Expression of the chondroitin sulphate proteoglycan, NG2, in paediatric brain tumours.

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Abstract

Background: While Neuron-glia 2 (NG2), is well characterized in the developing brain and in adult high grade gliomas, little is known about NG2 expression in paediatric brain tumours. Here, NG2 expression was examined in a range of paediatric brain tumours. Materials and methods: A retrospective immunohistopathological analysis of 57 paediatric brain tumour biopsies of various tumour types was carried out. Paediatric cell lines, including two medulloblastomas, and one dysembryoplastic neuroepithelial tumour, in addition to one adult high grade glioma, were also assessed for NG2 expression. Results: NG2 positive staining was seen in all DNETs examined; however only two of the fourteen medulloblastomas examined were NG2 positive. Compared to adult glioma, there was a lack of NG2 staining in the vasculature of paediatric brain tumours. Conclusions: NG2 expression in paediatric brain tumours differs depending upon type; and, unlike adult glioma, includes expression on lower grade tumours.

Introduction

Neuron-glia 2 (NG2), also known as CSPG4, is a chondroitin sulphate proteoglycan that has been well characterized in the developing human brain and in adult malignant primary brain tumours. Indeed, NG2 is often expressed in glioma cells which are characterised by increased proliferation rates (1), and exhibit high tumorigenic capabilities with an aggressive molecular phenotype (2). Moreover, its expression has been shown to correlate with the degree of malignancy in high grade glioma (3, 4). Both *in vitro* and *in vivo* data on malignant glioma in adult reveal that NG2 is over-expressed in tumour cells, endothelial cells and pericytes and its function involves tumour survival, growth, invasion and angiogenesis (3-7). NG2 has also been suggested to promote tumour vascularisation and to be involved with the recruitment of normal progenitor cells to the tumour mass and mediate the expansion of the transformed cell population (4). Additionally, NG2 has a role in glioma chemoresistance (8). NG2-dependent activation of α3β1 integrin effects cell survival due to increased signalling through the P13K/AKT pathway (8). In a spheroid model, gliomas were tested for sensitivity to the chemotherapeutic agents doxorubicin, etoposide and carboplatin, where a positive correlation was found between apoptosis resistance and NG2 expression levels (8). A study investigating GBM patient survival demonstrated that 50% of biopsies expressed NG2 on tumour cells and associated vessels and was linked to significantly shorter survival. In the NG2 positive samples, upregulation of an antioxidant, peroxiredoxin-1 (PRDX) and a reduction in products of oxidative stress were found in patients with the shortest survival times. These cells also showed resistance to ionising radiation and this may be mediated by induction of reactive oxygen species (ROS) scavenging enzymes and preferential DNA damage signalling (9).

While much is known about NG2 in adult high grade gliomas, very little is known concerning its expression in paediatric brain tumours, Which are known to be genetically and histologically distinct from their adult counterparts. The aim of this current investigation was to

identify and describe NG2 expression in human paediatric brain tumour sections and cell cultures derived from such neoplasms.

Methods

Ethics statement

Biopsies from glioma patients were obtained under Ethics permissions LREC 00-173 or KCH 11-094 or 11/SC/0048 in accordance with the National Research Ethics Service (NRES) and the study was approved through ethics committees for the University of Portsmouth and King's College Hospital, London. All patients consented to the use of biopsy material for research purposes. Consent forms were read to and duly signed by participating patients prior to surgery.

Tissue Sections

Archival paraffin embedded 10 μm sections of 57 paediatric brain tumour biopsies were used: 20 pilocytic astrocytomas, 9 astrocytomas, 5 ependymomas, 4 DNET (Dysembryoplastic Neuroepithelial Tumours), 14 medulloblastomas and 5 supratentorial PNETs. The patients were diagnosed at the Department of Neuropathology, Institute of Psychiatry, King's College London and were 16 years of age or younger. Paraffin embedded sections of an adult glioblastoma biopsy were also obtained from Charing Cross Hospital, Imperial College, London. The diagnosis was given by the neuropathologists working at King's College, London (AK and SAS) and at Charing Cross, London (RF).

Immunohistochemistry

Immunohistochemistry was performed on the paediatric cases using a standard immunoperoxidase protocol. Archival paraffin embedded 10μm sections were deparaffininized in xylene, washed in a graded series of ethanol and microwaved in citrate buffer for 10 minutes. The slides were subsequently incubated with an anti-NG2 chondroitin sulphate

proteoglycan antibody (Millipore, UK 1:50 dilution) overnight at 4^{o}C. An adult GBM case was also prepared for immunohistochemsity and incubated with an anti-NG2 human NG2/MCSP - clone LHM-2 antibody (R and D Systems, UK 1:33 dilution) overnight at 4^{o}C. Negative controls included an IgG isotype control and omission of the primary antibody. The secondary antibody used in the paediatric cases was swine anti-rabbit (DAKO, UK) and detected using an avidin-biotinylated enzyme complex (ABComplex DAKO, UK). For the GBM case, slides were incubated with a pre-diluted biotinylated pan-specific universal secondary antibody (Vector Labs, UK) and a streptavidin/peroxidase complex (Vector Labs, UK) and signal was visualized using diaminobenzidine tablets (Sigma-Aldrich, UK). Appropriate negative controls for both methods were used and no NG2 positivity was detected. Nuclei were counterstained with Harris' haematoxylin and examined using a Leica bright field microscope. For the paediatric cases, positive NG2 staining was determined by scoring the proportion of tumour sample covered in ten independent fields of views (objectives x5 and x40). Proportion of tumour cells greater than 50% is denoted in Table 1 as +++, 25% is denoted as ++, 10% denoted as + and less than 10% denoted as -.

Cell culture

Independent of the cases used for histological evaluation, the following cell cultures were examined for NG2 expression; two paediatric medulloblastomas (IN1008 and IN2072), one paediatric dysembryoplastic neuroepithelial tumour (IN1977), passage 4-18, and an 'in house' adult GBM (UP-007), passage 9-14. Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10-20% foetal bovine serum (FBS). Cells were routinely propagated in culture in a standard humidified incubator at 37^{o}C in a 5% carbon dioxide/95% air atmosphere, for up to fourteen serial passages. Paediatric cell lines were a kind gift from Dr Tracy Warr University of Wolverhampton.

Flow cytometry

A series of triplicate flow cytometric analyses were performed complementary to the procedures described above. Cells grown to confluency were harvested from T75 flasks, using cell dissociation solution, to avoid stripping cell surface antigens, for fifteen minutes. The cells were centrifuged at $200g_{av}$ for five minutes, disaggregated into a single-cell suspension and incubated with a human NG2/MCSP (clone LHM-2) antibody (R and D Systems, UK 1:33 dilution) at a dilution of 1:500 with PBS + 5% FBS for 30 minutes. This was followed by incubation with the secondary Alexa Fluor 488 antibody conjugate at a dilution of 1:500 with PBS + 5% FBS for 20 minutes. The negative controls were an IgG isotype control and omission of the primary antibody. Flow cytometric analysis was carried out on a FACSCalibur (BD Biosciences, Oxford, UK). The addition of propidium iodide allowed the live population of cells to be gated, to remove any false positive error from necrotic cells.

Immunocytochemistry

Fixation was achieved using 4% paraformaldehyde for two minutes. Cells grown to confluency on cover slips in duplicate were incubated with a human NG2/MCSP (clone LHM-2) antibody (R and D Systems, UK) at the required concentration of 4µg/ml for one hour. Cells being stained for Ki-67 were first permeabilised with 0.001% Triton X-100 for 2 mins, washed three times with phosphate buffered saline (PBS) and then incubated with 1:200 mouse monoclonal anti-human Ki-67 (Dako, UK) for one hour. For NG2, following three washes with PBS 1:400 of the secondary antibody conjugates anti-mouse Alexa Fluor's 488 or 568 (Invitrogen, UK) were added for thirty minutes in the dark followed by washing with PBS. The cells were then washed three times in PBS and counterstained with Hoechst Blue (10 µg/ml) for five seconds, as a nuclear counterstain. The cells were washed three times in PBS, mounted and examined using a Zeiss Axioimager epifluorescence microscope with excitation and barrier filters for

FITC, Texas Red and DAPI. The primary antibody was omitted in the negative controls. These data are shown in Table 2.

Statistics

Data is representative of three independent experiments carried out in triplicate and are expressed as mean values. Statistical analysis was performed on the data using a one way ANOVA followed by Tukey's multiple comparison post-test with a probability of < 0.05 being regarded as significant. The software package GraphPad Prism 3.02 was used to calculate the statistical tests.

Results

Immunohistochemical analysis of NG2 expression in paediatric brain tumours

A retrospective analysis of NG2 expression in 57 paediatric brain tumour biopsies using immunohistochemistry was performed. The intensity of NG2 positive staining varied among the different tumour types (Table 1). Of the nine astrocytomas four of the nine were positive for NG2, intriguingly, the three lower grade were highly positive and the higher grades were generally negative (Table 1). Seven of twenty pilocytic astrocytomas (35%) were NG2 positive, all of the DNETs were positive (n=4), only one of the five ependymomas examined expressed NG2, two of fourteen medulloblastomas expressed NG2 protein and three of five supratentorial PNETs demonstrated NG2 immunopositivity (Table 1). Localisation of NG2 positive staining differed between adult and paediatric brain tumours with the most striking difference being the lack of staining of vasculature (endothelial) structures in paediatric brain tumours (Figure 1a). In agreement with other reports, NG2 expression in adult high grade gliomas is localised in both tumour and vascular cells (Figure 1b and 1c). In pilocytic astrocytomas, NG2 expression is

seen at high power associated with elongated bipolar tumour cells (Figure 1c) which are characteristic of this tumour type.

Insert table 1 and figure 1

NG2 expression in paediatric cell lines.

We next examined NG2 expression in tumour cell cultures established from surgical tissue (passage 4-18). Flow cytometric analysis reveals a range of NG2 expression among the primary paediatric brain tumour cell lines with NG2 expression in medulloblastoma cultures being undetectable whereas in DNET, and adult high grade gliomas NG2 protein expression was detected (Figure 2). The highest NG2 expression was seen in the high grade glioma cell line, UP-007, (97.6%, Figure 2a) while with the medulloblastoma cell lines NG2 was barely detectable (< 1%, Figure 2c, d). These results are in agreement with the data obtained from patient biopsy material (Table 1) where only two of fourteen medulloblastomas examined demonstrated low NG2 staining. All of the DNETs examined expressed NG2. Because NG2 expression in adult GBMs is associated with proliferation we next sought to examine the co-expression of NG2 with that of Ki67 in primary paediatric brain tumour cell lines. In UP-007 cells, NG2 showed a positive correlation with Ki67 expression whereas in the medulloblastoma cell lines and the DNET cell line this was not the case (Table 2).

Insert figure 2 and table 2

Discussion

In adult high grade gliomas, NG2 expression is highly correlated with increased tumour cell proliferation, grade of tumour, poor prognosis and angiogenesis (5, 10). In this report, we demonstrate that NG2 protein expression in paediatric brain tumours is distinct from adult brain tumours and that in the context of childhood brain tumours, NG2 expression appears to be

varied amongst the different tumour types. Very few studies have reported the expression of NG2 in childhood brain cancers (11, 12). The present study aimed to elucidate the expression of NG2 in a range of paediatric brain tumours. According to our current knowledge the cells that express NG2 in the brain are oligodendrocyte precursor cells (OPCs), developing neurons, activated macrophage cells, (13) pericytes (10, 14-16) and endothelial cells (17). NG2 expressing brain endothelial cells *in situ* form a network of microvessels together with the pericytes and do not have processes (17).

In the present study, neoplastic cells within the highly vascularised PNETs were generally NG2 positive with the vascular cells devoid of NG2 expression (Figure 1). This differs greatly from the situation in high grade adult glioma, where vascular NG2 staining is generally prominent (3, 5, 11). Developing neurons rarely give rise to brain tumours, so with the possible exception of PNETs, NG2 expressing tumours may indicate a glial progenitor or earlier multi-potential glio-neuronal progenitor cell origin. Half of the cases of pilocytic astrocytoma and astrocytoma were found to express NG2. These results support previous reports purporting the notion therefore that some oligodendrogliomas, pilocytic astrocytomas and GBM's may arise from OPCs (18) and not necessarily from terminally differentiated astrocytes or oligodendrocytes. Ependymomas are thought to be derived from ependymal cells, which in their turn are thought to be derived from glial progenitor cells. Medulloblastomas arise in the posterior fossa although the histogenesis of these tumours is largely unknown, they are thought to be mainly of neuronal (cerebellar granule cell) origin. The origins of the histologically similar primitive neuroectodermal tumours (sPNET) that arise supratentorially are less clear. These tumours are both classified as embryonal and they have a similar histology but it has been shown that medulloblastomas are molecularly distinct from other brain tumours including the other embryonal tumours (19). Moreover, sPNETs and medulloblastomas display differences in response to various therapies and patient survival times differ (20, 21). The NG2 positivity in

sPNETs, pilocytic astrocytomas, low grade astrocytomas, and DNETs demonstrated here is therefore intriguing.

OPCs are present in the brain even after it is fully developed (22). Their role has been proposed to be that of production of new oligodendrocytes after a demyelinating insult. It is therefore possible that NG2 expression may represent proliferative active OPCs that may have accumulated in the area to attempt repair the damage caused by the tumour. We conclude, however, that from both our histological and *in vitro* results that the most likely explanation is that, neoplastic neural cells themselves express NG2. These cells may be derived from NG2 positive progenitors or may simply functionally express the NG2 antigen. However, the extent of staining for NG2 and the morphological appearance is consistent with the neoplastic cells themselves expressing NG2.

Previous studies of adult human glioma of various histological types have shown that NG2 is expressed more in high compared to low-grade gliomas (3). This study has indicated that the reverse may be true for paediatric tumours with increased NG2 expression in lower grade tumours compared with higher grades. In adult gliomas, it was also found using cell cycle analysis of astrocytoma cells that NG2 expressing cells were more proliferatively active than the NG2 negative cells and that NG2 is specifically expressed within the proliferative main mass and not the advancing edge in human glioma biopsies (11). Furthermore, Chekenya et al (23) have shown that GBM cells transfected with NG2 cDNA significantly enhanced growth rate *in vitro* compared with untransfected and sham transfected controls. It was also demonstrated that tumour spheroids consisting of the above NG2 transfected GBM cells increased the tumourigenicity of the cells *in vivo* compared to wild type and sham control. These results are, however, contradicted by the report from Shoshan and collegues (18) that oligodendrogliomas have higher NG2 expression than the highly proliferative glioblastomas. While it has been shown from the present study that there exist NG2 positive and NG2 negative groups of

pilocytic astrocytomas and astrocytomas and that sPNETs are generally NG2 positive while medulloblastomas are generally NG2 negative, the significance of these findings remains to be seen. It is tempting, however, to speculate that these subgroups might correspond to prognosis but a future study with accurate patient 'follow-ups' is required to establish this possibility, which is in particular, of the four molecular subgroups of medulloblastoma (24). NG2 expression has been shown to be greatest in the WNT signalling pathway group (personal communication; Adrian Dubuc, Brigham and the Women's Hospital, Boston, Massachusetts).

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Figure Legends

Figure 1. Immunohistochemical analysis of NG2 expression in brain tumours. NG2 staining (brown) and nuclei counterstained with Haematoxylin (blue/violet) a) s PNET (x5) staining for NG2 shows widespread staining although NG2 is absent around the vasculature conversely the adult GBM (x10) b) shows NG2 staining localised to the vasculature and widespread distribution c) (x20). The pilocytic astrocytoma demonstrates NG2 staining of the filamentous processes d) (x40).

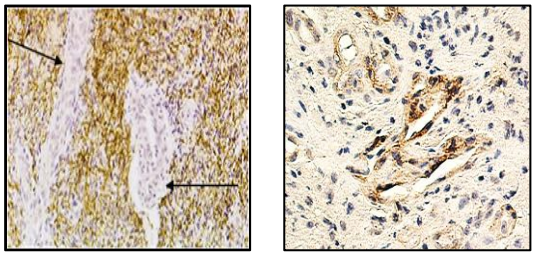
Figure 2. Flow cytometric analyses of NG2 expression in primary brain tumour cell lines. In an adult GBM primary cell line, UP-007, there was an average of 97.61% of the total population that is positive for NG2 a), IN1977 – a DNET cell line was 63.82% positive for NG2 b) IN1008

– a medulloblastoma cell line was 0.15% for NG2 c) and IN2072 – a medulloblastoma cell line was 0.13% positive for NG2 d).

FIGURES

Figure 1.

a) Paediatric sPNET b) Adult GBM



c) Adult GBM d) Pilocytic astrocytoma

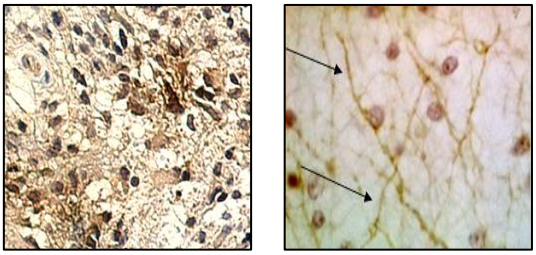
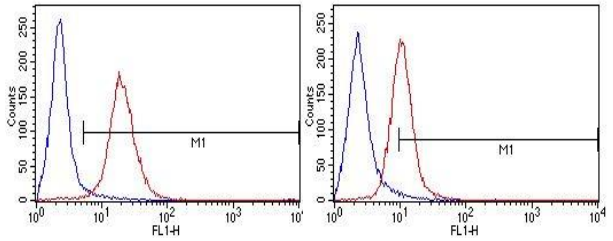
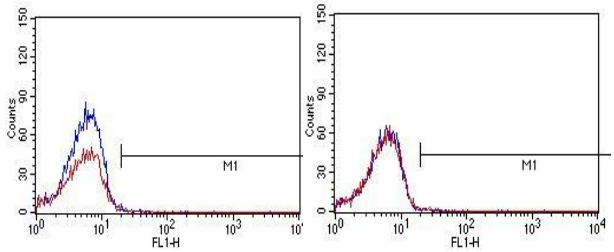


Figure 2.

a) UP-007 b) IN1977



c) IN1008 d) IN2072



TABLES

Table 1. A summary of patient details, diagnosis and NG2 status. NG2 expression is denoted as +++ (high), ++ (moderate), + (low) and – (no stain), this scoring is based upon the proportion of tumour cells greater than 50% is denoted in Table 1 as +++, 25% is denoted as ++, 10% denoted as + and less than 10% denoted as -.

Patient No.	Age	Sex	Grade	Histology	NG2
1	7	F	I	Astrocytoma	+++
2	1	M	I	Astrocytoma	+
3	1	M	I	Astrocytoma	++
4	7	F	I	Astrocytoma	-
5	9	F	I	Astrocytoma	-
6	11	F	II	Astrocytoma	-
7	7	M	II	Astrocytoma	-
8	7	F	III	Astrocytoma	+
9	3	F	III	Astrocytoma	-
10	4	F	I	Pilocytic Astrocytoma	+
11	3	F	I	Pilocytic Astrocytoma	-
12	6	M	I	Pilocytic Astrocytoma	-
13	9	F	I	Pilocytic Astrocytoma	-
14	15	M	I	Pilocytic Astrocytoma	-
15	3	F	I	Pilocytic Astrocytoma	-
16	11	F	I	Pilocytic Astrocytoma	-
17	13	M	I	Pilocytic Astrocytoma	-
18	11	M	I	Pilocytic Astrocytoma	++
19	9	M	I	Pilocytic Astrocytoma	+
20	9	M	I	Pilocytic Astrocytoma	+++
21	8	F	I	Pilocytic Astrocytoma	-
22	11	M	I	Pilocytic Astrocytoma	+
23	9	M	I	Pilocytic Astrocytoma	-
24	16	F	I	Pilocytic Astrocytoma	+
25	9.5	M	I	Pilocytic Astrocytoma	+
26	4	F	I	Pilocytic Astrocytoma	+
27	5	F	I	Pilocytic Astrocytoma	-
28	7	M	I	Pilocytic Astrocytoma	-
29	3	M	I	Pilocytic Astrocytoma	-
30	8	F	I	DNET	+++
31	1	M	I	DNET	+++
32	4	M	I	DNET	+
33	9	F	I	DNET	+++
34	1	M	II	Ependymoma	-
35	8	M	II	Ependymoma	-
36	12	F	II	Ependymoma	+
37	11	F	II	Ependymoma	-
38	2	M	II	Ependymoma	-
39	3	M	IV	Medulloblastoma	-
40	11	M	IV	Medulloblastoma	-
41	7	M	IV	Medulloblastoma	-
42	6	F	IV	Medulloblastoma	-
43	10	F	IV	Medulloblastoma	-
44	3	M	IV	Medulloblastoma	-
45	5	F	IV	Medulloblastoma	-
46	8	F	IV	Medulloblastoma	-
47	5	M	IV	Medulloblastoma	+
48	7	M	IV	Medulloblastoma	+
49	16	M	IV	Medulloblastoma	-
50	5	M	IV	Medulloblastoma	-
51	9	M	IV	Medulloblastoma	-
52	5	F	IV	Medulloblastoma	-
53	10	M	IV	sPNET	++
54	8	M	IV	sPNET	-
55	11	M	IV	sPNET	+++
56	10	M	IV	sPNET	+
57	4	F	IV	sPNET	-

Table 2. Antigen staining intensity of various glioma cell cultures as determined by immunocytochemistry. NG2 positive cells as determined by flow cytometry are denoted as percentage (FC). Staining intensity denoted as +++ (high), ++ (moderate), + (weak) and – (no stain).

Sample ID	NG2 ICC	NG2 FC %	Ki67 ICC
IN1008	-	0.15	+
IN2072	-	0.13	-
IN1977	++	63.0	+
UP-007	+++	97.0	+++

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