Advances in Molecular Diagnostics
ISSN: 2572-5073

Blanco et al., Adv Mol Diag 2017,2:1
DOI:10.4172/2572-5073.1000112

Research Article OMICS International

Double Expression of Epidermal Growth Factor Receptor and N-Glycolyl GM3 Ganglioside in Human Malignant Tumors: A Study in Four Different Clinical Scenarios

Rancés Blanco[1]*, Mercedes Cedeño[1], Charles E Rengifo[2], Damián Blanco[3], Milagros Frometa[1], Elizabeth Domínguez[4], Mayra Ramos-Suzarte[5], Adriana Carr[6] and Enrique Rengifo[1]

[1]*Center of Molecular Immunology, P.O. Box 16040, 216 St. and 15 Ave., Atabey, Playa, 11600 Havana, Cuba*
[2]*Department of Pathology, National Institute of Oncology and Radiobiology, 29 and F St., Vedado, Plaza de la Revolution, 10400 Havana, Cuba*
[3]*Department of Cell Biology and Tissues Banking, National Institute of Oncology and Radiobiology, 29 and F St., Vedado, Plaza de la Revolution, 10400 Havana, Cuba*
[4]*Laboratory of Biochemistry, Center of Molecular Immunology, P.O. Box 16040, 216 St. and 15 Ave., Atabey, Playa, 11600 Havana, Cuba*
[5]*Clinical Trials Direction, Center of Molecular Immunology, P.O. Box 16040, 216 St. and 15 Ave., Atabey, Playa, 11600 Havana, Cuba*
[6]*System Biology Direction, Center of Molecular Immunology, P.O. Box 16040, 216 St. and 15 Ave., Atabey, Playa, 11600 Havana, Cuba*

Abstract

The interaction between the epidermal growth factor receptor (EGFR) and N-glycolyl GM3 ganglioside (NeuGcGM3) and its contribution to the progression of tumors have been previously described. However, studies concerning to the double expression of EGFR and NeuGcGM3 in human tissues are scarce. Here, it was evaluated by immunohistochemistry the dual expression of EGFR and NeuGcGM3 in 153 samples of malignant tissues grouped as follow: primary tumors, metastasis, conventionally pre-treated samples and recurrent diseases. The presence of both molecules was evidenced in 60/101 (59.4%) and 18/26 (69.2%) of untreated primary tumors and metastasis, respectively. Remarkably, the dual of EGFR and NeuGcGM3 was statistically significant increase in high grade astrocytoma (22/31) when compared with low-grade astrocytomas (0/14) (p=0.000; Chi-square Test). In this type of samples, the tissue expression of EGFR was observed as a finely granular precipitate located in the plasmatic membrane of malignant cells, while NeuGcGM3 was located in the cell membrane but also in the cytoplasm of tumour cells. The double expression of these tissue antigens was detected in 14/20 (70.0%) of cases that received standard chemotherapy and/or radiotherapy schemes after the surgical removal of tumors. Interestingly, the expression pattern of EGFR and NeuGcGM3 after conventional treatment was similar to those observed in the rest of non-treated samples. Moreover, preliminary data revealed the dual appearance of these molecules in 4/6 (66.7%) of tumor recurrences. All these facts could support the potential design of combined immunotherapy schemes targeting simultaneously both EGFR and NeuGcGM3 in these clinical situations.

Keywords: EGFR; NeuGcGM3; Double expression; Primary malignant tumors; Metastasis

Abbreviations: EGFR: Epidermal Growth Factor Receptor; EGF: Epidermal Growth Factor; NeuGcGM3: N-Glycolyl GM3 Ganglioside; Mab: Monoclonal Antibody; HRP: Horseradish Peroxidase; IgG1: Immunoglobulin 1; DAB: 3,3'-Diaminobenzidine; NSCLC: Non-Small Cell Lung Cancer; HIF-1: Hypoxia-Inducible Factor-1

Introduction

Cancer constitutes one of the leading causes of both morbidity and mortality worldwide, with about 14 million new cases and 8.2 million cancer related deaths in 2012 [1]. Despite of the significant progresses in cancer therapy, the therapeutic options available for advanced/recurrent disease are insufficient Notably, most deaths from cancer are caused by metastasis and tumor relapse in which usually malignant cells display resistance to standard therapies [2]. This fact promotes the development and incorporation into clinical practice of alternative treatments using target molecules [3].

Among the most explored tissue antigens in cancer patients are the epidermal growth factor receptor (EGFR) [4-8] and the N-glycolyl GM3 ganglioside (NeuGcGM3) [9-14]. In some clinical trials, specific treatment targeting separately EGFR [15] and NeuGcGM3 [16] improved the overall survival of cancer patients. Nevertheless, the clinical benefits provided by these immunotherapeutic agents are not sufficient Consequently, combinations of immunotherapy strategies against EGF/EGFR system and NeuGcGM3 molecules are being currently proved [17].

On the other hand, the interaction between EGFR and GM3 ganglioside (the N-acetyl variant) has been previously described. GM3 diminishes the cancer cell proliferation inhibiting the EGFR tyrosine kinase and modulating the expression of cell cycle regulation proteins *in vitro* [18,19]. However, NeuGcGM3 is not able to inhibit the EGFR tyrosine kinase when compared with GM3 [20]. As a consequence, this binding could favor an uncontrolled EGFR system activation mediated by receptor ligands such as the epidermal growth factor (EGF). These results also provide a rational to the design a combined immunotherapy targeting simultaneously both EGFR and NeuGcGM3 tumor related antigens.

Recently, it was reported by our group the double expression of these tumor antigens in some primary malignant neoplasms [21,22]. Moreover, the co-expression of EGFR and NeuGcGM3 was also demonstrated in two different murine models of spontaneous lung metastasis: Lewis lung carcinoma (3LL-D122) and mammary carcinoma (4T1). Furthermore, combined immunotherapy using the

***Corresponding author:** Rancés Blanco, Laboratory of Recognition and Biological Activity Assays, Center of Molecular Immunology, P.O. Box 16040, 216 St. and 15 Ave., Atabey, Playa, 11600 Havana, Cuba, Tel: (537) 27179333464; E-mail: rances@cim.sld.cu

Received: January 20, 2017; **Accepted:** February 20, 2017; **Published**: February 28, 2017

Citation: Blanco R, Cedeño M, Rengifo CE, Blanco D, Frometa M, et al. (2017) Double Expression of Epidermal Growth Factor Receptor and N-Glycolyl GM3 Ganglioside in Human Malignant Tumors: A Study in Four Different Clinical Scenarios. Adv Mol Diag 2: 112. DOI:10.4172/2572-5073.1000112

murine 7A7 (anti-EGFR) and 14F7 (anti-NeuGcGM3) monoclonal antibodies (Mabs) synergistically reduced the weight of the metastatic lungs [22]. However, studies concerning the double expression of these two molecules in human tissues as well as its contribution to the biological behavior of tumors are scarce.

For these reasons, the aim of the present work was to evaluate the double expression of EGFR and NeuGcGM3 in four different clinical situations: (1) primary tumors, (2) lymph node and other sites metastasis, (3) samples obtained from conventionally pre-treated patients and (4) disease recurrence. The potential relation of the EGFR and NeuGcGM3 double positivity with the aggressive biological behavior of astrocytomas is also presented.

Materials and Methods

Tissue samples

It was retrospectively studied a total of 153 pathological specimens archived in the tissues collection of the Laboratory of Recognition and Biological Activity Assays (Center of Molecular Immunology, Havana, Cuba) between 2001 and 2016, after receiving the approved consent by the institutional Ethical Committee. Samples were formalin-fixed and paraffin-embedde following the standard histological procedures. Only cases with confirmed histopathological diagnosis were included in the present work.

The malignant tissues were grouped as follow: (1) primary tumors (n=101) divided in cervical carcinomas (n=29), malignant astrocytomas (n=45) and sarcomas (n=27), (2) lymph node and other site metastasis (n=26), (3) samples obtained from conventionally pre-treated patients (n=20) and (4) recurrence of primary malignancies (n=6). Primary tumors and metastasis belongs to patients without previous oncospecific treatments. Samples of anal canal carcinoma were obtained from patients pretreated with standard radiotherapy before the tumor surgical resection. The specimens of pancreatic adenocarcinoma and non-small cell lung cancer (NSCLC) were removed after the standard radio-chemotherapeutic regimens

Immunodetection of EGFR and NeuGcGM3

Five µm serial sections from each block were obtained in a Lizt 1512 micrometer and mounted on plus slides (Dako, S2024). All sections were attached to the slides by heating in a 60°C oven for 1 hour. Then, the slides were kept at room temperature and they were used within 30 days. The slides were dewaxed in xylene and rehydrated in decreasing ethanol series as usual. Two different tissue sections were used for the evaluation of EGFR or NeuGcGM3 molecules.

Afterward, the expression of EGFR was detected by mean of the EGFR PhamaDx™ kit (Dako, K1494) according to the manufacturer instructions. Briefly, sections were placed in a humid chamber and pretreated with Proteinase K solution for 5 minutes at room temperature. After that, the samples were incubated with the anti-EGFR antibody and then with the polymer-HRP for 30 minute each step. Between incubations, the slides were washed with the buffered solution (Dako, S3006). Regarding to the NeuGcGM3 expression, the samples were incubated with the murine version of the 14F7 Mab (a highly specific IgG1 anti-NeuGcGM3 ganglioside) [23] for 1 hour at room temperature. Afterward, the slides were incubated with EnVision-HRP (Dako, K4061) for 30 minutes. Between each step, the sections were washed with the above mentioned buffered solution.

For all samples, the enzymatic activity was visualized with a DAB (Dako, K3465) solution. Finally, the slides were counterstained with Mayer's Hematoxylin (Dako, S2020), dehydrated, and mounted.

Immunohistochemical evaluation

For EGFR expression, only the staining of cell membrane was evaluated while, the staining of both cell membrane and cytoplasm was considered as positive for 14F7 Mab. A semiquantitative scoring system was used to define levels of reactivity. Firstly, the most representative regions of each section were selected and the percentage of tumor cells showing immunostaining (0% to 100%) in them was subjectively estimated using the 10X objective lens. The intensity of reaction of each sample was judged as negative (0), weak (1), moderate (2) and strong (3). The results were considered according with two observers agreement. Then, the score was calculated for each specimen by multiplication of the intensity of reaction and the grade of positive cells, resulting in a score ranging from 0 to 300. Subsequently, these scores were grouped as follow: 0 (score 0); 1 (scores 1-100); 2 (scores 101-200), and 3 (scores 201-300).

Statistical analysis

Minitab® 16.1.0 software (2010 Minitab Inc. Pennsylvania, USA) was used for data analysis. Differences in the double expression of EGFR and NeuGcGM3 between low and high grade astrocytomas were analyzed using the Chi-square test. A p value <0.05 was considered statistically significant

Results

Immunohistochemical localization of EGFR and NeuGcGM3

In general, the tissue expression of EGFR was observed as a finely granular precipitate located in the plasmatic membrane of malignant cells. Similarity, the expression of NeuGcGM3 was observed as a finely granular reaction mainly located in the cell membrane but also in the cytoplasm of tumor cells as it was previously described in [24]. No reaction was observed in negative controls (See Supplemental Figure).

Double expression of EGFR and NeuGcGM3 in primary tumors

The double expression of EGFR and NeuGcGM3 was evidenced in 25/29 (86.2%), 13/27 (48.1%) and 22/31 (71.0%) of cervical carcinomas (Table 1; Figure 1A and 1B), sarcomas (Table 2; Figure 1C and 1D) and high grade astrocytomas (III-IV) (Table 3; Figure 1E and 1F), respectively. However, low-grade astrocytomas (I-II) showed no dual expression of EGFR and NeuGcGM3 (0/14). Consequently, the simultaneous presence of these tissue antigens was statistically significant associated with the increase in the histological grade of astrocytic tumors (p=0.000; Chi-square Test).

Double expression of EGFR and NeuGcGM3 in metastatic sites

The double positivity of EGFR and NeuGcGM3 antigens in a variety of metastatic tissues is summarized in Table 4 and Figure 2. Overall, the

EGFR scores	NeuGcGM3 scores (n=29)			
	0	1	2	3
0	1	0	2	1
1	0	2	0	1
2	0	1	1	1
3	0	2	4	13
Immunohistochemical evaluation: 0 (score 0); 1 (scores 1-100); 2 (scores 101-200), and 3 (scores 201-300)				

Table 1: Double expression of EGFR and NeuGcGM3 in primary cervical carcinomas.

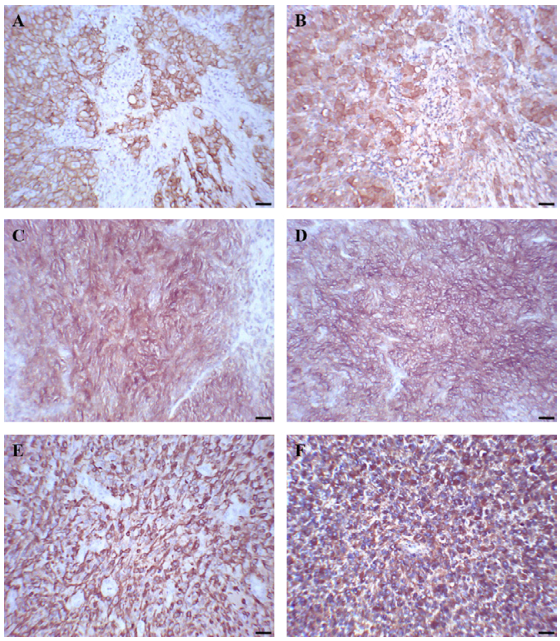

Figure 1: Double expression of EGFR and NeuGcGM3 in primary tumors. A and B: cervical carcinoma. C and D: malignant hemangiopericytoma. E and F: high-grade astrocytoma (glioblastoma). Left and right columns correspond to EGFR and NeuGcGM3 expression, respectively (brown color). The presence of EGFR was evidenced in cell membrane of malignant cells, while NeuGcGM3 was detected in both cell membrane and cytoplasm of these cells. Counterstaining with Mayer's Hematoxylin (blue color). Black bar=100 μm.

EGFR scores	NeuGcGM3 scores (n=27)			
	0	1	2	3
0	5	2	2	2
1	0	0	2	1
2	0	0	0	1
3	3	1	0	8

Immunohistochemical evaluation: 0 (score 0); 1 (scores 1-100); 2 (scores 101-200), and 3 (scores 201-300)

Table 2: Double expression of EGFR and NeuGcGM3 in sarcomas.

EGFR scores	NeuGcGM3 scores (n=14)			
	0	1	2	3
0	11	1	1	0
1	0	0	0	0
2	0	0	0	0
3	1	0	0	0
EGFR scores	**NeuGcGM3 scores (n=31)**			
	0	1	2	3
0	7	0	0	0
1	1	6	3	1
2	0	0	2	2
3	1	1	1	6

Low grade, astrocytomas grades I-II; High grade, astrocytomas grades III-IV. Immunohistochemical evaluation: 0 (score 0); 1 (scores 1-100); 2 (scores 101-200), and 3 (scores 201-300)

Table 3: Double expression of EGFR and NeuGcGM3 in low grade (upper part) and high grade (lower part) malignant astrocytomas.

dual expression of EGFR and NeuGcGM3 was detected in 18/26 (69.2%) of human metastasis without taking into consideration the tumor origin or the metastatic site. Of interest, higher levels (scores 2 and 3) of both molecules was evidenced in 13/18 (72.2%) of double positive cases.

Double expression of EGFR and NeuGcGM3 in samples obtained from previously treated patients

In general, the double expression of EGFR and NeuGcGM3 was detected in 14/20 (70.0%) of conventionally pre-treated samples (Table 5 and Figure 3). The simultaneous expression of EGFR and NeuGcGM3 was increased (scores 2 and 3) in 10/14 (71.4%) of positive cases independently of the histopathological classification of tumors and the previous therapy modality. No evident alterations in the expression pattern of both molecules when compared with those previously described for primary tumors were observed.

Double expression of EGFR and NeuGcGM3 in tumor recurrence

In this preliminary study, both the EGFR and NeuGcGM3 were expressed at the same time in 4/6 (66.7%) of tumor recurrence (Table 6). The double expression of these tumor antigens was increased (scores 2 and 3) in 3/4 (75.0%) of positive cases.

Discussion

The expression of EGFR and NeuGcGM3 in a variety of malignant tumors as compared with matched healthy tissues [13,25], as well as their role in the aggressive biological behavior of tumors [21,26,27], converts these molecules in attractive targets for cancer immunotherapy. Furthermore, the double expression of EGFR and NeuGcGM3 was recently reported in a variety of primary malignant neoplasms [21,22].

Sample number	Histopathological diagnosis	Immunohistochemical score	
		EGFR	NeuGcGM3
1	LNM of lung adenocarcinoma	3	1
2	LNM of lung adenocarcinoma	2	3
3	LNM of breast adenocarcinoma	1	3
4	LNM of breast adenocarcinoma	0	0
5	LNM of gastric adenocarcinoma	2	2
6	LNM of gastric adenocarcinoma	3	3
7	LNM of colon adenocarcinoma	0	2
8	LNM of colon adenocarcinoma	1	3
9	LNM of colon adenocarcinoma	0	0
10	LNM of colon adenocarcinoma	3	3
11	LNM of colon adenocarcinoma	0	3
12	LNM of colon adenocarcinoma	3	3
13	LNM of colon adenocarcinoma	3	3
14	LNM of colon adenocarcinoma	2	2
15	LNM of colon adenocarcinoma	0	3
16	LNM of colon adenocarcinoma	0	0
17	LNM of colon adenocarcinoma	1	2
18	LNM of unknown primary tumor	3	2
19	LNM of unknown primary tumor	3	1
20	LNM of unknown primary tumor	3	3
21	PM of germinal cells tumor	0	3
22	PM of colon adenocarcinoma	3	3
23	VM of anal canal carcinoma	3	3
24	CM of unknown primary tumor	3	3
25	OM of colon adenocarcinoma	3	0
26	BM of renal cell carcinoma	3	3

LNM, lymph node metastasis; PM, pulmonary metastasis; VM, vaginal metastasis; CM, cerebral metastasis, OM, ovary metastasis; BM, brain metastasis. Immunohistochemical evaluation: 0 (score 0); 1 (scores 1-100); 2 (scores 101-200), and 3 (scores 201-300)

Table 4: Double expression of EGFR and NeuGcGM3 in lymph node and other sites metastasis.

However, in most of tumors only preliminary results concerning to the dual expression of these molecules were reported [22].

In this work, it was augmented the number of cases in cervical carcinoma, astrocytomas and sarcomas. The expression patterns of EGFR and NeuGcGM3 antigens were similar than those previously observed by our group in other primary tumors [21,22]. These results confirmed that dual expression of EGFR and NeuGcGM3 is a frequent phenomenon in human tumors, irrespective to the malignant cell lineage. Moreover, this fact provides a rational for the design of combined strategies of treatment targeting simultaneously both EGFR and NeuGcGM3 in these malignancies. In fact, promising clinical results were obtained using combined immunotherapy with CIMAvax-EGF® (a molecular vaccine that induces anti-EGF antibodies neutralizing the EGF/EGFR binding) and racotumomab (an anti-idiotypic vaccine related to NeuGcGM3) in patients with advanced NSCLC [17].

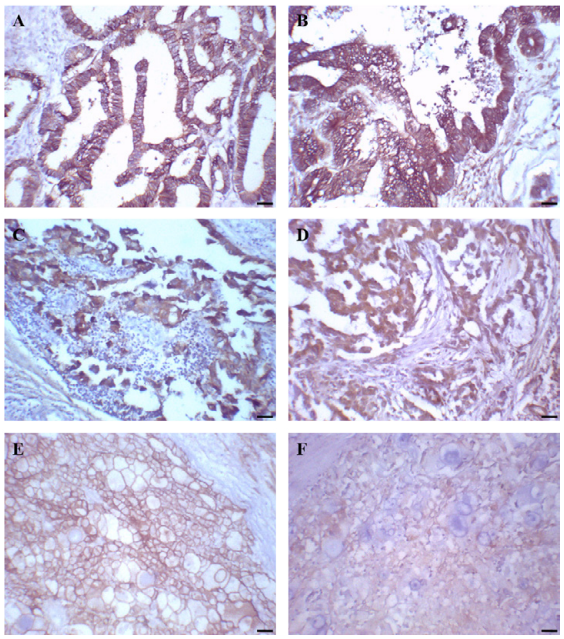

Figure 2: Dual expression of EGFR and NeuGcGM3 in metastatic sites. A and B: pulmonary metastasis of colon adenocarcinoma. C and D: lymph node metastasis of lung adenocarcinoma. E and F: lymph node metastasis of unknown primary tumor. Left and right columns correspond to EGFR and NeuGcGM3 expression, respectively (brown color). EGFR was detected in cell membrane of metastatic cells. Observe the expression of NeuGcGM3 located in plasmatic membrane and cytoplasm of malignant epithelial cells. Counterstaining with Mayer's Hematoxylin (blue color). Black bar=100 µm.

Sample number	Histopathological diagnosis	Immunohistochemical scores	
		EGFR	NeuGcGM3
1	Anal canal carcinoma	3	1
2	Anal canal carcinoma	3	2
3	Anal canal carcinoma	3	3
4	Anal canal carcinoma	3	3
5	Anal canal carcinoma	3	0
6	Pancreatic adenocarcinoma	3	3
7	Pancreatic adenocarcinoma	3	3
8	Pancreatic adenocarcinoma	3	3
9	Pancreatic adenocarcinoma	3	3
10	Pancreatic adenocarcinoma	3	3
11	Lung squamous carcinoma	1	3
12	Lung squamous carcinoma	3	1
13	Lung adenocarcinoma	0	1
14	Lung adenocarcinoma	0	2
15	Lung adenocarcinoma	0	3
16	Lung adenocarcinoma	3	2
17	Lung adenocarcinoma	0	3
18	Large cell lung carcinoma	0	1
19	Large cell lung carcinoma	2	1
20	Lung adenosquamous carcinoma	3	3
Immunohistochemical evaluation: 0 (score 0); 1 (scores 1-100); 2 (scores 101-200), and 3 (scores 201-300)			

Table 5: Double expression of EGFR and NeuGcGM3 ganglioside in previously treated samples.

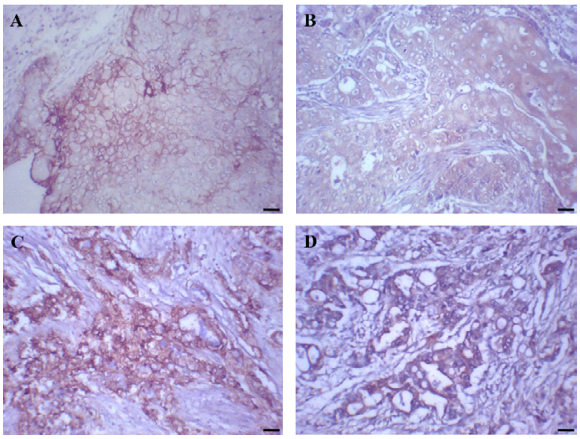

Figure 3: Double positivity for EGFR and NeuGcGM3 in samples obtained from previously treated patients. A and B: adenosquamous carcinoma of the lung. C and D: pancreatic adenocarcinoma. Left and right columns correspond to EGFR and NeuGcGM3 expression, respectively (brown color). EGFR was evidenced in cell membrane of malignant epithelial cells, while NeuGcGM3 was detected in both cell membrane and cytoplasm of these cells. Note: these patterns of expression were similar to those previously described for primary tumors. Counterstaining with Mayer's Hematoxylin (blue color). Black bar=100 µm.

Sample number	Tumor recurrence	Immunohistochemical scores	
		EGFR	NeuGcGM3
1	Nasopharyngeal carcinoma	0	2
2	Grade IV astrocytoma	0	3
3	Cervical carcinoma	2	3
4	Uterine adenocarcinoma	3	3
5	Breast adenocarcinoma	2	3
6	Breast adenocarcinoma	1	3
Immunohistochemical evaluation: 0 (score 0); 1 (scores 1-100); 2 (scores 101-200), and 3 (scores 201-300)			

Table 6: Double expression of EGFR and NeuGcGM3 in tumor recurrences.

Remarkably, the double expression of EGFR and NeuGcGM3 was increased in high grade malignant astrocytomas when compared with low grade tumors. In a previous study, NeuGcGM3 was incapable to inhibit the EGFR tyrosine kinase when compare with the N-acetylated variant of this ganglioside (GM3) [20]. In addition, patients displaying the $EGFR^+/EGF^+/NeuGcGM3^+$ immunophenotype of NSCLC displayed higher index of cells proliferation as well as an increased risk of tumor recurrence. Consequently, the expression of both targets in the same tumor sample was significantly associated with a poor overall survival in NSCLC patients [21]. In this context, the results obtained in this study could suggest that EGF/EGFR system and NeuGcGM3 may coordinately contribute to the aggressive biological behavior of high grade astrocytomas.

Metastatic disease is responsible for the vast majority of cancer patient deaths and constitutes one of the most important clinical

challenges of solid tumor oncology [28,29]. Here, it was reported the double expression of EGFR and NeuGcGM3 in 69.2% of both lymph node and other metastatic sites. The overexpression of EGFR in metastatic lymph node and other metastatic sites has been extensively reported [4,8,30]. Additionally, it was published that overexpression of EGFR is associated with neoplastic spread through lymph node involvement and metastasis to other organs [5,31]. Furthermore, increased expression of EGFR in metastatic lymph nodes was also associated with a poor survival [30], suggesting the association of this molecules with the aggressiveness of tumors.

Similarity, Blanco et al. found the aberrant presence of NeuGcGM3 in most metastasis of some primary tumors [14]. Of interest, Labrada et al. demonstrated an increased expression of NeuGcGM3 from primary tumors to metastatic lesions in a murine model [32]. While de León et al. reported the immunosuppressive properties of NeuGcGM3 [33,34]. In line with this, the dual expression of NeuGcGM3 and EGFR could be a mechanism "misused" by malignant cells to generate both immunosuppression [33,34] and cell proliferation [20,21], favoring the metastatic process. Interestingly, preclinical data showed that combined immunotherapy against these two molecules using 7A7 and 14F7 murine Mabs has a potent anti-metastatic effect in two different murine models of lung metastasis [22].

On the other hand, the overexpression of EGFR in patients that underwent surgery after chemo and/or radiotherapy was previously published. Moreover, the increased in EGFR expression was found in patients with NSCLC [35] and rectal adenocarcinoma after these conventional treatments [36], supporting the role of this molecule in the repair of cellular damage induced by radiation. Similarly, it was demonstrated that the absence of NeuGcGM3 expression was not associated with the use of preoperative polychemotherapy in neuroblastoma patients [37]. In addition, the presence of NeuGcGM3 in Wilms tumors and pediatric retinoblastomas after chemotherapy was reported [38,39], suggesting the potential use of this molecule for targeted therapies after conventional treatment

In the present study, it was evidenced that NeuGcGM3 and EGFR expression persisted in the tumor sections after radio and/or chemotherapy, without any noticeable reduction in the intensity of reaction and in the percentage of positive cells. In fact, the double expression of EGFR and NeuGcGM3 was detected in the 70% of cases after preoperative therapy, similar to that observed in the rest of non-treated samples. These results suggest that radio-chemotherapy are not able to significantly diminish the dual expression of EGFR and NeuGcGM3, as it was previously described for these molecules separately [35,39]. Furthermore, this data permit to identify a population of conventionally pre-treated patients in whom the effect of the combined immunotherapy schemes could be potentially explored.

It is known that recurrence after curative resection of tumors and postoperative chemotherapy remains one of the major problems in cancer management [40,41]. In this phase of the disease, malignant cells display resistance to standard therapies, in part, due to the hypoxic conditions of tumors. Hypoxia-resistant cells are recognized to have diminished response to radio and chemotherapy [42]. Here, it was reported a preliminary study regarding the dual expression of EGFR and NeuGcGM3 in tumor recurrences. This phenomenon was evidenced in the 66.7% of tumor relapses. In this way, although the number of cases is small, it would be interesting to extend the evaluation of the double expression of these molecules in tumor recurrences in order to assess the potential use of combined immunotherapy in this advanced disease.

Finally, it was reported the relation between hypoxia-inducible factor-1 (HIF-1) with the EGFR expression [43]. Moreover, hypoxia promote EGFR signaling via caveolin-1 [44]. In the same way, under hypoxic conditions tumor cell are able to increase the expression of N-glycolyl containing gangliosides [45,46]. Remarkably, interaction of NeuGcGM3 with the extracellular domain of EGFR allows to an up regulation of the tyrosine kinase function [20]. In this respect, the potential contribution of EGFR and NeuGcGM3 double expression to the recurrence of tumors after conventional treatments should be investigated.

In summary, we reported the double expression of EGFR and NeuGcGM3 in four different cancer conditions: human primary malignancies, metastasis, samples obtained from conventionally pretreated patients and recurrences. This fact could permit to consider the design of combined immunotherapeutic schemes targeting simultaneously both molecules in each scenario. Additionally, the increased frequency of EGFR and NeuGcGM3 dual expression in high grade astrocytomas could suggest the contribution of this phenomenon to the aggressive biological behavior of these tumors. Further studies to compare the dual expression of NeuGcGM3 and EGFR in paired tumor samples (before and after chemotherapy and radiotherapy) are recommended.

Acknowledgments

The authors want to thank Mr. Rolando Domínguez for his valuable English assistance in the edition of this manuscript. The authors also express grateful to all medical doctors, histotechnicians and researchers involved in the collection or processing of tissue samples. Financial support was provided by the Center of Molecular Immunology.

Conflict of Interest

The authors declare that they have no conflict of interest

Authors' Contribution

Rancés Blanco and Mercedes Cedeño authors contributed equally to this work. Adriana Carr and Enrique Rengifo co-supervised this study.

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