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# Immunoreaction of 14F7 Mab Raised against N-Glycolyl GM3 Ganglioside Correlates with High Histological Grade in some Tumors of Neuroectodermal and Epithelial Lineage

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## Abstract

The aberrant expression of N-glycolyl GM3 ganglioside (NeuGcGM3) has been reported in a variety of malignant tumors. Nevertheless, the relationship between NeuGcGM3 expression and aggressive biological behavior still remains unclear for the majority of malignancies. In this article the tissue reactivity of the 14F7 monoclonal antibody, a highly specific IgG1 against NeuGcGM3, in breast cancer, urinary bladder tumors and malignant gliomas of adult patients is shown as well as its relation with the histological grade of these malignancies. The expression of NeuGcGM3 was detected in 92/155 (59.3%) of tumors independently of the histopathological classification. However, a preferential expression of NeuGcGM3 was detected in: infiltrating ductal carcinoma (77.1%) vs. infiltrating lobular carcinoma (15.9%) (p=0.024), grade III (94.9%) vs. grade II (77.8%) and grade I (63.8%) transitional cell carcinoma (p=0.042) and high-grade astrocytomas (78.6%) vs. low-grade astrocytic tumors (10.0%) (p=0.026). The results achieved suggest the relationship between the tissue expression of NeuGcGM3 and the more aggressive biological behavior of these malignancies, regardless of the tumor cell lineage. The data obtained also support the continuous use of NeuGcGM3 as a target for immunotherapy in malignancies expressing this molecule as well as that of 14F7 monoclonal antibody for the selection of candidate patients for these specific therapies.

**Keywords:** N-glycolyl GM3 ganglioside; 14F7 Mab; Tissue reactivity; Neuroectodermal and epithelial tumors; High histological grade

**Abbreviations:** CD: Cluster of Differentiation; CMP-NeuAc Hydroxylase: Cytidine Monophospho-N-Acetylneuraminic Acid Hydroxylase; DAB: 3,3-Diaminobencidine; DCs: Dendritic Cells; GT: Glycosyltransferase; IDC: Infiltrating Ductal Carcinoma; IgG1: Immunoglobulin Subclasse 1; ILC: Infiltrating Lobular Carcinomas; LSAB: Labelled-Streptavidin Biotin System; Mab: Monoclonal Antibody; NeuAc: N-Acetylneuraminic Acid; NeuGc: N-Glycolylneuraminic Acid; NeuGcGM3: N-Glycolyl GM3 Ganglioside; NOS: No Otherwise Specified; OMRS: Other Minorly Represented Subtype; STMR: Subtypes Minor Represented; TMA: Tissue Micro Arrays; VSSP: Very Small Size Proteoliposomes

## Introduction

Gangliosides are sialic acid-bearing glycosphingolipids engaged in a variety of biological events that occur at vertebrate´s cell membrane [1-3]. During malignant transformation, the composition of gangliosides changes at both quantitative and qualitative levels. Among the molecules contributing to tumor-associated carbohydrate structures of glycosphingolipids, sialic acid is considered one of the most important [4,5].

The two major sialic acid variants in mammals are N-acetylneuraminic acid (NeuAc) and N-glycolylneuraminic acid (NeuGc). NeuAc is the biosynthetic precursor of NeuGc, which is a component of gangliosides in most animal species. The conversion of NeuAc to NeuGc is catalyzed by the cytidine monophospho-NeuAc hydroxylase enzyme. However, in humans this enzyme is inactive [6]. In this sense, the aberrant expression of the NeuGc residue in humans has been associated with its incorporation from dietary sources to the altered metabolism of malignant cells [4,7], also favored by the hypoxic conditions of tumors [8].

Unusual glycolylated gangliosides have been identified by immunohistochemistry in a variety of human malignancies. Specifically, N-glycolyl GM3 ganglioside (NeuGcGM3) has shown limited expression in human normal tissues; however, it has been detected in several tumors [9-14] by means of 14F7 monoclonal antibody (Mab) reactivity. This Mab is a mouse IgG1 specific for NeuGcGM3 [15]. As a consequence, NeuGcGM3 has become an attractive target for both active and passive immunotherapy of these malignancies.

Interestingly, *in vitro* and *in vivo* models demonstrated the immunosuppressive effects of NeuGcGM3 as well as its contribution to tumor cells progression [16-18]. In line with these results, the expression of NeuGcGM3 was associated with a more aggressive disease in colon adenocarcinoma [19] and non-small cell lung cancer [20]. However, opposite results were also reported [21,22]. In addition, differential expression of NeuGcGM3 has been detected in relation with the histological classification of tumors [10,23]. Nevertheless, the biological significance of this ganglioside still remains unclear for the majority of malignant tumors.

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In the present work the tissue expression of NeuGcGM3 in breast cancer, urinary bladder tumors and malignant gliomas of adult patients as well as its relation with the histological grade of these neoplasms is shown.

## Materials and Methods

### Monoclonal antibody

14F7 Mab (IgG1), a highly specific anti-NeuGcGM3 ganglioside antibody, produced by the Center of Molecular Immunology (Havana, Cuba) was used. This Mab was generated with the methodology previously described in ref. [15]. 14F7 Mab is able to detect the carbohydrate antigenic determinant of NeuGcGM3 ganglioside after formalin fixation and routine tissue processing [20,24].

### Study design

A retrospective, observational study of a consecutive series of patients diagnosed with breast cancer (n=46), urinary bladder malignant tumors (n=55) and malignant gliomas (n=54) was performed. All tissue samples were received from the pathology departments of both National Institute of Oncology and Radiobiology (Havana, Cuba) and the National Institute of Neurology and Neuropathology (Havana, Cuba), after obtaining approved consent from the institutional ethical committees.

### Tissue specimens and previous processing

For urinary bladder tumors, tissue micro arrays (TMA) were made. Briefly, each block of paraffin-embedded tumor material was cut into 5 μm-thick sections and placed on glass slides (Dako, S2024). Slides were stained with Hematoxylin and eosin and an expert pathologist verified the presence of tumor cells and marked the tumor area. Two mm diameter cylinders were taken from each block and they were included in recipient tissue array blocks using a precision tissue array instrument (Thermo Scientifics, TMA-001).

For all samples, five micrometer serial sections from each paraffin block were obtained in a microtome (Lizt 1512, Germany). All sections were attached to the slides by heating at 60°C in oven for 1 hour. Afterward, the slides were kept at room temperature until they were used. The slides were dewaxed in xylene and rehydrated in graded ethanol series following the commonly used procedure for this operation and endogenous peroxidase activity was blocked with the peroxidase-blocking reagent (Dako, K5207) for 10 minutes. All sections were rehydrated in distilled water for 10 minutes and then rinsed with wash buffer (Dako, K5207).

### Immunohistochemical staining

Slides were placed in a humid chamber and incubated with 14F7 Mab for 1h at room temperature. Negative controls were performed substituting the primary antibody for the washing buffer. Sections of ductal breast carcinoma were taken as positive control [11].

After two rinses in wash buffer, the slides were incubated with the reagents of LSAB detection system (Dako, K0690) for 30 minutes in each step. Between incubations, samples were washed with wash buffer for 10 minutes. Enzymatic activity was visualized with DAB substrate chromogen solution (Dako K3465). Slides were counterstained with Mayer´s Hematoxylin (Dako S2020), dehydrated and mounted with a synthetic medium.

### Evaluation of results

The intensity of the reaction of each sample was qualitatively estimated and expressed as follows: negative (0), weak (1), moderate (2) and intense (3). Then, in each specimen, the percentage of positive tumor cells in the most representative areas was measured using a 100X magnification. Results obtained by two independent observers were taken to do the final evaluation of the analysis. Afterward, the percentage of positive cells and the intensity of reaction were multiplied in each specimen, resulting in a score ranging from 0 to 300. Finally, these scores were classified in this manner: low expression (scores $< 150$) and high expression (scores $\geq 150$) to obtain the final score (F-score) as previously described in ref. [20].

### Statistical analysis

GraphPad Prism 5 software (2007 GraphPad Software Inc. La Jolla, CA USA) was used for data analysis. The relation between 14F7 Mab immunoreactivity and histological grade of malignant tumors was analyzed using the Chi-square test. Correlations were assessed by Spearman ranks correlation coefficients. A p value $< 0.05$ was considered statistically significant.

## Results

### Immunohistochemical localization of NeuGcGM3

In general, the reactivity of 14F7 Mab was observed on both plasmatic membrane and cytoplasm of malignant cells with a homogeneous and finely granular pattern of staining as previously described in ref. [24]. According to the F-score, the expression of NeuGcGM3 was detected in 92/155 (59.3%) of tumors independently of the histopathological classification.

### Breast cancer

A weak to intense reactivity with 14F7 Mab was observed in 45/46 (97.8%) of breast carcinomas, regardless of the histological subtype of tumors (Table 1). Only, one case of infiltrating lobular carcinoma was not reactive to this Mab. In all positive cases, the expression of NeuGcGM3 was observed in more than 95% of malignant epithelial cells. However, only 31/46 (67.4%) of breast carcinomas showed high levels of NeuGcGM3 expression. The staining of 14F7 Mab according to the histological subtype of tumors followed this distribution: 13/15 (86.7%) of no otherwise specified (NOS) infiltrating ductal carcinomas (IDC), 14/20 (70.0%) of other minorly represented subtype (OMRS) infiltrating ductal carcinomas and 4/11 (15.9%) of infiltrating lobular carcinomas (ILC).

A preferential expression of this ganglioside was detected in IDC when compared with ILC (77.1% vs 15.9%). A statistically significant difference was evidenced when the immunoreaction with 14F7 Mab in these tumors was compared ($p=0.024$; Chi-square test) (Figure 1). But, no difference was obtained when the expression of NeuGcGM3 was compared in NOS infiltrating ductal carcinomas and OMRS infiltrating ductal carcinomas.

### Urinary bladder tumors

The tissue expression of NeuGcGM3 was observed in 49/55 (89.1%) cases and different intensities of reaction and percentage of positive cells were obtained. However, according to the F-score 46/55 (83.6%) of urinary bladder tumors showed high levels of 14F7 Mab staining (Table 1). Compared with other urinary bladder tumors, transitional cell carcinoma exhibited increased levels of NeuGcGM3 expression in 45/53 (84.9%) cases without taking into consideration the degree of cellular atypia gradation.

The tissue expression of NeuGcGM3 increased progressively together with the histological grade of tumors 63.6% (7/11), 77.8% (7/9)

| Histopathological classification | 14F7 Mab reactivity | | Positive cases (%) |
|---|---|---|---|
| | F-score | | |
| | Low | High | |
| Breast cancer | | | |
| Infiltrating lobular carcinoma | 7 | 4 | 15.9 |
| Infiltrating ductal carcinoma | | | |
| *No otherwise specified (NOS)* | 2 | 13 | 86.7 |
| *Other subtype minor represented* | 6 | 14 | 70.0 |
| Total | 15 | 31 | 67.4 |
| Urinary bladder tumors | | | |
| Urothelial cell carcinoma | | | |
| *Grade I* | 4 | 7 | 63.6 |
| *Grade II* | 2 | 7 | 77.8 |
| *Grade III* | 2 | 31 | 93.9 |
| Squamous cell carcinoma | 0 | 1 | 100 |
| Undifferentiated tumor | 1 | 0 | 0 |
| Total | 9 | 46 | 83.6 |
| Malignant gliomas | | | |
| Astrocytomas | | | |
| *Grade I* | 4 | 0 | 0.0 |
| *Grade II* | 5 | 1 | 16.7 |
| *Grade III* | 9 | 4 | 30.8 |
| *Grade IV* | 5 | 7 | 58.3 |
| Other low-grade gliomas | 16 | 3 | 15.8 |
| Total | 39 | 15 | 27.8 |

Legend. %, percentage; F-score: Low expression of NeuGcGM3 (F-score<150); High expression of NeuGcGM3 (F-score ≥ 150).

**Table 1:** Immunoreaction of 14F7 Mab according to the histological classification of malignant tumors.

and 93.9% (31/33) for grade I, II and III transitional cell carcinoma, respectively. Although, only a statistical trend was obtained (p=0.079, Spearman coefficient=0.260; Spearman Test). A statistically significant association was evidenced when the expression of NeuGcGM3 was compared in grade I, II and grade III transitional cell carcinoma (p=0.042; Chi-square test) (Figure 2).

## Malignant gliomas

In general, high levels of NeuGcGM3 expression was evidenced in 15/54 (27.8%) of all malignant gliomas (Table 1). Low-grade astrocytoma (I-II) showed a limited reactivity with 14F7 Mab (1/10). But, the recognition of 14F7 Mab gradually was associated with the progressive increase in the malignancy of malignant astrocytomas with 0.0% (0/4), 16.7% (1/6), 30.8% (4/13) and 58.3% (7/12) of positive cases for grade I, II, III and IV astrocytomas, respectively (p=0.013, Spearman coefficient=0.414; Spearman Test). Consequently, the reactivity of 14F7 Mab was greater in high-grade astrocytomas as compared to low-grade tumors (p=0.026; Chi-square Test).

In addition, the reaction with 14F7 Mab was evidenced in 3/19 (15.8%) cases of other low-grade gliomas. A statistically significant difference was obtained when the staining with this Mab was compared in other low-grade gliomas and high-grade astrocytomas (p=0.015; Chi-square Test) (Figure 3). However no statistically significant difference was evidenced between the expression of NeuGcGM3 in low-grade astrocytomas and other low-grade gliomas.

## Discussion

Changes in the cell surface glycolipids composition that take place during malignant transformation have been previously described [5]. The appearance of specific carbohydrate epitopes in certain tumors have been reported to affects their progression, invasive and metastatic potential [25,26]. In particular, some studies have shown the relevance of NeuGcGM3 in cancer progression. NeuGcGM3 appeared to be involved in tumor-induced DC suppression [21], in the down-modulation of CD4 expression in T lymphocytes [16] as well as, in the proliferation and differentiation of $CD4^{+}CD25^{-}$ T lymphocytes [17], abrogating an anti-tumor-specific immune response.

Breast tumour tissues were shown to be distinct from normal mammary tissues in terms of both ganglioside and glycosyltransferase (GT) genes composition. The gangliosides GM3 (the N-acetyl variant), GD3 and the derivatives 9-*O*-acetyl-GD3 (CDw60 antigen) and 9-*O*-acetyl-GT3, which show a very restricted expression in normal breast tissues, are over-expressed in IDC [27]. It was also suggested that GM3 or GD3 synthase (ST8Sia I) overexpression may contribute to increasing the malignant properties of breast cancer cells by mediating cell proliferation and migration [28]. ST8Sia I over-expression was associated with both poor histopathological grading in oestrogen receptor-negative tumors and lower survival of patients [29].

Marquina et al. reported that the expression of NeuGcGM3 in breast tumors using mass spectrometry analysis [27]. Later, Carr et al. reported the generation of 14F7 Mab, a highly specific IgG against NeuGcGM3 that recognizes breast infiltrating ductal carcinoma and cutaneous melanoma by immunohistochemistry on both frozen [15] and formalin-fixed and paraffin embedded samples [11,14]. The ability of 14F7 Mab labelled with 99mTc to recognize breast tumors *in vivo* by the radioimmunoscintigrafic technique was also demonstrated [30]. Nevertheless, no information about the potential role of NeuGcGM3 in the biological behavior of breast cancer has been found in the literature consulted.

In this paper, differences in the levels of NeuGcGM3 expression according to the histological subtype of breast carcinoma are reported for the first time. These results were obtained by means of the application of a novel score that integrates the percentage of 14F7-positive cells with the intensity of the immunohistochemical reaction (F-score) [20]. A preferential expression of NeuGcGM3 was evidenced in IDC as compared with ILC, indicating a more aggressive biological behavior

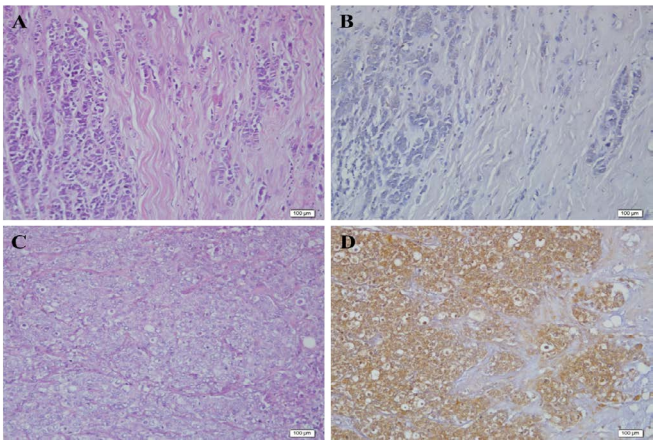


**Figure 1:** Immunoreactiviy of 14F7 Mab in breast cancer. A and C: Hematoxylin and eosin staining of infiltrating lobular and ductal carcinomas, respectively. Observe: the very weak expression of NeuGcGM3 in the infiltrating lobular carcinoma section (B) while an intense reaction with 14F7 Mab was evidenced in infiltrating ductal carcinoma (D) (Brown color). Counterstaining with Mayer's Hematoxylin (Blue color). White bar=100 µm.

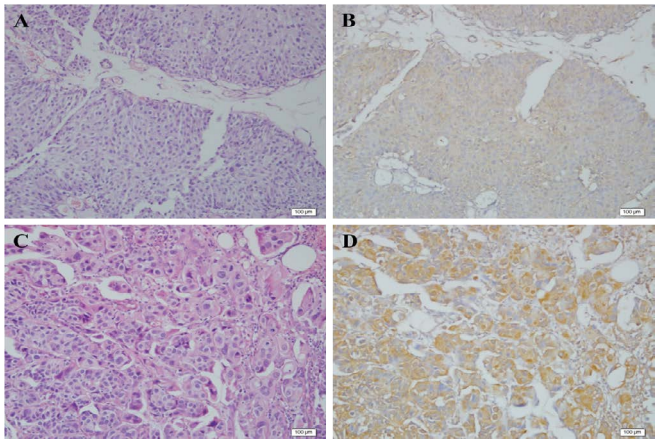


**Figure 2:** Immunostaining of 14F7 Mab in urinary bladder tumors. A and C: Hematoxylin and eosin staining of grade I and grade III transitional cell carcinomas, respectively. Observe: a low expression of NeuGcGM3 (Brown color) in the grade I transitional cell carcinoma sample (B). By the contrary, high level of this ganglioside was detected in grade III tumor (D). Counterstaining with Mayer's Hematoxylin (Blue color). White bar=100 µm.

of these malignancies. In line with these results, it was demonstrated that the metastatic potential and the stage-matched prognosis is worse for patients with IDC than ILC [31].

In a previous work, Blanco et al. reported the expression of NeuGcGM3 in lymph node metastasis of breast cancer as well as its incorporation to the cell membrane of peritumoral lymphocytes [14]. A clear association between NeuGcGM3 insertion in lymphocyte plasma membranes and the CD4 down-modulation effect was also documented [16]. In this way, our results and those concerning to the high expression of NeuGcGM3 in lymph node metastasis of breast infiltrating carcinoma suggest that higher levels of this ganglioside could contribute to a more accelerated cancer progression and metastasis of IDC. In line with this, the expression of ST6GalNAc V, a sialyltransferase involved in the biosynthesis of α-series gangliosides, enhances the adhesion of breast cancer metastatic cells to brain endothelium and their passage through the blood-brain barrier [32].

Differential patterns of gangliosides expression and distribution have been also found in malignant bladder tumors [33,34]. Blanco et al. previously reported a preliminary study of NeuGcGM3 expression in transitional cell carcinoma (n=9) [11]. In the present work, the study of the reactivity of 14F7 Mab was extended to 55 cases of bladder tumors. The expression of NeuGcGM3 was evidenced in the 83.6% of them, independently of the histological grade. Interestingly, it was revealed a direct correlation between the amounts of NeuGcGM3 expressed and the histological grade of these malignancies. In fact, the 14F7 Mab staining was significantly increased in grade III transitional cell carcinoma tumors as compared with grade I-II.

Todeschini et al. reported the relation of GM2, GM3 and/or GM2/GM3 complexes in cell motility and growth in bladder cancer [33]. In addition, over-expression of GM3 induces apoptosis and reduces the malignant potential in murine bladder cancer [33]. Interestingly, a greater presence of GM3 in nonmuscle invasive papillary tumors of urinary bladder was detected as compared with invasive tumors [35]. Furthermore, GM3 diminishes the cancer cell proliferation by inhibiting EGFR tyrosine kinase [36] and modulating the expression of cell cycle regulation proteins [37]. However, NeuGcGM3 variant is not able to inhibit the EGFR tyrosine kinase as much as the N-acetylated GM3 variant [38]. Aberrant accumulation of NeuGcGM3 in transitional cell carcinoma could diminish the anti-tumor effects of GM3, contributing to the potential invasion of these tumors. In this sense, our results suggest that the amount of NeuGcGM3 expressed may serve as an indicator of the invasive potential of bladder tumors.

Recently, Scursoni et al. reported the expression of NeuGcGM3 in pediatric neuroblastoma using formalin-fixed and paraffin-embedded tissues [39]. Also, the 14F7 Mab reaction was demonstrated by Blanco et al. in both pediatric and adult gliomas, although the number of adult gliomas samples evaluated in that study was limited (n=8) [13]. In this study, the expression of NeuGcGM3 was detected in about 30% of malignant gliomas of adult patients regardless of the histological grade of tumors. Nevertheless, malignant gliomas expressing higher levels of NeuGcGM3 were preferably high-grade tumors rather low-grade tumors.

It is known that the progression of malignant brain tumors is associated with altered gangliosides composition and distribution. The literature reports a decrease in the total amount of more complex gangliosides (polysialylated) joint to increased proportions of simpler mono-and disialogangliosides, such as GM3, GM2, GD3 and GD2 [40-42]. GM3 is able to inhibit angiogenesis, proliferation and invasion of glioma cells [41,43]. The accumulation of GM3 has been evidenced in a variety of brain malignancies [40,42]. Nevertheless, the preferential accumulation of NeuGcGM3 in high-grade gliomas seems to be in agreement with the more aggressive biological behavior of these tumors. In this sense, our results are consistent with a previous report of Scursoni et al., who found a correlation between the expression of NeuGcGM3 with a more aggressive form of pediatric neuroblastomas [39].

Tumor hypoxia has been considered one of the most important causes of both the aggressive course of malignant gliomas and the resistance to conventional therapies. Malignant glioma cells produce a number of different immunosuppressive mediators that inhibit anti-tumor immunity, contributing to their rapid proliferation even in a hypoxic environment [8,44]. Tumor hypoxia is considered responsible of NeuGcGM2 ganglioside expression in human cancer cells through the incorporation of NeuGc. The effect of hypoxia could be to expedite

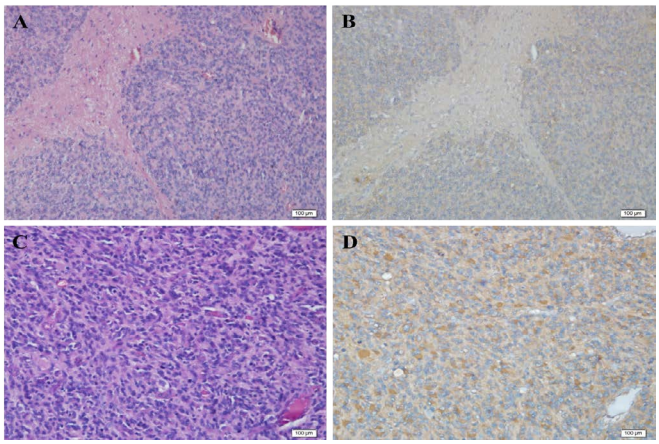


**Figure 3:** Immunoreaction of 14F7 Mab in malignant gliomas. A and C: Hematoxylin and eosin staining of ependimoma (grade II) and glioblastoma (grade IV), respectively. Observe: a weak reactivity of 14F7 Mab in ependimoma section (Brown color). Nevertheless, an increased expression of NeuGcGM3 was evidenced in the glioblastoma cells (D). Counterstaining with Mayer's Hematoxylin (Blue color). White bar=100 µm.

sialic acid transport from the external medium, due to the increase observed in sialin expression (a sialic acid molecule transporter) [8]. Moreover, higher levels of NeuGcGM2 and NeuGcGM3 gangliosides were detected in U87-MG glioblastoma cells that grew as a xenograft in severe combined immunodeficiency (SCID) mice (*in vivo*) as compared to these cells in cultures (*in vitro*) [45]. Further experiments in order to explore the potential relation between tumor hypoxia and the expression of NeuGcGM3 in malignant gliomas are being planned by our group.

In Cuba, some clinical trials have been performed for the treatment of cancer patients using two immunotherapeutic approaches targeting NeuGcGM3: a molecular vaccine (NeuGcGM3/VSSP) [46] and an anti-idiotype antibody-based vaccine (racotumomab, 1E10) [47]. Preliminary results suggested that racotumomab and NeuGcGM3/VSSP induce immune response to NeuGcGM3 antigen, improving the survival of treated patients [46,47]. However, other immune mechanisms could be involved in this survival benefits. Additionally, other clinical trials are currently being designed with a humanized version of 14F7 Mab. In preclinical scenarios, this Mab was able to induce potent antitumor activity both *in vitro* and *in vivo* on a solid mouse myeloma model [48,49]. The data obtained in this study could permit to consider the design of newer clinical trials with these immunotherapeutic agents in urinary bladder tumors and malignant gliomas that express NeuGcGM3, along or combined with the established modalities of treatment.

In summary, we confirmed the presence of NeuGcGM3 ganglioside in breast cancer, urinary bladder tumors and malignant gliomas. Interestingly, increased expression of NeuGcGM3 is correlated with the high histological grade in these malignancies. Moreover, the preferential expression of this ganglioside accompanied the disease progression in urinary bladder neoplasms and malignant astrocytomas. Our results suggest the relationship between the tissue expression of NeuGcGM3 and the more aggressive biological behavior of these malignancies, regardless of the tumor cell lineage. Our data also support the continuous use of NeuGcGM3 as a target for immunotherapy of malignancies expressing this molecule as well as the use of the murine version of 14F7 Mab for the selection of candidate patients for these specific therapies.

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