panel of six UM cell lines (data not shown) to determine baseline expression of this protein and identified several UM cell lines with high levels of nestin expression (e.g. 92.1, Mel270 and MP41), which may then be used to create isogenic cell lines for this purpose.

In conclusion, our study has shown that nestin positive tumour cells are found in UM patient tissue. A high proportion of nestin expressing cells correlates with poor survival. MUM also express nestin, 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. The primitive neuroectodermal CSCs that may be responsible for metastasis and poor prognosis may be identified using nestin.

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The authors declare that there is no conflict of interest.

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