Acta Ophthalmologica

Immunoreactivity of the 14F7 Mab raised against *N*-Glycolyl GM3 Ganglioside in retinoblastoma tumours

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ABSTRACT.

Introduction: **The identification of molecules expressed selectively on the surface of retinoblastoma cells would allow applying targeted therapies. The Ganglioside, *N*-Glycolyl-GM3 (NeuGc-GM3), is an attractive candidate, as it has been detected in other paediatric neuroectodermic tumours, and it is not expressed in human normal tissues. The 14F7 antibody recognizes specifically the ganglioside NeuGc-GM3.**

Purpose: **To characterize the expression of NeuGc-GM3 in retinoblastoma cell lines and in retinoblastoma tumours using the 14F7 monoclonal antibody.**

Methods: **We studied WERI-Rb1 and Y79 cell lines, 24 retinoblastoma primary tumours from unilateral and bilateral cases and two bone marrow biopsies from metastatic retinoblastoma. Tumours were classified into three groups: non-invasive (n = 13), invasive (n = 9) and metastatic (n = 2). Three eyes enucleated because of non-tumoural conditions were used as controls. Cell lines and tumour sections were studied by immunohistochemistry using the 14F7 antibody. NeuGc-GM3 expression was evaluated by analysing the percentage of positive tumoural cells and the staining intensity. These parameters were analysed comparatively among the three groups.**

Results: **Both retinoblastoma cell lines showed immunoreactivity to NeuGc-GM3 but WERI-Rb1 presented higher intensity than Y79. All the tumours studied showed strong immunoreactivity to NeuGc-GM3 with no significant differences among groups. In both bone marrow specimens, NeuGc-GM3 immunoreactivity was observed in retinoblastoma cells. In bilaterally enucleated cases, NeuGc-GM3 immunoreactivity was not altered before and after chemotherapy. Non-tumoural retinas were negative.**

Conclusions: **NeuGc-GM3 is highly expressed in retinoblastoma cell lines, tumours and metastatic cells to the bone marrow, and it is not detectable in control eyes. There were no significant differences in the immunoreactivity to 14F7 among tumours from different disease stages. Its immunoreactivity did not change after chemotherapy.**

Key words: ganglioside – metastatic retinoblastoma – *N*-Glycolyl-GM3 – retinoblastoma tumour – targeted therapy

Acta Ophthalmol.

doi: 10.1111/aos.12578

Introduction

Retinoblastoma is the most common primary intraocular malignancy of childhood, and it is highly curable when is diagnosed in its early intraocular stages, as is the case in developed countries. However, its survival rates in developing countries are significantly lower. Late diagnosis, poor treatment compliance and limitations for access to treatment, leading to extraocular dissemination, are the major causes for these poor results (Chantada et al. 2011; Navo et al. 2012).

In developing countries, most affected patients still need enucleation of the affected eye, and some of them need postoperative chemotherapy to reduce the risk of extraocular relapse (Chantada et al. 2009, 2010). Extraocular dissemination is still the most common cause of death in affected patients in that setting (Chantada et al. 2006a). In developed countries, however, most children are frequently treated with an eye-sparing approach (Abramson 2005; Abramson et al. 2008). In these cases, children with retinoblastoma harbouring a germ line mutation of the Rb1 gene are at increased risk of secondary malignancies associated with the mutagenic effect of treatment. Hence, targeted therapies capable of delivering specific treatment agents selectively to the tumour cells, sparing the normal tissue would be ideal for treatment of this tumour. One possibility for targeted

therapy includes the use of monoclonal antibodies able to recognize molecules of the surface of tumour cells. This approach was reported for other ocular tumours (Missotten et al. 2010) and other paediatric neuroectodermic tumours such as neuroblastoma. In neuroblastoma, passive immunotherapy with monoclonal antibodies directed to the ganglioside GD2, present in the cell surface, is used for the treatment of disseminated disease (Seeger 2011; Cheung et al. 2012; Ahmed & Cheung 2014), but as disseminated disease is uncommon in developed countries, there is little experience in retinoblastoma.

GD2 is the most extensively studied ganglioside in paediatric malignancies. It is also expressed abundantly in retinoblastoma tumour cells, so it was used as a suitable marker for identification of suspicious tumour cells in the bone marrow or cerebrospinal fluid of affected patients (Seeger 2011; Laurent et al. 2013). However, low levels of GD2 expression in some normal tissues such as the peripheral nerves, neurons (Mennel et al. 1992), skin melanocytes (Hersey 1991) and bone marrow stem cells (Rasini et al. 2013) limit the widespread use of this antigen for delivering targeted therapy to the eye because of anticipated toxicity, as evident when used for immunotherapy of neuroblastoma (Sorkin et al. 2010).

The glycolylated variant of the monosialic acid GM3 (NeuGc-GM3) has also been found to be abundantly expressed in many human tumours (Marquina et al. 1996; Carr et al. 2003; Alfonso et al. 2007; Scursoni et al. 2010, 2011; Blanco et al. 2011, 2012, 2013; Zhong et al. 2012; Lahera et al. 2014). This variant contains glycolylated sialic acid, which is not detected in normal human tissues and fluids, including the healthy retina (Higashi et al. 1988; Marquina et al. 1996; Scursoni et al. 2010, 2011; Blanco et al. 2011, 2012, 2013; Zhong et al. 2012; Lahera et al. 2014). Human cells lack of the enzyme cytidine monophospho-*N*-acetylneuraminic acid hydroxylase, which regulates its synthesis (Irie & Suzuki 1998; Malykh et al. 2001). The limited expression of NeuGc-GM3 in human normal tissues, as well as its presence in different tumours, indicates that this variant of GM3 could be a good candidate for immunotherapy to different tumours (Neninger et al. 2007; Fernandez et al. 2010; Scursoni et al. 2011). Thus, the 14F7 antibody that recognises this epitope represents a promising alternative to immunotherapy treatments. Using the 14F7 antibody, our group reported that neuroblastoma and other neuroectodermic tumours express NeuGc-GM3 in a high proportion of cases (Scursoni et al. 2010, 2011). There is limited published evidence reporting the presence of NeuGc-GM3 in retinoblastoma cell lines (Higashi et al. 1988), and none of these studies has evaluated its expression and cell distribution on histopathological grounds in different disease stages and clinical situations. Also, 14F7 is the first IgG1 highly specific against NeuGc-GM3 reported in the literature (Carr et al. 2000) available for clinical purposes. Thus, if retinoblastoma cells selectively expressed this ganglioside and if it was effectively recognized by the 14F7 antibody, this finding could have clinical and therapeutic implications.

Therefore, the aim of this study was to describe the presence and cellular distribution of NeuGc-GM3 in retinoblastoma tumour cells as a new potential target to develop immunotherapy strategies against retinoblastoma.

Material and Methods

The 14F7 antibody

The monoclonal antibody 14F7 was generously provided by the Center of Molecular Immunology (La Habana, Cuba) and used at a final concentration of 20 μg/ml. The 14F7 antibody is a mouse IgG1 that specifically recognizes the ganglioside NeuGc-GM3, as previously described (Guthmann et al. 2006).

Retinoblastoma cell lines

We started our study by analysing the immunoreactivity of commercially available retinoblastoma cell lines WERI-Rb-1 and Y79 (American Type Culture Collection, Manassas, VA, USA) to the 14F7 antibody by immunocytochemistry. We used the X63 murine myeloma cell line as positive control (Segatori et al. 2012). The cells were cultured in RMPI medium supplemented with pyruvic acid, glucose and sodium bicarbonate in an atmosphere of 5% CO_2 at 37°C. The cells were collected by centrifugation and fixed with paraformaldehyde 4% for 10 min, and then they were washed with sodium phosphate buffer (PBS) and incubated 1 hr with the antibody 14F7 mouse anti-NeuGc-GM3. Afterwards, they were washed and incubated with secondary antibody conjugated with Flourescein iso-thiocyanate (FITC) and with 4′,6-diamidino-2-phenylindole (DAPI) as nuclear staining.

Archival cases

We reviewed paraffin-embedded pathological specimens from 24 eyes enucleated from 21 patients with retinoblastoma treated at our centre. We selected from our database cases encompassing many different clinical situations including laterality, invasiveness, prior therapy and tissue availability of high quality specimens. For the purpose of this study, we classified the eyes into three groups: (i) Non-invasive retinoblastoma which included those cases enucleated that showed only intraretinal invasion or prelaminar and/or focal choroidal invasion (n = 13 eyes). (ii) Invasive retinoblastoma including those eyes showing invasion to other ocular coats such as the massive choroidal invasion, postlaminar optic nerve or scleral invasion (n = 9 eyes). (iii) Metastatic retinoblastoma comprising eyes enucleated due to tumour cells has dispersed and has settled distant metastasis (n = 2 eyes).

Six eyes had received prior therapy before enucleation; three of them because of a conservative attempt with systemic chemoreduction and the remaining three had overt extraocular retinoblastoma or massive buphthalmia treated with neoadjuvant chemotherapy and planned enucleation after tumour reduction. Seventeen cases were unilateral, and the remaining patients had bilateral retinoblastoma. Also, we included the bone marrow of two cases of two additional patients with metastatic retinoblastoma. Three eyes from children enucleated because of trauma were included as controls.

Immunohistochemistry

Sections of 5 μm from formalin-fixed, paraffin-embedded tumour samples were used. After reaction of primary antibodies, sections were incubated with a peroxidase-labelled polymer conjugated to secondary anti-mouse

antibodies using the EnVision+ System-HRP (DAB) (DakoCytomation, Copenhagen, Denmark) and developed with 3,3′-diaminobenzidine as chromogen. Proper positive and negative controls were made in every staining battery. Briefly, in the same slice, there were sections of retinoblastoma tumour and breast carcinoma (this last one as positive control of ganglioside detection) (Fernandez-Marrero et al. 2011). Two negative controls were made, one omitting the primary anti-NeuGc-GM3 antibody and the other with the isotype control antibody (mouse IgG1). We have previously demonstrated that the routine technique did not extract antigenic carbohydrate determinants of gangliosides, thus allowing immunohistochemical detection in tumour sections (Alonso et al. 1999).

Immunohistochemical evaluation

NeuGc-GM3 expression was semi-quantitatively evaluated, and results were scored by two independent pathologists. In the rare event of divergent evaluation, a consensus was obtained by discussing the cases.

In all the samples studied, we quantified the intensity of the immunoreaction to NeuGc-GM3 and the amount of positive cells to it. We graded the intensity from 0 to 3, meaning 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = intense staining. We also graded the percentages of immunoreactive cells to NeuGc-GM3 in each tumour, sorting them in discrete values from 0 to 4. These values mean 0 = when 0–19% of cells were immunoreactive, 1 = when 20–39% of cells were immunoreactive, 2 = when 40–59% of cells were immunoreactive, 3 = when 60–79% of cells were immunoreactive and 4 = when 80–100% of cells were immunoreactive to NeuGc-GM3. Both parameters were measured in five high-power fields, by optic microscopy. These two values (the intensity and the percentage) allowed us to calculate an immunoreactive score (IRS) as previously described (Remmele & Stegner 1987; Beck et al. 1994; Fujimura et al. 2012). This score was calculated for each specimen by the multiplication of the staining intensity (0–3) and the amount of positive cells (0–4), resulting in a score ranging from 0 to 12 as described elsewhere (Beck et al. 1994; Matsuo et al. 2011). With this score, we could classify tumours in positive and negative for the expression of NeuGc-GM3. Positive tumours were considered when the score value was at or above 5 that was the cut-off, being negatives those under this value (Scursoni et al. 2011).

Statistical analysis

We compared with the Mann–Whitney test the IRS between the non-invasive cases with those of the invasive plus metastatic retinoblastoma.

Results

NeuGc-GM3 in retinoblastoma cell lines

All cell lines analysed were immunoreactive to the 14F7 antibody. The positive control cells (X63 cells) showed an intense immunoreactivity to 14F7 mainly restricted to the cell membrane. WERI-Rb1 cells showed intense immunoreactivity to 14F7, but the label was spread on all over the cell surface and also and with stronger immunolabelling in round-shape speckles presented in the cytoplasm. Y79 cells were also immunoreactive to 14F7 and with similar pattern than WERI-Rb1 cells, but with much weaker intensity (Fig. 1).

NeuGc-GM3 in the different clinical groups of retinoblastoma

The presence of NeuGc-GM3 ganglioside was evident in all specimens analysed (Table 1), as detected by immunohistochemistry using the specific monoclonal antibody 14F7. The immunoreactivity in the tumour cells was evident in the membrane and the cytoplasm.

In non-invasive cases, (Fig. 2) while all the tumour cells were immunoreactive to NeuGc-GM3, there was some heterogeneity in the intensity of the expression and some areas showed less intense immunoreaction than others. We were not able to identify any specific pattern to explain this variation as it was not correlated with necrosis, differentiation or any specific tumour location inside the eyeball.

In invasive and metastatic tumours, when tumour cells microscopically or massively invaded the choroid, sclera and the postlaminar optic nerve, tumour cells infiltrating these tissues were also immunoreactive to NeuGc-GM3 in all cases (Fig. 3). The presence

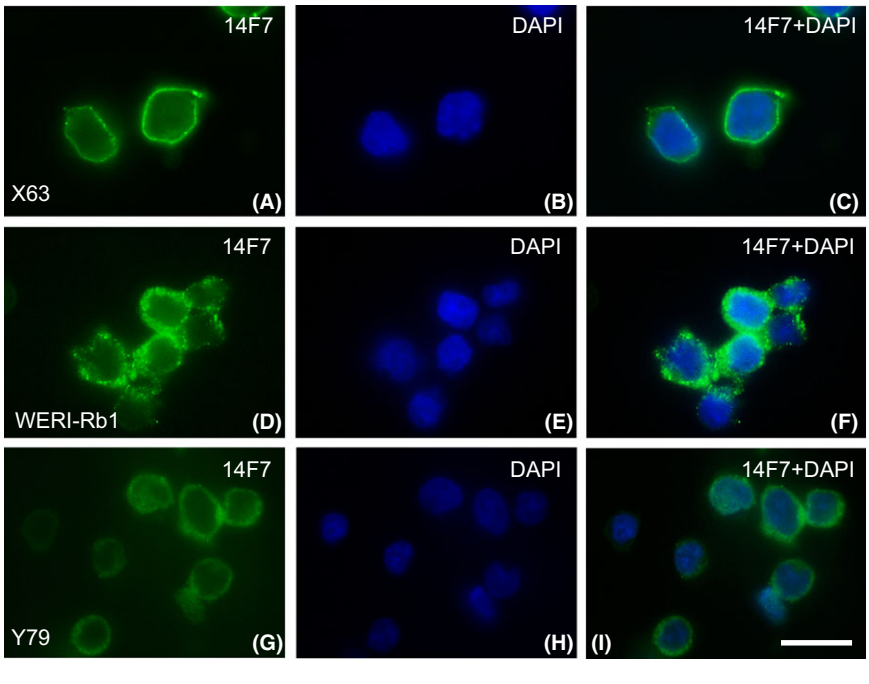

Fig. 1. (A–I) Immunohistochemical detection of NeuGc-GM3 ganglioside with the 14F7 antibody, in positive control cells, X63 murine myeloma cell line and retinoblastoma cells, WERI-Rb1 and Y79. (A–C) Positive control shows intense immunoreactivity to 14F7 selectively localized in the cell membrane. (D–F) WERI-Rb1 cells are also positive to the 14F7, but the immunolabel is located in small rounded structures resembling lipid rafts. (G–I) Y79 cells are also positive to 14F7, showing the same distribution of immunolabel than WERI-Rb1, but with less intensity than the other one. Nuclear staining: DAPI. Calibration bar: 20 μm.

Table 1. Table showing that all the specimens analysed are positive (IRS above 5) to 14F7 antibody, indicating the presence of NeuGc-GM3 in this tumor, independently of their IRSS Stage, group or prior therapy treatment.

Patient	IRSS stage	TNM	Extra-retinal microstages	Group	Laterality	Prior therapy	IRS
1	I	pT2aN0M0	C1-N0-S0	Non-invasive	Bilateral	No	12
1	I	pT2aN0M0	C0-N1-S0	Non-invasive	Bilateral	Yes	8
2	I	pT2bN0M0	C1-N1-S0	Non-invasive	Bilateral	No	12
2	I	pT2aN0M0	C1-N1-S0	Non-invasive	Bilateral	Yes	8
3	I	pT2aN0M0	C1-N0-S0	Non-invasive	Bilateral	No	12
3	I	pT2aN0M0	C0-N1-S0	Non-invasive	Bilateral	Yes	6
4	I	pT2aN0M0	C1-N0-S0	Non-invasive	Bilateral	No	9
5	I	pT2aN0M0	C0-N1-S0	Non-invasive	Unilateral	Yes	12
6	I	pT1N0M0	C0-N0-S0	Non-invasive	Unilateral	No	12
7	I	pT3bN0M0	C2-N0-S0	Non-invasive	Unilateral	No	12
8	I	pT1N0M0	C0-N0-S0	Non-invasive	Unilateral	No	12
9	I	pT3aN0M0	C1-N2-S0	Non-invasive	Unilateral	No	6
10	I	pT2bN0M0	C1-N1-S0	Non-invasive	Unilateral	No	12
11	I	pT3aN0M0	C1-N2-S0	Invasive	Unilateral	No	6
12	I	pT3bN0M0	C2-N2-S0	Invasive	Unilateral	No	6
13	II	pT4bN0M0	C2-N3-S1	Invasive	Unilateral	No	6
14	II	pT4bN0M0	C2-N3-S1	Invasive	Unilateral	No	12
15	II	pT4bN0M0	C2-N3-S0	Invasive	Unilateral	No	8
16	II	pT4bN0M0	C2-N3-S0	Invasive	Unilateral	No	8
17	II	pT4bN0M0	C2-N3-S1	Invasive	Unilateral	No	12
18	III	pT3bN0M0	n/a	Invasive	Unilateral	Yes	6
19	III	pT4bN0M0	n/a	Invasive	Unilateral	Yes	8
20	IVa	pT4bN0M1a	n/a	Metastatic	Unilateral	No	12
21	IVa	Pt4bn0m1a	n/a	Metastatic	Unilateral	No	6

IRSS = International Retinoblastoma Staging System. IRSS and extra-retinal micro-stages according to Chantada et al. (2006a). TNM = Union for International Cancer Control/American Joint Committee on Cancer (UICC/AJCC) Tumor, Node, Metastasis (TNM) categories and stages (7th Edition). IRS = Immunoreactive score, n/a = not applicable.

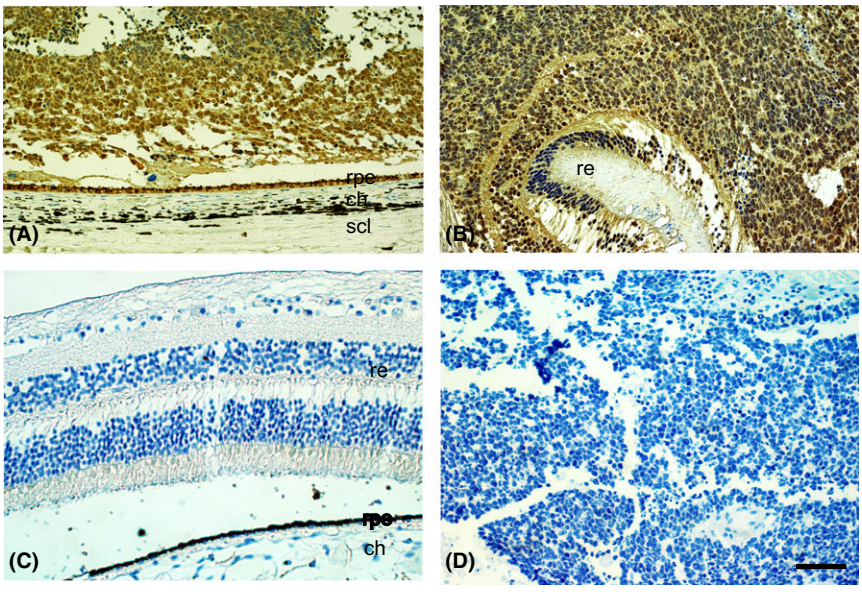

Fig. 2. (A–D) Microphotographies showing the immunoreactivity to 14F7 antibody against NeuGc-GM3 ganglioside in two sections of non-invasive retinoblastomas, non-tumoural retina and the negative isotype control in a section of retinoblastoma tumour. (A) In this section of non-invasive retinoblastoma, the tumour cells have replaced the retina (re). All the tumour cells are positive to 14F7 in this type of retinoblastoma tumour. The retinal pigment epithelium (rpe) conserves its integrity, and the choroids and the sclera are free of tumour cells. Neither the choroids (ch) nor the sclera (scl) are positive to 14F7. (B) Non-invasive retinoblastoma where the retina has been almost completely supplanted by tumour, just remaining a little-folded piece of it. The tumour cells are positive to NeuGc-GM3, independently of their differentiation degree. (C) In the non-tumoural retina, all the layers are not immunoreactive to 14F7, neither the choroids nor the pigment retinal epithelium. (D) Negative isotype control is completely absence of immunoreactivity to 14F7. Calibration bar: 50 μm.

of immunoreactivity on tumour cells that have reached the different layers of the eye indicates that these infiltrating cells also contain this ganglioside in their membrane and cytoplasm.

The retina surrounding the tumour was faintly immunoreactive for NeuGc-GM3, suggesting shedding of gangliosides from cancer cells, as described in renal tumours (Scursoni et al. 2010) and with other gangliosides in retinoblastoma (Portoukalian et al. 1993), but the retina in distant sites and that of control eyes was uniformly negative (Fig. 2) as it was also demonstrated by other authors in normal retina and other tissues (Table 2).

All the cases presented an elevated IRS, above or similar to 6 points (Table 1), which indicate a high level of expression of NeuGc-GM3 in all groups of retinoblastoma tumour studied.

NeuGc-GM3 in previously treated eyes

There were three cases with bilateral retinoblastoma, in which we evaluated one eye enucleated at diagnosis and the fellow eye enucleated after failure of conservative therapy (six eyes in total). Another two eyes from patients with

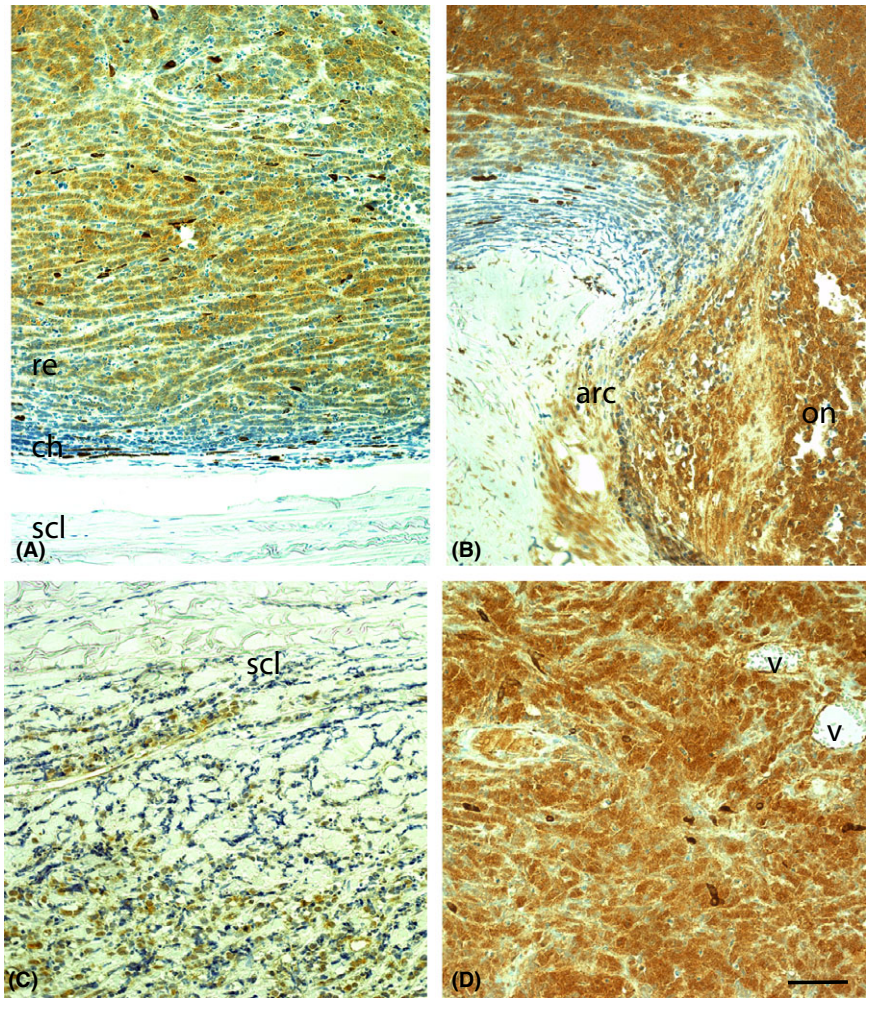

Fig. 3. (A–D) Microphotographies showing the immunoreactivity to 14F7 antibody against NeuGc-GM3 ganglioside in sections of a metastatic retinoblastoma. (A) In this microphotography, tumour cells have invaded the retina (re) and choroids (ch). All the tumour cells are positive to 14F7. The sclera (scl) remains free of tumour cells. (B) This microphotography shows tumour cells that have invaded the optic nerve (on) in the lamina cribrosa area and also in the arachnoid space (arc). The tumour cells are also positive to NeuGc-GM3 in these areas. (C) Microphotography showing tumour cells positive to 14F7 that have reached the sclera. (D) Area of the tumour showing blood vessels (v) negative to 14F7, surrounded by tumour cells positive to it. Calibration bar: 50 μm.

Table 2. Table summarizing the articles where the expression of NeuGc-GM3 in normal tissues using the 14F7 antibody has been analyzed.

Normal tissue	Expression of NeuGc-GM3 using the 14F7 antibody	References
Colon	Negative	Lahera et al. (2014)
Normal lymphocytes of axillary lymph nodes and colon local lymph nodes	Negative	Blanco et al. (2013)
Melanotic nevi of the oral mucosa	Negative	Zhong et al. (2012)
Normal lung	Negative	Blanco et al. (2012)
Stomach, large intestine, pancreas and liver	All negative except some peritumoural areas of stomach	Blanco et al. (2011)
Adrenal neural tissue	Negative	Scursoni et al. (2011)
Normal mammary tissues	Negative	Marquina et al. (1996)
Normal retinal tissues	Negative	Higashi et al. (1988)
Non-tumoral fetal kidney	Negative	Scursoni et al. (2010)

massively disseminated tumours receiving neo-adjuvant chemotherapy were also studied. In these cases, we observed that NeuGc-GM3 immunoreaction persisted after the chemotherapy in the tumour cells of the eye enucleated thereafter treatment, without any perceivable reduction in the intensity and in the amount of immunoreactive cells. We could assume that chemotherapy did not modify the presence and localization of this ganglioside.

NeuGc-GM3 in metastatic sites

Bone marrow biopsy specimens, belonging to two patients with metastatic retinoblastoma, with almost complete replacement of the normal bone marrow by tumour cells were evaluated. In these cases, tumour cells were also positive for NeuGc-GM3 (Fig. 4) showing intense immunoreaction in cytoplasm and membrane to NeuGc-GM3. The surrounding tissue was negative.

Statistical analysis

We compared the immunoreactivity to 14F7 between non-invasive forms of retinoblastoma with invasive plus metastatic forms. We did not find any statistically significant difference between the IRS of these two situations ($p = 0.08$).

Discussion

Our data showed consistent immunoreactivity to the 14F7 monoclonal antibody, a highly specific IgG1 raised against NeuGc-GM3 as a tumour-associated antigen in retinoblastoma. It showed a pattern of membrane and cytoplasm staining possibly corresponding to lipids rafts or endocytotic vesicles (Miranda et al. 2011). All our retinoblastoma cases and cell lines were immunoreactive, including non-invasive with or without pathology risk factors associated with tumour invasiveness (Sastre et al. 2009), also invasive and metastatic, those previously treated and in two cases of metastatic dissemination to the bone marrow. As all tumours studied showed immunoreactivity to the 14F7 antibody, it was not possible to establish any correlation between the expression and any clinical finding or prognosis. Our data confirm the absence of NeuGc-GM3 in the normal retina and the lack of any

cross-reaction of the 14F7 antibody to normal retinal cells (Higashi et al. 1988). This highly consistent and specific expression may have clinical relevance in at least three scenarios.

(1) As a new marker for immunodiagnosis: As this ganglioside is virtually not expressed in any human healthy tissue, its identification may be useful for characterization of tumour cells for immunodiagnosis. Immunological characterization of tumour cells is, however, seldom needed for evaluating enucleated eyeballs with retinoblastoma because of the limited number of tumours that may involve the eye of children. Nevertheless, this may be important when tumour disseminates to other ocular structures or outside the eye as very few markers have been identified for immunodiagnosis and the differential diagnosis are important. In these situations, immunodiagnosis may help in differentiating retinoblastoma tumour cells from normal cells or from other neuroectodermic tumours. Following an initial description of its expression in enucleated eyeballs, our group previously reported the expression of another ganglioside (GD2) in retinoblastoma cells metastatic to the bone marrow (Chantada et al. 2006b). As retinoblastoma cells may resemble immature hematopoietic cells on microscopic examination, immunological characterization would be also useful in cases where suspicious tumour cells invade the choroid (Sastre et al. 2009). A similar use may be pursued for the CSF where normal pleocytosis may occur making the differential diagnosis difficult. However, as most tumours of neuroectodermal origin express this and other tumour-specific gangliosides in a similar pattern, lineage-specific markers such as the cone-rod homeobox-containing transcription factor (CRX) are preferable in cases when distinction between, for example a tumoural bone lesion arising from a metastatic retinoblastoma or a secondary bone tumour of neuroectodermal origin is needed (Terry et al. 2012).

(2) As a target-specific delivery of therapeutic agents for the treatment of retinoblastoma: In recent years, increased interest in local drug delivery was evident for retinoblastoma, including subconjunctival, intravitreal routes and intra-arterial chemotherapy (Abramson et al. 2008; Bartuma et al. 2013; Venturi et al. 2013; Parareda et al. 2014). To our knowledge, only conventional chemotherapy agents were given by these routes for retinoblastoma in the clinic. However, there are many examples of conjugated agents, including nanoparticles, bound to molecules expressed in the surface of retinoblastoma cells in order to improve the precision of delivery to tumour cells, sparing the normal structure of the eye. NeuGc-GM3 is an attractive molecule for this potential use as it was expressed in the surface of all evaluated retinoblastoma tumours, as opposed to other reported molecules used in retinoblastoma such as EpCam (Krishnakumar et al. 2004) and it is not expressed in other retinal cells. In addition, our results indicate that NeuGc-GM3 is also expressed in tumours after chemotherapy and radiotherapy treatment in a consistent fashion.

(3) As a candidate target for immunotherapy for disseminated disease: The positivity to 14F7 suggesting expression of NeuGc-GM3 in metastatic retinoblastoma as well as in both cases in which we evaluated the bone marrow makes it attractive for this use. However, further study of NeuGc-GM3 expression in metastatic retinoblastoma cells is warranted as we could only evaluate two patients because of the rarity of metastatic retinoblastoma in our centre limiting the availability of specimens for study.

In conclusion, the high and tumour-selective expression of NeuGc-GM3 as recognized by 14F7 monoclonal antibody in retinoblastoma warrants its future study as a tool for immunodiagnosis and for delivering targeted therapy to intraocular tumours or systemic immunotherapy of disseminated disease.

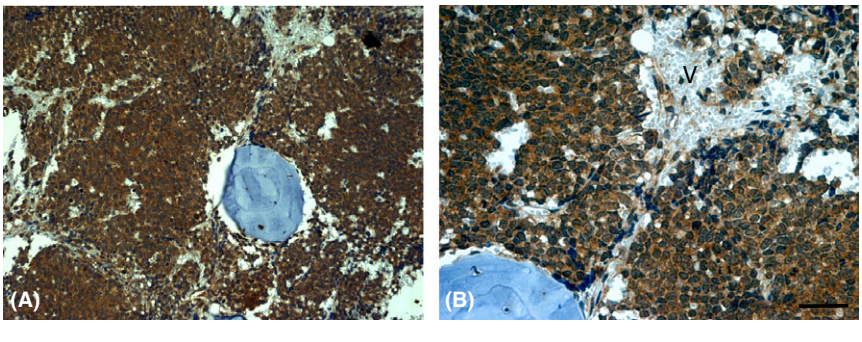

Fig. 4. (A, B) Microphotographies showing the immunoreactivity to 14F7 antibody against NeuGc-GM3 ganglioside in sections of bone marrow biopsy of a patient with metastatic retinoblastoma. (A) The tumour cells have completely replaced the normal bone marrow. All the tumour cells are positive to 14F7. (B) A bigger magnification of the bone marrow shows a blood vessel (v) that is not immunoreactive to 14F7, surrounded by tumour cells strongly positive to 14F7. Calibration bar: 100 and 50 μm (A and B respectively).

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Received on April 27th, 2014.
Accepted on September 23rd, 2014.

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The study was carried out at the Garrahan Pediatric Hospital and the Quilmes National University. V. Segatori is a research fellow and A.V. Torbidoni, M. Gabri and D. Alonso are members of CONICET (Argentina). Financial support: Grants from the National Agency of Scientific and Technological Promotion from Argentina. Preliminary results of this work were presented at the annual meeting of the Association for Research in Vision and Ophthalmology (ARVO) 2012, Fort Lauderdale, Florida, United States. No conflicting relationship exists for any of the authors.