

LABORATORY SCIENCE

Human uveal melanoma expresses NG2 immunoreactivity

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Background/aims: NG2 is the rat homologue of the human melanoma proteoglycan (HMP), also known as the high molecular weight melanoma associated antigen. Most cutaneous melanomas, as well as glioblastomas, chondrosarcomas, and some leukaemias express NG2 immunoreactivity, recognised using monoclonal antibody (mAb) 9.2.27. This antibody has also been used for molecular targeting in targeted α therapy for melanoma. The purpose of this study was to evaluate the expression of NG2 immunoreactivity in human uveal melanoma and normal ocular tissue using mAb 9.2.27.

Methods: Enucleated eyes from 26 patients with choroidal or ciliary body melanoma (n=26) were available as paraffin sections, and stained with haematoxylin and eosin to assess for tumour cell type and histopathology. Additional slides were investigated for NG2 immunoreactivity using mAb 9.2.27 and alkaline phosphatase anti-alkaline phosphatase (APAAP) immunostaining. Two independent observers graded immunostaining using a semiquantitative scale from 0 (negative) to 3 (strong).

Results: Immunostaining for mAb 9.2.27 could not be graded in 7/26 cases with dense pigmentation of the tumour. For the remaining cases, grade 2 (moderate) or more immunostaining was seen in 18/19 tumours (95%). The retina, retinal pigment epithelium (RPE), and choroid displayed weak immunostaining (grade 0.5–1.5) in the majority of melanoma affected eyes. Normal retina and choroid (n=5) appeared negative for mAb 9.2.27. Optic nerve axon bundles in both control and melanoma affected eyes displayed moderate immunostaining.

Conclusion: In the present study, the majority of human uveal melanomas expressed NG2 immunoreactivity, as detected using mAb 9.2.27. This antibody may be a suitable candidate for radioimmunotherapy to target ocular melanoma.

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Uveal melanoma, affecting iris, ciliary body, and choroid, is the most common primary ocular malignancy in adults, with an average incidence of 8 per 1 000 000. Smaller primary lesions may be controlled with local resection, laser photocoagulation, proton therapy, or plaque radiotherapy. However, these treatments are often associated with sight threatening complications including radiation retinopathy, macula oedema, scarring, and haemorrhaging.¹ Recurrence of the primary tumour after local resection is also commonly observed, with recurrence rates for iris, ciliary body, and choroidal tumours between 11% and 32%.^{2–4} Treatment of recurrent disease usually involves enucleation of the eye.

Even with effective early treatment of the primary tumour, uveal melanoma displays an unpredictable clinical course during which clinically evident metastases may occur after a prolonged disease free interval.¹ Metastatic disease from primary uveal melanoma usually appears in 10–40% of patients within 10 years, mostly in the liver, and with an average survival of 5.4 months following diagnosis.^{5,6} Development of metastatic disease is currently untreatable. Chemotherapy is generally ineffective because of the restricted period of action during the therapeutic cycle. Immunotherapy is still in the experimental stage and its effects remain elusive. However, α radioimmunotherapy is worth consideration as a potential treatment of primary and metastatic ocular melanoma. For example, the radionuclide bismuth-213 (²¹³Bi) ($t_{1/2}$ = 46 minutes), which has been chelated to some monoclonal antibodies, emits an α particle with short range (80 μ m) and high linear energy transfer radiation which is about 100 times greater than for β particles.⁷

mAb 9.2.27 recognises a Mr 250 000 glycoprotein NG2, associated with a chondroitin sulphate proteoglycan^{8,9} and is expressed on most human cutaneous melanomas and some

gliomas.¹⁰ The epitope for antibody binding has high in vivo expression and has been detected on >90% of melanoma cells in cutaneous lesions,¹¹ with high cell surface specificity for human cutaneous melanoma cells. The expression of mAb 9.2.27 has not yet been investigated in human ocular melanoma. mAb 9.2.27 has also been used extensively in vitro and in vivo as a tumour marker for therapeutic purposes,^{12–15} and has also been tested in a limited phase I clinical trial.¹⁶ Our group has recently chelated the α emitting radioisotope ²¹³Bi to mAb 9.2.27 to form an α immunoconjugate (AIC), which is highly specific and cytotoxic to cutaneous melanoma cells in vitro¹⁷ and can completely regress tumour growth in a xenograft mouse model after local injection.¹⁸ Preliminary results from our phase I clinical trial also demonstrate that AIC can target cutaneous melanoma cells after local injection (unpublished data).

As a first step in the selection of a possible cell surface target antigen for AIC therapy of uveal melanoma, we examined 26 cases of pathologically confirmed uveal melanomas for NG2 immunoreactivity, using mAb 9.2.27 immunohistochemistry.

MATERIALS AND METHODS

Specimens

Enucleated eyes from 26 patients with choroidal or ciliary body melanoma were routinely fixed and paraffin embedded (n = 26). The age at enucleation ranged from 41 to 90 years (mean 64.7 years). The tumours were predominantly posterior choroidal (69%), with smaller numbers of equatorial tumours (12%) and mixed anterior choroidal/ciliary body tumours (19%). Following informed consent, control normal eyes from the Lions NSW Eye Bank (n = 5) were also fixed and paraffin embedded. The study protocol was approved by the University

Table 1 Uveal melanomas: cell type and mAb immunostaining*

Specimen	Cell type	Melanoma-IR	Retina-IR	Choroid-IR
1	Spindle B	2.5	1	0.5
2	Mixed	2	0.5	0
3	Spindle B	2.5	0.5	0.5
4	Mixed, mostly spindle	2	1.5	1.5
5	Spindle B	NA	NA	NA
6	Mixed, mostly spindle	2.5	1	1
7	Mixed, mostly spindle	2.5	0.5	1
8	Mixed, mostly spindle	2.5	0.5	0.5
9	Mixed	2.5	1	1
10†	Mixed	NA	NA	NA
11†	Spindle	NA	NA	NA
12†	Spindle	2.5	1	0.5
13	Mixed, mostly epithelioid	NA	NA	NA
14	Mixed	2.5	0	0
15†	Spindle	2	1	0
16	Mixed	2.5	0	0
17†	Spindle	NA	NA	NA
18	Mixed	2.5	0.5	0.5
19	Mixed	NA	NA	NA
20	Mixed	3	1	0.5
21†	Mixed	2.5	0.5	0.5
22	Epithelioid	1.5	0.5	0.5
23	Mixed	2	1.5	1.5
24	Mixed, mostly epithelioid	2	1	0
25†	Mixed	NA	NA	NA
26	Mixed	3	0	0

IR = immunoreactivity; NA = not assessed.

*Semi-quantitative scale for immunostaining: 0 = negative; 1 = weak; 2 = moderate; 3 = strong.

†¹²⁵I plaque therapy.

of Sydney human ethics committee and followed the tenets of the Declaration of Helsinki.

Tissue samples were sectioned (8 µm thick) in a sagittal plane often including the optic nerve head and cornea. Paraffin sections routinely stained with haematoxylin and eosin were assessed for tumour cell type and histopathology.

Antibodies

Mouse anti-human melanoma IgG2a monoclonal antibody (mAb 9.2.27) was used as the primary antibody (kindly provided by the Professor Hersey, Royal Newcastle Hospital, Newcastle, Australia). Rabbit anti-mouse IgG and alkaline phosphatase anti-alkaline phosphatase (APAAP) complex were purchased from Dakopatts (Glostrup, Denmark). Sheep anti-mouse biotinylated IgG was purchased from Amersham Pharmacia Biotech (Bucks, UK). ExtrAvidin peroxidase was purchased from Sigma (MO, USA).

Immunohistochemistry

NG2 immunoreactivity in uveal melanoma sections was detected using the APAAP method.¹⁹ Paraffin embedded sections (8 µm) were incubated for 20 minutes at 60°C, deparaffinised in xylene and graded alcohols, and rehydrated in TRIS buffered saline (TBS, pH 7.5). The optimal primary antibody concentration was determined based on positive labelling of known target cells (cutaneous melanoma cells) and minimal background labelling of stromal tissue. Briefly, sections were incubated in mAb 9.2.27 (25 µg/ml) overnight in a humidified chamber at 4°C, washed three times with TBS and incubated with rabbit anti-mouse IgG (1:100 dilution) at room temperature for 1 hour. Sections were then washed three times in TBS and incubated for 1 hour in APAAP complex (1:100 dilution) at room temperature. After washing three times with TBS, immunoreactivity was detected by the addition of freshly prepared alkaline phosphatase substrate (0.2 mg/ml naphthol AS-MX phosphate containing 0.1 mg/ml fast red TR and levamisole in 0.1M TRIS-HCl (pH 8.2)) for 10 minutes. The sections were counterstained in Harris's haematoxylin for 1 minute and mounted in glycerol gelatin under glass coverslips.

NG2 immunoreactivity was also examined in some uveal melanoma sections using an indirect peroxidase method as follows. Sections were initially deparaffinised through xylene and alcohols then incubated in mAb (9.2.27) for 72 hours at 4°C. After rinsing in TBS, sections were incubated in biotinylated sheep anti-mouse IgG (1:100 dilution) for 1 hour at room temperature, rinsed and incubated in ExtrAvidin peroxidase (1:250 dilution) for 1 hour at room temperature. After rinsing in TBS bound antibody was visualised with Vector NovaRED substrate (Vector Laboratories, CA, USA) substrate. The sections were dehydrated and mounted in DePeX and coverslipped.

Negative controls included omission of the primary antibody or incubation of sections with an irrelevant IgG2a antibody. Positive control sections from cutaneous melanoma were also included.

Assessment of immunostaining

Two independent observers (YL and RMC) assessed immunostained sections of ocular melanomas and control eyes using a Leica light microscope at a final magnification of 400×. mAb 9.2.27 immunostaining was graded for each section using a semi-quantitative scale: 0 = negative, 1 = weak, 2 = moderate, or 3 = strong. An average of the grades for the two observers was taken.

RESULTS

Ocular melanoma histopathology

Uveal melanoma size (maximum diameter (mm) × maximum thickness (mm)) ranged from 5 mm to 24 mm × 1 mm to 14 mm respectively. From haematoxylin and eosin stained sections the mitotic rate was <5 mitoses/high powered field in 58% of cases; extraocular invasion was observed in 50% of cases. Approximately 75% of tumours displayed mixed spindle/epithelioid or epithelioid morphology with ~25% tumours displaying spindle cell morphology (Table 1).

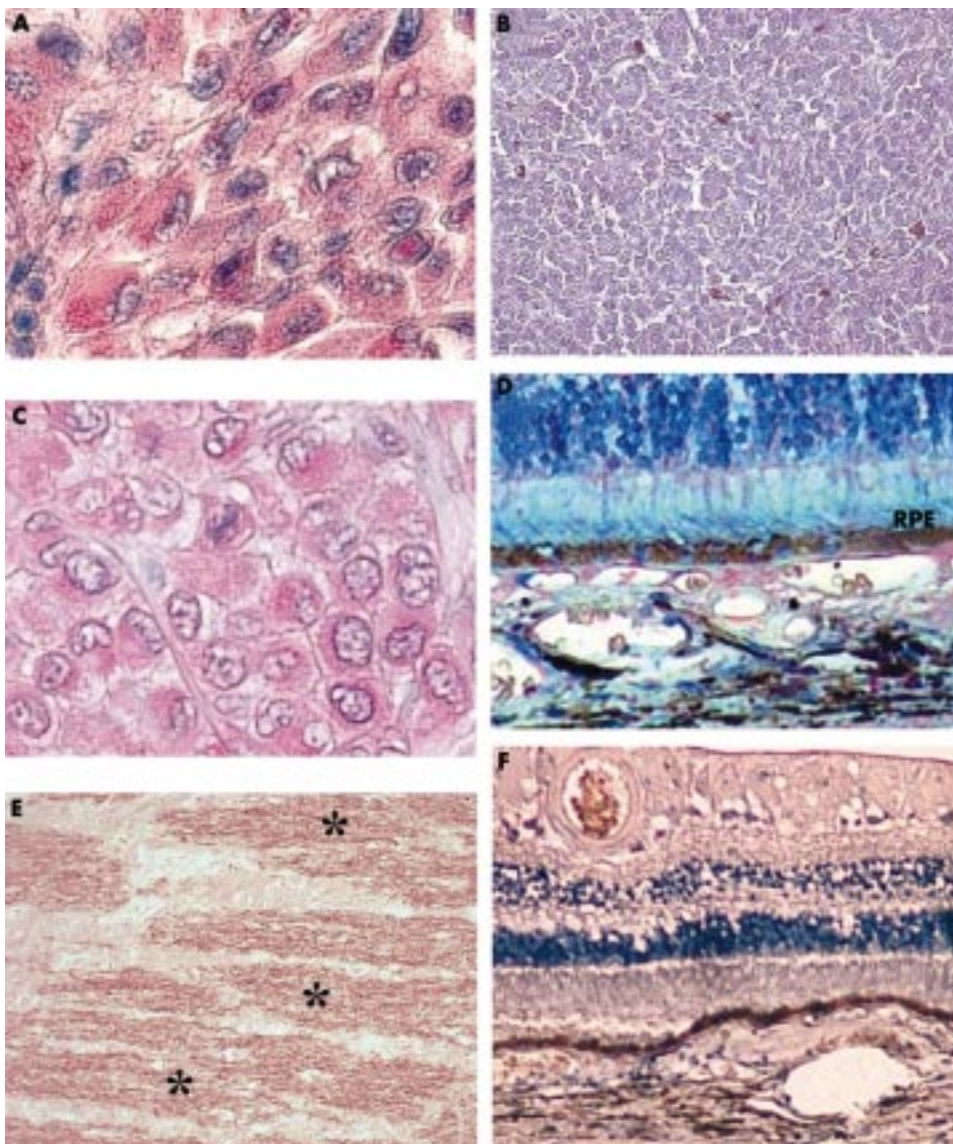


Figure 1 (A) Light micrograph illustrating grade 3 alkaline phosphatase (AP) immunostaining (pink) in a uveal melanoma; nuclei are counterstained blue ($\times 400$). (B) Negative control (no primary mAb) showing no apparent immunoreactivity in this uveal melanoma section ($\times 250$). (C) Cutaneous melanoma cells (positive control) clearly illustrate grade 3 AP immunostaining ($\times 400$). (D) Outer retina, retinal pigmented epithelium (RPE), and choroid in the retina of a uveal melanoma eye. Weak AP immunostaining is seen in the RPE and moderate immunostaining in the underlying choriocapillaris/choroid ($\times 250$). (E) Longitudinal section of optic nerve showing NG2 immunoreactivity post-laminar axon bundles (asterisk), the septa between bundles show no apparent immunoreactivity (Vector NovaRED) ($\times 250$). (F) An example of normal retina and choroid showing minimal NG2 immunoreactivity ($\times 100$).

Immunohistochemistry

Uveal melanomas

For 7/26 cases immunostaining could not be assessed because of the dense pigmentation of the tumour. Of the remaining 19 cases, 18 (~95%) uveal melanomas displayed grade 2 (moderate) or greater immunostaining for mAb 9.2.27 (Table 1; Fig 1A). Isotype or negative control sections displayed no apparent staining (Fig 1B). Cutaneous melanoma cells (positive control) were strongly immunolabelled with mAb 9.2.27 (grade 3) (Fig 1C).

Retina, choroid, and optic nerve

In melanoma affected eyes, weak immunostaining was seen in the retina of most cases (grade 0.5 to 1.5; Table 1). Immunostaining was predominantly localised to the nerve fibre layer, and inner and outer plexiform layers, and RPE appeared to display weak immunostaining (Fig 1D). Weak immunoreactivity was also noted in the choroid of 13/19 specimens assessed (Table 1). Optic nerve axon bundles close to the lamina cribrosa showed weak mAb 9.2.27 immunostaining, and obvious NG2 immunoreactivity myelinated axon bundles were seen posterior to the lamina cribrosa (Fig 1E). At higher power (not shown) this immunostaining appeared to be localised to the myelin sheaths surrounding the axons. The septa between axon bundles did not show immunoreactivity for mAb 9.2.27. In normal control eyes, the retina and choroid displayed minimal immunoreactivity (Fig

1F). Optic nerve axon bundles displayed moderate mAb 9.2.27 immunostaining similar to the distribution seen in melanoma affected eyes.

DISCUSSION

Melanoma associated chondroitin sulphate proteoglycan is a protein expressed on the surface of human malignant melanoma cells and recognised by mAb 9.2.27.⁹ Cloning and sequence analysis have revealed an 80% homology of this molecule with the rat NG2 molecule,^{20, 21} and melanoma associated chondroitin sulphate proteoglycan is now referred to as human NG2.

NG2 is widely expressed in various tumours including chondrosarcomas, glioblastomas, and some leukaemias.^{10, 22-24} Numerous studies have also found NG2 expression in cutaneous melanoma,¹¹ although the expression of NG2 immunoreactivity in human uveal melanoma and other ocular tissues remains to be fully described. In the present study, using immunohistochemistry, we found that most of the uveal melanomas in the series examined displayed cell surface mAb 9.2.27 immunostaining; normal retinas and choroid displayed low level immunoreactivity. We also observed moderate NG2 immunoreactivity in post-laminar myelinated axon bundles (but not septa) of optic nerves from control and melanoma affected eyes, consistent with reports of NG2 positive classes of oligodendrocytes in CNS white matter tracts including optic nerve.²⁵

Several studies have found that new vessels in normal developing tissue, in wound healing and in tumours express NG2 immunoreactivity,²⁶ initially reported predominantly in pericytes.²⁷ However, more recent studies have also found NG2 expression in normal capillary endothelium,¹⁰ pericytes,^{2, 27, 28} and microglia.²⁹ In the present study, mAb 9.2.27 immunostaining of uveal melanoma vasculature was not obvious and is currently being investigated. Additionally, the expression of NG2 has been associated with an enhanced malignant potential in cancer cells, including melanoma cells.³⁰ Overall, these observations suggest that NG2 may have a functional role in both angiogenesis and tumour development, although this remains to be studied in uveal melanoma.

The localisation of NG2 in tumours (including uveal melanoma) suggests a potential use for targeted delivery of therapeutic agents to control tumour growth. Our in vitro and preclinical studies show ²¹³Bi-9.2.27 AIC can kill NG2 positive metastatic skin melanoma cells in vitro and regress tumour growth in a subcutaneous xenograft model. However, given the potential for a human anti-mouse antibody (HAMA) response,³¹ a humanised anti-NG2 antibody would be best to use to deliver various agents aimed at destroying human cancer cells. Interestingly, the results from the St George Hospital phase I clinical trial for skin melanoma have not shown a significant HAMA response, using 450 µCi ²¹³Bi-9.2.27 AIC (unpublished data). Alternatively, NG2 proteoglycan binding peptides have been shown to target NG2 positive tumour vasculature in wild type mice with melanoma xenografts, with subsequent reduction in tumour growth; this effect was not seen in xenografted NG2 null mice.³² These peptides may also be useful to specifically target NG2 expressing tumour cells.³²

In conclusion, the majority of uveal melanomas in the present study showed moderate to strong immunostaining for mAb 9.2.27. This may be of therapeutic significance for local administration of α radioimmunoconjugate therapy for ocular melanoma.

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