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ABSTRACT

Gangliosides are glycolipids present on the cell surface. The N-glycolylated ganglioside NeuGc-GM3 has been described in some neoplasms, such as breast carcinoma and melanoma, but is usually not detected in normal human cells. Our aim was to evaluate the presence of NeuGc-GM3 in Wilms tumor by immunohistochemistry. Postchemotherapy tumors were grouped into different histologic subtypes considering the main preserved component. Formalin-fixed, paraffin-embedded tumor samples were cut into 5-μm sections. The monoclonal antibody 14F7, a mouse IgG1 that specifically recognizes NeuGc-GM3, and a peroxidase-labeled polymer conjugated to secondary antibodies were used. Sections from breast carcinoma were employed as positive controls. Presence of NeuGc-GM3 was evident in 22 of 25 (88%) cases. The staining was stronger in the epithelial component, with a membrane pattern and cytoplasmic diffusion. The stromal component expressed cytoplasmic NeuGc-GM3 in cells with rhabdomyoblastic differentiation. Tubules of adjacent renal tissue were also positive, but no expression of NeuGc-GM3 was detected in nontumoral fetal kidney. Until now, the expression of N-glycolylated gangliosides in pediatric solid tumors has not been investigated. The present study evidenced the expression of NeuGc-GM3 in a high proportion of Wilms tumors, suggesting its potential utility as a specific target of immunotherapy.

Key words: gangliosides, GM3, immunohistochemistry, immunotherapy, N-glycolyl neuraminic acid, nephroblastoma

INTRODUCTION

Gangliosides comprise a family of complex glycosphingolipids present on the cell surface, which have 1 or many sialic acid residues. They are abundant in the neuron plasma membrane, although they are also present in most cellular types in much smaller quantities [1]. Several gangliosides are involved in the communication of cells with tissue microenvironment and may participate in tumor-induced immunosuppression and malignant cell behavior [2].

Neosynthesis is one of the major concepts for cancer-associated alterations of cell surface carbohydrates, formulated by Hakomori and Kannagi 25 years ago based on observation of changes in glycolipid composition [3]. The most common sialic acids in mammals are N-acetylneuraminic (NeuAc) and N-glycolylneuraminic (NeuGc) acids. The key step in the biosynthesis of NeuGc is the conversion of NeuAc to NeuGc, which is catalyzed by the cytidine monophospho-N-acetylneuraminic acid hydroxylase [4]. NeuGc-containing gangliosides are normal components of cell membranes in all mammals except human beings. The lack of expression of NeuGc in humans is due to inactivation by a deletion of the hydrolase gene [5]. However, neosynthesis of carbohydrate determinants and expression of NeuGc gangliosides was observed in human cancer, possibly by diet incorporation of nonhuman sialic acid [6]. The N-glycolylated ganglioside NeuGc-GM3 has been described in some neoplasms, including breast carcinoma [7] and melanoma [8], but is usually not detected in normal human cells. This fact defines NeuGc-GM3 as an interesting target for immunotherapy [9].

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Hypoxia has a profound effect on the cell surface expression of carbohydrate determinants [10]. It was recently reported that tumor hypoxia induces a strong expression of NeuGc-gangliosides in human cancer cells. Tumor hypoxia stimulated the expression of sialin, a sialic acid transporter that enhances incorporation of the nonhuman sialic acid NeuGc [11].

In this regard, adult carcinoma tissues frequently express NeuGc-gangliosides, such as GM2 or GM3, specifically in the hypoxic areas in cancer nests [7,10]. On the contrary, NeuGc-gangliosides are virtually absent in nonmalignant epithelia.

In 1991, Sakakibara and colleagues [12] chromatographically analyzed the glycolipid patterns in Wilms tumor. They detected an increase of ganglioside GM3 in cancer tissues as compared with uninvolved tissues. However, the expression of the particular neo-antigen NeuGc-GM3 has not been previously investigated and there is little information about the presence of NeuGc-gangliosides in pediatric tumors.

Wilms tumor is one of the most common solid tumors of childhood and dramatic improvements have occurred as a result of advances in multimodality treatment [13]. It is considered an ''embryonic tumor'' of the kidney, being a mimicry of various elements in normal or abnormal nephrogenesis and presenting a diverse spectrum of histologic appearance. In this regard, Wilms tumor gives the unique opportunity to learn about the expression of NeuGc-gangliosides in diverse transformed cell lineages, comprising epithelial, stromal, and blastemal elements [13]. Furthermore, characterization of specific tumor antigens could be important to design novel immune-based treatment strategies, in order to reduce long-term complications of current protocols.

Previously, we reported that the novel monoclonal antibody racotumomab (formerly known as 1E10), an anti-idiotype vaccine that is able to induce a specific response against NeuGc-containing gangliosides, can activate significant antitumor effects in preclinical mouse tumor models [14]. Racotumomab showed promising results in clinical trials in patients with advanced breast carcinoma [15,16] and melanoma [17]. More recently, adults with non-small cell lung cancer were vaccinated after receiving standard chemotherapy, and no evidence of unexpected or serious adverse effects was reported [18].

The assessment of WT1 and p53 suppressor proteins can help the physician to perform an immunohistochemical characterization of Wilms tumor. The suppressor gene *WT1* plays an important role in normal development of the kidney and it is mutated in about 10% of Wilms tumors [19]. Interestingly, it is known that the anaplastic variant often expressed undetectable levels of *WT1* [20]. The immunohistochemical determination of p53 nuclear accumulation is not a direct indicator of the presence of mutations, but has demonstrated to be associated with poor prognosis in many tumors [21]. Accordingly, Sredni and colleagues [22] reported that p53 immunopositivity seems to be associated with aggressive disease and relapse.

The central aim of the present work was to evaluate the presence of the N-glycolylated ganglioside NeuGc-GM3 in Wilms tumor. Postchemotherapy tumors were grouped in different histologic subtypes, considering the main preserved component, and analyzed by immunohistochemistry. Additionally, WT1 and p53 proteins were detected as reference markers, providing complementary information about tumor biology.

METHODS

Archival cases and tissue processing

We retrospectively reviewed pathological specimens from 25 patients with a diagnosis of Wilms tumor treated at the Garrahan Pediatric Hospital (Buenos Aires, Argentina). All patients underwent surgical removal of the renal tumor after administration of 4 weeks of chemotherapy (weekly vincristine 1.5 mg/m^2 and biweekly actinomycin D 45 μg/kg). Histologic assessment, pathologic staging, and treatment protocols were in accordance with the SIOP 2001 guidelines from the International Society of Pediatric Oncology [23]. Guidelines only considered cases with more than one third of residual viable tumor after chemotherapy for classification in different histologic subtypes. Regressive and necrotic types were not considered in this study, since the viable part of the tumor involves less than one third of the tumor mass. Hence, suitable cases were selected and grouped in 5 subtypes, as follows: mixed subtype (at least 2 components including blastemal, epithelial, and/or stromal, none of them exceeding 66% of the residual viable tumor), epithelial subtype (>66% of epithelial component in the residual viable tumor and scattered foci of blastemal areas not exceeding 10%), stromal subtype (>66% of stromal component in the residual viable tumor and blastemal areas not exceeding 10%), blastemal subtype (>66% of blastemal component in the residual viable tumor and variable proportions of other components), and anaplastic subtypes (tumors with histologic criteria of anaplasia, either in focal or diffuse patterns). According to the SIOP 2001 Working Classification of renal tumors of childhood, the mixed, epithelial, and stromal subtypes belong to the intermediate-risk tumors, while blastemal and anaplastic subtypes fit into high-risk tumors [23]. Ten of the cases analyzed also included adjacent nontumoral renal tissue, and 3 samples of normal fetal kidney were also examined. Sections of 5 μm from formalin-fixed, paraffin-embedded tumor samples were used. We previously demonstrated that the routine technique did not extract antigenic carbohydrate determinants of gangliosides, thus allowing immunohistochemical detection in tumor sections [24].

Primary antibodies

The antiganglioside monoclonal antibody 14F7 was produced by the Center of Molecular Immunology (La

Table 1. NeuGc-GM3, WT1, and p53 immunopositivity in the different histological subtypes of Wilms tumor

Histological subtype	NeuGc-GM3 Positive cases[a] (%)	NeuGc-GM3 Positive tumor cells[b] (%)	NeuGc-GM3 Predominant intensity[c]	WT1 Positive cases[a] (%)	p53 Positive cases[a] (%)
Epithelial	5/5 (100)	80 ± 5.5	3+	4/5 (80)	0/5 (0)[d]
Mixed	5/5 (100)	78 ± 7.3	3+	5/5 (100)	1/5 (20)[d]
Anaplastic	3/5 (60)	52 ± 21.3	2+	2/5 (40)	5/5 (100)[d]
Blastemal	4/5 (80)	60 ± 15.5	1+	3/5 (60)	3/5 (60)[d]
Stromal	5/5 (100)	36 ± 7.5[e]	2+	2/5 (40)	1/5 (20)[d]
Total	22/25 (88)	61 ± 6.3	2+	16/25 (64)	10/25 (40)

[a]Positive/total cases.
[b]Values are means ± SEM.
[c]Intensity of the positive staining was scored as 1+ = mild; 2+ = moderate; 3+ = intense.
[d]Significantly inversely correlated with NeuGc-GM3 immunopositivity, within the different histological subtypes; $r = -0.97$, $P < 0.01$ (Pearson test).
[e]Significantly different from epithelial and mixed subtypes; $P < 0.05$ (Kruskal-Wallis test with Dunn posttest).

Habana, Cuba) and used at a final concentration of 20 μg/mL. The 14F7 antibody is a mouse IgG1 that specifically recognizes NeuGc-GM3, as previously described [8]. Detection of WT1 protein was performed using the specific mouse monoclonal antibody 6F-H2 (DakoCytomation, Carpinteria, CA, USA) at a dilution of 1:50. The p53 protein was detected by the mouse monoclonal antibody DO-7 (DakoCytomation) at a dilution 1:50. The DO-7 antibody recognizes both normal p53 protein and p53 oncoprotein.

Immunohistochemistry

After reaction of primary antibodies, all sections were incubated with a peroxidase-labeled polymer conjugated to secondary anti-mouse antibodies using the DakoCytomation EnVision+ System-HRP(DAB) and developed with 3,3′-diaminobenzidine as chromogen. Proper positive and negative controls were made in every staining battery. Sections from breast carcinoma were employed as positive controls of NeuGc-ganglioside detection [7]. Immunohistochemical expression was semiquantitatively evaluated. We scored the intensity of the staining from 0 to 3: 0 = no staining; 1+ = mild; 2+ = moderate; 3+ = intense. In addition, the percentage of positive tumor cells was measured for each specimen. Tumors were classified as negative when no staining was observed or only <20% of cells were positive. Results were scored by 3 independent pathologists.

RESULTS

Presence of NeuGc-GM3 ganglioside was evident in 22 of 25 (88%) cases of Wilms tumors, as detected by immunohistochemistry using the specific 14F7 monoclonal antibody. All cases corresponding to epithelial, stromal, or mixed histologic subtypes were positive, whereas some negative cases occurred in anaplastic or blastemal tumors. On average, more than 50% of tumor cells were positive in all histologic subtypes, with the exception of the stromal tumors, which showed a significantly lower percentage ($P < 0.05$) of NeuGc-GM3–positive tumor cells (Table 1).

NeuGc-GM3 staining was moderate to intense in most cases, although blastemal tumors tended to show a mild staining. Representative pictures of the different histological subtypes are shown in Figure 1. The staining was stronger in the epithelial component, with a membrane pattern and cytoplasmic diffusion. The stromal component expressed cytoplasmic NeuGc-GM3 in cells with rhabdomyoblastic differentiation. In addition, blastemal and stromal components showed some NeuGc-GM3 membrane expression. Tubules of adjacent renal tissue were positive for NeuGc-GM3 in the cytoplasmic compartment in all cases analyzed (Fig. 2A), suggesting the shedding of gangliosides from tumor cells. In this regard, no expression of NeuGc-GM3 was detected in all samples of nontumoral kidney tissue from fetal autopsy (Fig. 2B).

As shown in Table 1, most cases of Wilms tumors of the epithelial and mixed subtypes were positive for WT1 protein. In the case of the mixed subtype, all epithelial components expressed WT1, while only some blastemal components were positive. Regarding p53 protein accumulation, immunodetectable expression was particularly evident in all cases of anaplastic tumors and also in most cases of blastemal tumors. A significant inverse correlation ($P < 0.01$) was found within the different histological subtypes between the percentage of positive cases for p53 expression and the percentage of positive cases for NeuGc-GM3 (see also Table 1).

DISCUSSION

To the best of our knowledge, this is the first report on the expression of N-glycolylated gangliosides in pediatric solid tumors. Our immunohistochemical study using a specific monoclonal antibody evidences NeuGc-GM3 expression in 88% of cases of Wilms tumor analyzed. The strongest expression was found in the epithelial component of Wilms tumor and the lower percentage of positive tumor cells was observed in the stromal subtype.

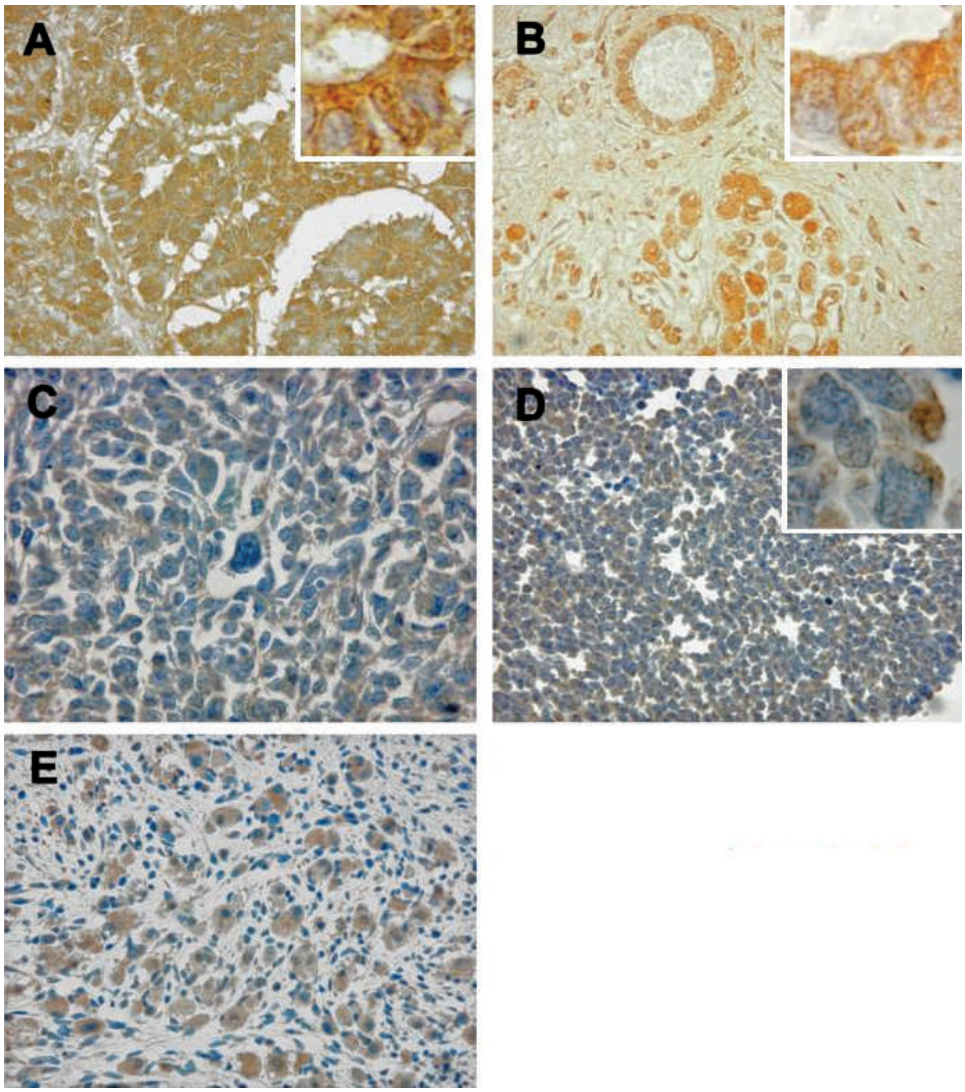

Figure 1. Immunohistochemical detection of NeuGc-GM3 ganglioside in the different histological subtypes of Wilms tumor. **A.** Epithelial. **B.** Mixed. **C.** Anaplastic. **D.** Blastemal. **E.** Stromal. Insets show intense membrane expression with cytoplasmic diffusion in epithelial components (**A** and **B**), and mild staining in some blastemal cells (**D**). Original magnification ×400 (insets ×1000). A color version of this figure is available online.

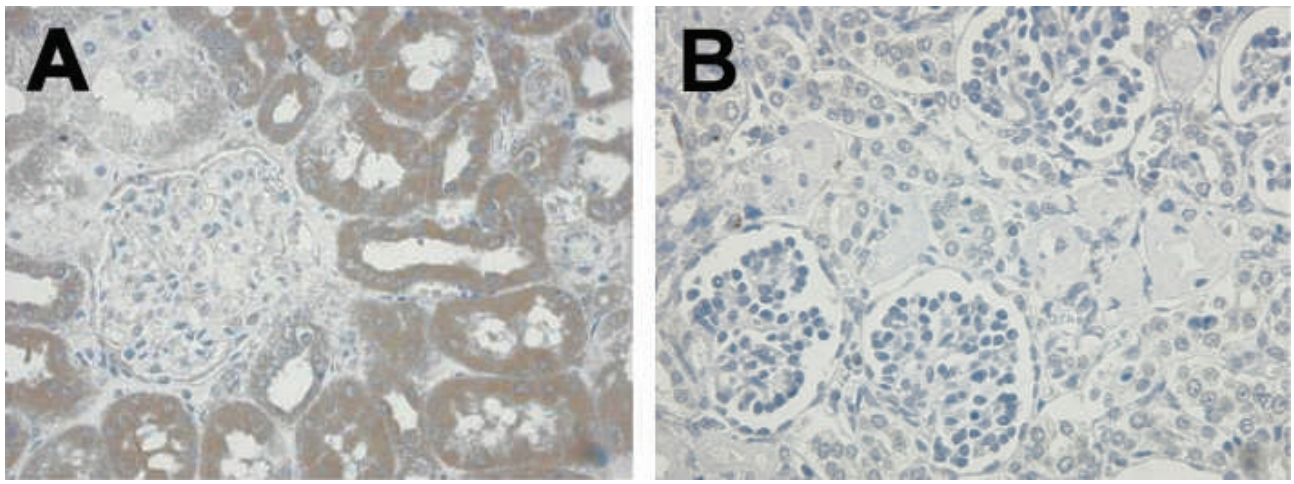

Figure 2. Immunohistochemical analysis of NeuGc-GM3 expression in nontumoral renal tissue. **A.** Adjacent non-tumoral renal tissue in Wilms tumor. **B.** Normal kidney from fetal autopsy. Original magnification ×400. A color version of this figure is available online.

In this regard, it is known that complex glycosphingolipids are abundant in cells of neuroectodermal origin and are also present in some epithelial cells [25]. We have obtained similar results in a preliminary study with the P3 monoclonal antibody, a less-specific IgM that recognizes different NeuGc-containing gangliosides and sulfatides, including NeuGc-GM3 [26]. More than 70% of Wilms tumors showed a positive staining for NeuGc residues using the P3 antibody (data not shown).

Results demonstrated that nonneoplastic fetal kidney tissue was negative for NeuGc residues, supporting the idea that NeuGc-GM3 may be considered a neo-antigen associated with renal transformation. Detection of the gangliosides in the tubules of renal tissue adjacent to the tumor mass may indicate the shedding of NeuGc from transformed cells. Gangliosides can be actively shed from the cell surface of 1 cell and taken up by other cells by insertion of their lipid anchors into the membrane [27]. Tumor cells apparently misuse this process for their own advantage, but its real physiological functions remain to be discovered.

Mammalian cells are covered by a dense glycocalyx, composed of glycolipids, glycoproteins, glycophospholipid anchors, and proteoglycans. Sialic acids attached to cell surface glycoconjugates play important roles in many physiological and pathological processes, including microbe binding that leads to infections, regulation of the immune response, and progression and spread of human malignancies [28]. The possibility that NeuGc-containing glycoconjugates are taken up directly from the diet must be taken into account. However, the potential role of alternative biosynthetic pathways of NeuGc in human neoplasias is not known [29].

Our data showed that most cases of Wilms tumors of the epithelial and mixed subtypes were positive for normal WT1 protein and negative for abnormal p53 accumulation. The same histologic subtypes demonstrated the higher levels of immunohistochemical expression of NeuGc-GM3. Detection of abnormal p53 protein expression was clearly evident in all anaplastic tumors, an observation that is in agreement with previous studies on primary nephrectomy specimens and recurrent tumors [30]. We observed that anaplastic tumors also presented a lower immunopositivity for NeuGc-GM3. Considering the histologic subtypes analyzed, a significant inverse correlation between p53 accumulation and detection of NeuGc-GM3 was found. This may suggest an abundance of the ganglioside associated with intact suppressor proteins in Wilms tumor.

The use of preoperative chemotherapy changes the distribution pattern as well as the prognostic value of the different histologic subtypes compared to tumors treated with immediate surgery. Viable tumors after preoperative chemotherapy with blastemal-predominant histology or diffuse anaplasia have a poorer prognosis than epithelial, stromal, or mixed subtypes [23,31]. As hypoxia-resistant cancer cells are known to have diminished response to chemotherapy, it is important to find potential target molecules for novel antitumor strategies [10]. In this context, resistant cancer cells could overexpress NeuGc-containing gangliosides under hypoxic conditions [11].

The absence of NeuGc-containing gangliosides in normal human tissues makes these gangliosides immunogenic. In fact, antibodies that recognize gangliosides containing NeuGc residues appear after administration of animal serum to humans [32]. Although the number of cases is small, the present characterization of a specific neo-antigen in Wilms tumor may be of value for the design of immunotherapeutic protocols. Complementary therapy with an antitumor vaccine may reduce the need for high doses of the cytotoxic drugs used in current protocols or improve chemotherapy results in resistant patients. Future studies with larger series of Wilms tumors, as well as other pediatric solid tumors, could correlate the immunohistochemical expression of NeuGc-GM3 with established clinical variables, such as staging, treatment, and disease progression.

REFERENCES

1. Bitton RJ, Guthmann MD, Gabri MR, et al. Cancer vaccines: an update with special focus on ganglioside antigens. Oncol Rep 2002; 9:267–276.
2. Patra SK. Dissecting lipid raft facilitated cell signaling pathways in cancer. Biochim Biophys Acta 2008;1785:182–206.
3. Hakomori S, Kannagi R. Glycosphingolipids tumor–associated and differentiation markers: a guest editorial. J Natl Cancer Inst 1983; 71:231–251.
4. Schlenzka W, Shaw L, Kelm S, et al. CMP-N-acetylneuraminic acid hydroxylase: the first cytosolic Rieske iron-sulphur protein to be described in Eukarya. FEBS Lett 1996;385:197–200.
5. Irie A, Suzuki A. CMP-N-acetylneuraminic acid hydrolase is exclusively inactive in humans. Biochem Biophys Res Comun 1998;248:330–333.
6. Tangvoranuntakul P, Gagneux P, Diaz S, et al. Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid. PNAS 2003;100:12045–12050.
7. Marquina G, Waki H, Fernandez LE, et al. Gangliosides expressed in human breast cancer. Cancer Res 1996;56:5165–5171.
8. Carr A, Mullet A, Mazorra Z, et al. Mouse IgG1 monoclonal antibody specific for N-glycolyl GM3 ganglioside recognized breast and melanoma tumors. Hybridoma 2000;19:241–247.
9. Fernandez LE, Alonso DF, Gomez DE, Vazquez AM. Ganglioside-based vaccines and anti-idiotype antibodies for active immunotherapy against cancer. Expert Rev Vaccines 2003;2:817–823.
10. Kannagi R, Yin J, Miyazaki K, Izawa M. Current relevance of incomplete synthesis and neo-synthesis for cancer-associated alteration of carbohydrate determinants-Hakomori's concepts revisited. Biochem Biophys Acta 2008;1780:525–531.
11. Yin J, Hashimoto A, Izawa M, et al. Hypoxic culture induces expression of sialin, a sialic acid transporter, and cancer-associated gangliosides containing non-human sialic acid on human cancer cells. Cancer Res 2006;66:2937–2945.
12. Sakakibara N, Gasa S, Kamio K, et al. Distinctive glycolipid patterns in Wilms' tumor and renal cell carcinoma. Cancer Lett 1991;57:187–192.
13. Kaste SC, Dome JS, Babyn PS, et al. Wilms tumour: prognostic factors, staging, therapy and late effects. Pediatr Radiol 2008;38:2–17.
14. Vázquez AM, Gabri MR, Hernández AM, et al. Antitumor properties of an anti-idiotypic monoclonal antibody in relation to N-glycolyl-containing gangliosides. Oncol Rep 2000;7:751–756.
15. Diaz A, Alfonso M, Alonso R, et al. Immune responses in breast cancer patients immunized with an anti-idiotype antibody mimicking NeuGc-containing gangliosides. Clin Immunol 2003;107:80–89.

16. Guthmann MD, Castro MA, Cinat G, et al. Cellular and humoral immune response to N-glycolyl-GM3 elicited by prolonged immunotherapy with an anti-idiotypic vaccine in high-risk and metastatic breast cancer patients. J Immunother 2006;29:215–223.
17. Alfonso M, Diaz A, Hernandez AM, et al. An anti-idiotype vaccine elicits a specific response to N-glycolyl sialic acid residues of glycoconjugates in melanoma patients. J Immunol 2002;168:2523–2529.
18. Alfonso S, Díaz RM, De la Torre A, et al. 1E10 anti-idiotype vaccine in non-small cell lung cancer: experience in stage IIIb/IV patients. Cancer Biol Ther 2008;6:1847–1852.
19. Ramani P, Cowell JK. The expression pattern of Wilms' tumour gene (WT1) product in normal tissues and paediatric renal tumours. J Pathol 1996;179:162–168.
20. Hazen-Martin DJ, Re GG, Garvin AJ, Sens DA. Distinctive properties of an anaplastic Wilms' tumor and its associated epithelial cell line. Am J Pathol 1994;144:1023–1034.
21. Quinlan DC, Davidson AG, Summers CL. Accumulation of p53 protein correlates with poor prognosis in human lung cancer. Cancer Res 1992;52:4828–4831.
22. Sredni ST, Camargo B, Lopes LF, Teixeira R, Simpson A. Immunohistochemical detection of p53 protein expression as a prognostic indicator in Wilms tumor. Med Pediatr Oncol 2001;37:455–458.
23. Vujanic GM, Sandstedt B, Harms D, et al. Revised International Society of Pediatric Oncology (SIOP) working classification of renal tumors of childhood. Med Pediatr Oncol 2002;38:79–82.
24. Alonso DF, Gabri MR, Guthmann MD, Fainboim L, Gomez DE. A novel hydrophobized GM3 ganglioside/*Neisseria meningitidis* outer membrane protein complex vaccine induces tumor protection in B16 murine melanoma. Int J Oncol 1999;15:59–66.
25. Simons K, van Meer G. Lipid sorting in epithelial cells. Biochemistry 1988;27:6197–6202.
26. Vazquez AM, Alfonso M, Karlsson A, et al. Generation of a murine monoclonal antibody specific for N-glycolylneuraminic acid containing gangliosides that also recognizes sulfated glycolipids. Hybridoma 1995;14:551–556.
27. Lauc G, Heffer-Lauc M. Shedding and uptake of gangliosides and glycosylphosphatidylinositol-anchored proteins. Biochim Biophys Acta 2006;1760:584–602.
28. Varki NM, Varki A. Diversity in cell surface sialic acid presentations: implications for biology and disease. Lab Invest 2007;87:851–857.
29. Malykh YN, Schauer R, Shaw L. N-glycolylneuraminic acid in human tumours. Biochimie 2001;83:623–634.
30. Hill DA, Shear TD, Liu T, Billups CA, Singh PK, Dome JS. Clinical and biologic significance of nuclear unrest in Wilms tumor. Cancer 2003; 97:2318–2326.
31. Vujanic GM, Harms D, Sandstedt B, Weirich A, de Kraker J, Delemarre JF. New definitions of focal and diffuse anaplasia in Wilms tumor: the International Society of Paediatric Oncology (SIOP) experience. Med Pediatr Oncol 1999;32:317–323.
32. Livingston PO. Approaches to augmenting the immunogenicity of melanoma gangliosides: from whole melanoma cells to ganglioside-KLH conjugate vaccines. Immunol Rev 1995;145:147–166.