IMMUNODETECTION OF N-GLYCOLYL GM3 GANGLIOSIDE IN LUNG CARCINOMA BY IMMUNOHISTOCHEMISTRY: A TECHNICAL STUDY USING FROZEN AND FORMALIN-FIXED AND PARAFFIN-EMBEDDED TISSUES.

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ABSTRACT
N-glycolyl GM3 ganglioside (NeuGcGM3) expression in lung carcinomas using formalin-fixed and paraffin-embedded (FFPE) samples has been recently demonstrated. In the present work, it was confirmed the tissue expression of NeuGcGM3 in lung carcinomas by mean of two different mAbs, P3 (anti-NeuGc-containing gangliosides and sulfated glycolipids) and 14F7 (a highly specific for NeuGcGM3) using FFPE samples. In addition, it was reported the tissue expression of NeuGcGM3 in frozen lung carcinoma sections, also supported by the chemical extraction of this ganglioside with organic solvents such as: ethanol, methanol and chloroform/methanol. Moreover, the murine, chimeric and humanized versions of 14F7 mAb showed a similar pattern of immunostaining in this kind of samples. It was also demonstrated that formalin fixation prevent the damage and/or extraction of the antigenic determinant recognized by 14F7 mAb. Consequently, the detection of NeuGcGM3 in frozen samples and their FFPE counterparts was comparable. Our data seems to be in agreement with the potential use of chimeric and/or humanized versions of 14F7 mAb for the passive immunotherapy of lung carcinoma expressing NeuGcGM3. The reactivity of 14F7 murine mAb in FFPE tissues permits to consider it as a useful tool in the selection of patients for specific therapies.

Keywords: Lung cancer, N-Glycolyl GM3, immunohistochemistry, 14F7 mAb, frozen tissues.

INTRODUCTION

Lung cancer is one of the leading causes of cancer-related deaths worldwide [1]. In patients with lung tumors, some genetic and regulatory abnormalities usually limit the survival benefit provided by the standard therapeutic options available. Therefore, drugs targeting abnormal pathways could lead to more effective treatments for this difficult disease [2].

Gangliosides are sialic acid-containing glycosphingolipids engaged in many biological events that take place at vertebrate's cell membrane [3]. Commonly, malignant cells exhibit aberrant overexpression of gangliosides present or not in normal adult tissues [3-5]. These changes allow considering some gangliosides as tumor-associated antigens [5,6]. Unusual glycolylated gangliosides have been identified by immunohistochemical methods in a variety of human malignancies becoming attractive targets for immunotherapy [7,8].

The expression of N-Glycolyl GM3 (NeuGcGM3) ganglioside in breast tumors by chemical analysis was previously reported [5]. In addition, the reactivity of the 14F7, a highly specific IgG1 anti-NeuGcGM3 ganglioside mAb, against breast infiltrating ductal carcinoma and melanomas was documented by immunohistochemistry using frozen tissues [8]. The ability of 14F7 mAb labelled

with 99mTc to recognize breast tumor *in vivo* by the radioimmunoscintigrafic technique was also demonstrated [9].

More recently, some authors published the tissue expression of NeuGcGM3 in lung carcinomas by mean of 14F7 and GMR8 mAbs [10-12]. These studies were restricted to formalin-fixed and paraffin-embedded (FFPE) samples which is the most common specimen available for immunohistochemical assays on tissue after the histopathological diagnosis. Nevertheless, gangliosides are usually extracted after the routine treatment of tissues with organic solvents [13].

In the last few years a chimeric and humanized variant of 14F7 mAb were developed in our center. The resulting recombinant mAbs exhibited a similar capacity to bind NeuGcGM3 and to induce cytotoxicity no dependent of complement as compared with the murine counterpart [14,15]. Nevertheless, the potential use of these mAbs in future clinical trials requires their careful characterization, particularly with respect to their tumor specificity as well as the cross-reactivity and binding properties to normal tissues [16].

In the present paper, it was evaluated the tissue reactivity of P3 (anti-NeuGc-containing gangliosides and sulfated glycolipids) [7] and 14F7 [8] mAbs in lung cancer using FFPE samples. The immunoreactivity of 14F7 mAb in these malignancies using frozen tissues as well as a preliminary study of the chemical nature of the antigen determinant recognized by this Mab was also assessed. Additionally, a comparability study of the immunostaining pattern of the murine, chimeric and humanized versions of 14F7 mAb was performed in the same system. Moreover, the expression of NeuGcGM3 ganglioside by mean of 14F7 mAb recognition in frozen tissues and their FFPE counterparts were also compared.

MATERIALS AND METHODS

2.1 Monoclonal Antibodies

The P3 mAb (IgM, anti-NeuGc-containing gangliosides that also recognizes sulfated glycolipids) [7] and the 14F7 mAb, a murine IgG1 highly specific against N-glycolyl GM3 ganglioside [8] were used. The chimeric [14] and humanized [15] versions of 14F7 mAb were also used. All mAbs were purified by protein A affinity chromatography (Pharmacia) and analyzed by SDS-PAGE under reducing conditions. The murine, chimeric and humanized versions of 14F7 mAb were biotinylated as described [17]. Briefly, the antibodies were extensively dialyzed with 0.2 M pH 8.5 borate buffer and then diluted to a concentration of 1 mg/mL. Afterward, they were incubated with 100 mg/mL of biotin N-hydroxysuccinimide ester (Sigma-Aldrich H1759) for 4 h with gentle agitation at room temperature. Finally, the unreacted biotin was removed and the monoclonal antibodies were dialyzed against phosphate buffer saline (PBS).

Additionally, a polyclonal antibody anti-von Willebrand factor (FVIII) (Dako A0082) and a monoclonal antibody (MN-116) that detects an epitope common to many cytokeratins (Dako M0821) were used to verify the quality of the tissues.

2.2 Tissue samples

Fresh samples of 18 lung carcinomas were obtained by conventional intra-operatory biopsy at the “Hermanos Ameijeiras” General Hospital. The study was carried out with the approval of the institutional ethical committee. All tissue samples were embedded in OCT compound and quickly frozen by immersion in liquid nitrogen and stored at -70ºC until sectioning. Five micrometer sections were obtained in a cryostat and slides were stored at -20ºC until they were used. Additionally, a number of 36 routinely processed, FFPE archival samples, including 10 matched counterparts of the frozen tissues were studied. For FFPE

tissues, five micrometer serial sections from each block were obtained and the slides were processed and stained as it was previously described [11]. For cytokeratin evaluation, the sections were pre-treated with 10mM sodium citrate buffer at pH 6.0 for 10 minutes in a microwave oven at 600 W. After pre-treatment, the slides were washed in distilled water for 10 minutes and then were rinsed with TBS (Tris/saline buffer solution) for 5 minutes.

2.3 Immunohistochemical staining

For FFPE samples, the sections were incubated with P3, 14F7 and MN-116 mAbs for 1h at room temperature. Negative controls were performed by substituting primary antibody for the negative control included in the EGFR pharmDx™ kit (Dako K1494) and sections of colonic adenocarcinoma of known positivity for these antigens were taken as positive control. After two rinses in washing solution the slides were incubated with a polymer/HRP (Dako E0354) for 30 minutes. Between incubations, slides were washed for 10 minutes.

For frozen samples, the sections were fixed in 4% paraformaldehyde during 20 minutes at room temperature. All samples were washed in tap water and rehydrated in distilled water for 10 minutes and TBS for 5 minutes. Slides were incubated with an endogenous peroxidase inhibition solution for 10 minutes (Dako X2003) followed by incubation during 2 hours with the biotinylated antibodies (1 mg/mL) diluted 1:50 at room temperature. After washing for 10 minutes with TBS, the sections were incubated with streptavidin/peroxidase (Dako K0690) during 1 hour. Slides containing an NGcGM3-positive cell line (P3X63Ag8.653, CRL-1580) were taken as positive control [14]. Negative controls were performed by replacing the primary antibody with biotinylated human immunoglobulins (intacglobin®, Cuba).

In all cases, the reaction was developed using diaminobenzidine (Dako K3465). Finally, the slides were counterstained with Mayer's Hematoxylin (Dako S2020), dehydrated and mounted with a synthetic medium.

2.4 Extractability of NeuGcGM3 in frozen and FFPE tissues. In frozen NSCLC tissues, the sections were treated before or after 4% neutral buffered formaldehyde (Spectrum, F0110) fixation according to the following protocols: ethanol (Spectrum, E1028), methanol (Spectrum, M1240) and chloroform/methanol (2:1 v/v) for 30 minutes at 2-8ºC each one. In FFPE samples, the sections were treated with the organic solvents as it was previously described. Positive and negative control samples were included in each staining procedure.

2.5 Evaluation of immunoreaction

Staining of both cell membrane and cytoplasm was considered as positive for P3 and 14F7 mAbs. The intensity of reaction of each tumor tissues was considered as negative (0), weak (1), moderate (2) and strong (3). The most representative regions of each section were selected and the percentage of stained tumor cells was estimated using the 10x objective lens (0-100%). Afterward, a score was calculated for each specimen by multiplication of the intensity of reaction and the percentage of positive cells, resulting in a score ranging from 0 to 300. Subsequently, these scores were grouped as follow, 0 (score 0); 1 (scores <100), 2 (scores 100-200) and 3 (scores ≥200) and considered as the final score (F-score). All microscopic analyses were performed by two different observers.

RESULTS

3.1 Evaluation of tissues preservation. In order to verify the quality of the frozen tissues, it was firstly evaluated the immunohistochemical reactivity of an anti-von Willebrand factor (FVIII) polyclonal antibody.

Concerning to formalin-fixed and paraffin-embedded tissues, a monoclonal antibody that detects an epitope common to many cytokeratins (MN-116) was used. The samples remained well preserved, conserving both the molecular antigenic determinants and the tissue morphology.

3.2 The P3 mAb reacted intensely in FFPE sections of lung carcinoma. The tissue reactivity of P3 mAb was evidenced in 35/36 (97.2%) lung cancer samples independently of the type of carcinoma. The intensity of reaction of P3 mAb varied from weak to intense, but in 25/36 (69.4%) samples a moderate to intense reactivity with this mAb was evidenced. Only one case including in the group of other minor types represented showed no staining with P3 mAb (Table 1).

The pattern of reaction of P3 mAb was homogeneous, finely granular and it was located in both cell membrane and cytoplasm of malignant cells (Figure 1). An additional nuclear staining was detected in 2/36 (5.5%) cases.

3.3 High levels of 14F7 murine mAb reactivity were also detected in FFPE sections. The reaction of 14F7 mAb was evidenced in 28/30 (93.3%) lung cancer samples independently of the type of carcinoma (Table 2). Only 1/2 (50.0%) large cell carcinoma as well as 1/6 (16.7%) lung carcinoma including in the group of other minor types represented displayed no reactivity with 14F7 mAb.

The intensity of reaction of 14F7 mAb varied from weak to intense, but 22/28 (78.6%) of lung carcinomas showed a moderate to intense recognition. The pattern of staining of 14F7 mAb was finely granular and was located on both cell membrane and cytoplasm of malignant cells (Figure

Table 1. Immunostaining of P3 mAb in formalin-fixed and paraffin-embedded tissues.

Histopathological type	F-score			
	0	1	2	3
	no. cases (%)	no. cases (%)	no. cases (%)	no. cases (%)
Squamous cell carcinoma	0/6	2/6 (33.3)	2/6 (33.3)	2/6 (33.3)
Adenocarcinoma	0/17	3/17 (17.6)	2/17 (11.8)	12/17 (70.6)
Large cell carcinoma	0/2	0/2	1/2 (50.0)	1/2 (50.0)
Carcinoid tumor	0/3	3/3	0/3	0/3
Others*	1/8 (12.5)	2/8 (25.0)	3/8 (37.5)	2/8 (25.0)

Legend. No., number; %, percentages; *group of tumors minority represented; F-score, 0 (score 0); 1 (scores <100), 2 (scores 100-200) and 3 (scores ≥200).

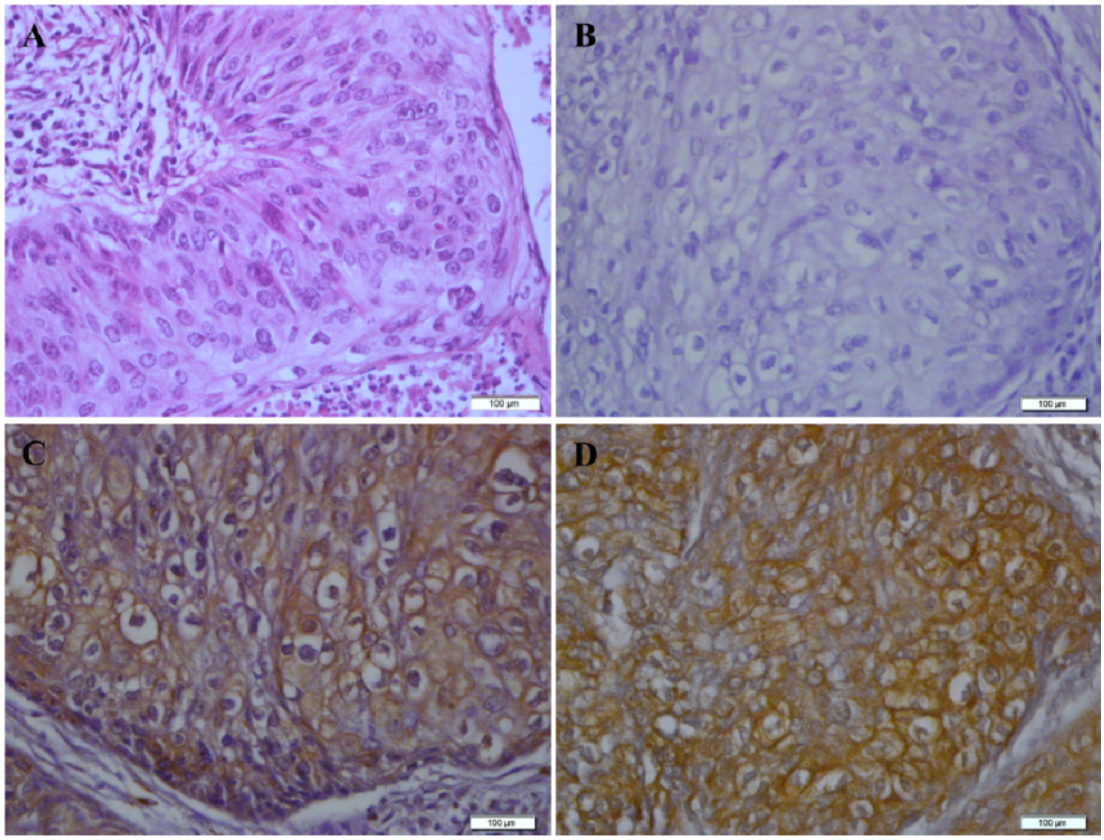

Fig. 1. Microphotographs of FFPE non-small cell lung carcinoma sections. A: Hematoxylin and eosin staining. B: Negative control. Note: the absence of immunostaining. C and D: Intense tissue staining of P3 and 14F7 mAbs, respectively, located in both cell membrane and cytoplasm of malignant cells (Brown color). Counterstaining with Mayer's Hematoxylin (Blue color). White bar=100µm.

Table 2. Immunostaining of 14F7 mAb in formalin-fixed and paraffin-embedded tissues.

Histopathological type	F-score			
	0	1	2	3
	no. cases (%)	no. cases (%)	no. cases (%)	no. cases (%)
Squamous cell carcinoma	0/6	1/6 (16.7)	3/6 (50.0)	2/6 (33.3)
Adenocarcinoma	0/14	2/14 (14.3)	2/14 (14.3)	10/14 (71.4)
Large cell carcinoma	1/2 (50.0)	1/2 (50.0)	0/2	0/2
Carcinoid tumor	0/2	1/2 (50.0)	0/2	1/2 (50.0)
Others*	1/6 (16.7)	1/6 (16.7)	1/6 (16.7)	3/6 (50.0)

Legend. No., number; %, percentages; * group of tumors minority represented; F-score, 0 (score 0); 1 (scores <100), 2 (scores 100-200) and 3 (scores ≥200).

1). In one case (3.6%) an additional nuclear staining of 14F7 mAb was detected (Figure 2).

A statistically significant correlation was evidenced when the reactivity of P3 and 14F7 mAbs were compared (n=29) ($p<0.0001$, $r=1.000$; Spearman test).

3.4 The immunoreaction of 14F7 murine mAb was confirmed in frozen sections after 4% paraformaldehyde fixation. The recognition of 14F7 mAb was evidenced in 18/18 lung carcinomas. The pattern of staining was homogeneous, finely granular and mainly located on the cell membrane, although the cytoplasm was also decorated. No nuclear staining with 14F7 mAb was evidenced. The intensity of reaction with 14F7 mAb varied from weak to intense, but at least 13/18 (72.22%) lung carcinomas showed a moderate to intense recognition. The staining was evidenced in more than 50% of malignant cells although, in 3/18 (16.67%) cases the reaction was mainly observed in cell clusters. No immunostaining was obtained in sections incubated with intacglobin®, used as negative controls.

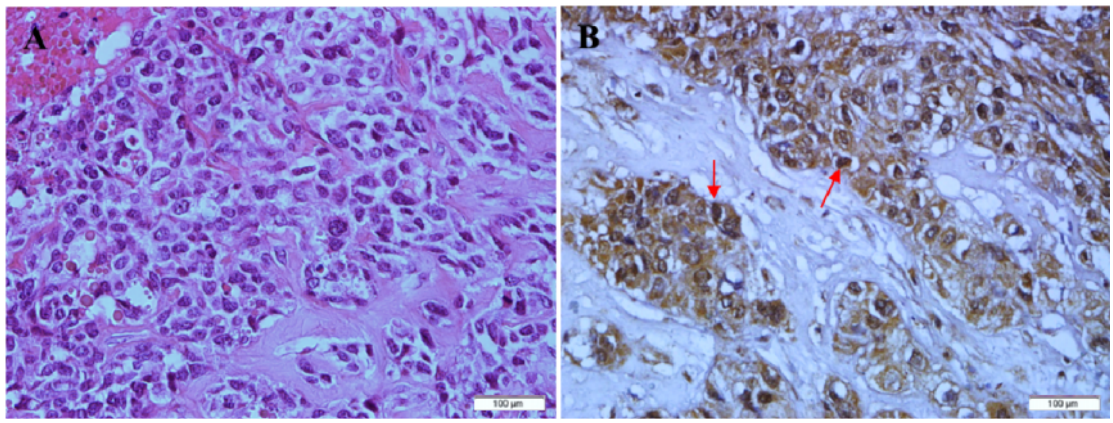

Fig. 2. Microphotographs of FFPE non-small cell lung carcinomas. A: Hematoxilin and eosin staining. B: Intense immunoreactivity of 14F7 mAb located in both cell membrane and cytoplasm of malignant cells (Brown color). Observe the additional nuclear immunostaining of 14F7 mAb (red arrows). Counterstaining with Mayer's Hematoxylin (Blue color). White bar=100µm.

Table 3. Comparison of 14F7 murine mAb immunostaining in frozen and formalin-fixed and paraffin-embedded sections.

No.	Samples	F-score	
		Frozen	FFPE
1	SCC	2	3
2	SCC	2	2
3	SCC	3	3
4	SCC	3	2
5	SCC	2	2
6	ADC	3	3
7	ADC	2	1
8	ADC	3	0
9	MEC	3	2
10	SCLC	3	1

Legend. No., number; SCC, ADC and MEC, squamous carcinoma, adenocarcinoma and mucoepidermoid carcinoma, respectively; SCLC, small cell lung carcinoma; F-score, 0 (score 0); 1 (scores <100), 2 (scores 100-200) and 3 (scores ≥200).

3.5 The reaction of 14F7 murine mAb in frozen sections matched with that observed for their FFPE counterparts. In general, the staining in FFPE samples matched to that seen in frozen tissues (p=1.0000; Fisher's exact test) (Table 3). Only one case of frozen tissues was positively stained with 14F7 mAb whereas the FFPE counterpart resulted negative.

The immunostaining with 14F7 was located on both cell membrane and cytoplasm of malignant cells (Figure 3). However, a weak dissolving and/or a distorting in the localization of the antigenic determinant recognized by

14F7 mAb were evidenced as compared with frozen tissues.

Table 4. Effects of some chemical treatments in the tissue extractability of NeuGcGM3 by mean of the 14F7 mAb reaction.

Kind of samples	Chemical treatment	F-score
Frozen	No treatment	3
	Before 4% NBF fixation	
	Ethanol	0
	Methanol	0
	Chloroform-methanol	0
	After 4% NBF fixation	
	Ethanol	3
	Methanol	3
	Chloroform-methanol	1*
FFPE	No treatment	3
	Ethanol	3
	Methanol	3
	Chloroform-methanol	0

Legend. NBF, neutral buffered formalin; FFPE, formalin-fixed and paraffin-embedded tissues; F-score, 0 (score 0); 1 (scores <100) and 3 (scores ≥200); * less than 25% of positive cells.

3.6 The effect of a variety of organic solvents in the tissue extractability of NeuGcGM3 was evaluated in NSCLC sections (see Materials and Methods). An intense, homogeneous and finely granular reactivity of 14F7 mAb in frozen NSCLC sections without treatment was evidenced (Table 4, Figure 4). By the contrary, the treatment of tissues with ethanol, methanol and chloroform/methanol, before 4% NBF fixation, significantly removed the staining with 14F7 mAb.

Comparable results were also obtained with P3 mAb, used as control (Data not shown). No immunoreaction, independently of the chemical treatment, was detected in sections used as negative controls.

On the other hand, no changes in the pattern of staining with 14F7 mAb were detected in ethanol and methanol-treated samples after 4% NBF (neutral-buffered formalin) fixation. But, the reactivity of 14F7 Mab was significantly eliminated with chloroform/methanol treatment after 4% NBF fixation. Similar results were obtained in FFPE tissues after the treatment with the organic solvents following protocols as it was previously mentioned (Data not shown).

3.7 The murine, chimeric and humanized versions of 14F7 mAb showed a similar pattern of staining in frozen samples. The comparison of the murine, chimeric and humanized versions of 14F7 mAb was performed in 17 cases. The recognition of the tissues by these mAbs was variable in the intensity of reaction but similar patterns of staining and percentage of positive cells were observed in 16/17 (94.12%) cases (Table 5, Figure 5). Only one case (No. 3) showed a weak staining with both murine and chimeric versions but was negative to the humanized mAb.

No immunostaining was obtained with the biotinylated human immunoglobulins used as negative control.

When the reactivity of the 14F7 murine mAb was compared with those from the chimeric and humanized versions statistically significant correlations were observed ($p<0.0001$, $r=0.8696$ and $p<0.0003$, $r=0.7745$; Spearman test, respectively). Similar results was obtained when the reaction of chimeric and humanizad variants of 14F7 mAb were compared ($p<0.0001$, $r=0.8492$; Spearman test).

Table 5. Comparison of 14F7 mAbs immunostaining in frozen sections.

No.	Samples	F-score		
		Murine	Chimeric	Humanized
1	SCC	2	2	2
2	SCC	2	ND	3
3	SCC	1	1	0
4	SCC	3	3	3
5	SCC	2	2	2
6	SCC	2	2	3
7	SCC	2	1	2
8	SCC	3	3	3
9	SCC	3	3	3
10	SCC	2	2	2
11	ADC	3	ND	3
12	ADC	2	2	3
13	ADC	3	3	3
14	ADC	3	3	2
15	ADC	2	2	2
16	MEC	3	3	3
17	SCLC	3	3	3

Legend. No., number; SCC, ADC and MEC, squamous carcinoma, adenocarcinoma and mucoepidermoid carcinoma, respectively; SCLC, small cell lung carcinoma; F-score, 0 (score 0); 1 (scores <100), 2 (scores 100-200) and 3 (scores ≥200); ND, not done.

DISCUSSION

It is well known that normal human cells are incapable of synthesizing NeuGc due to a specific inactivating mutation in the cytidine monophospho-N-acetylneuraminic acid hydroxylase (CMP-NeuAc hydroxylase) gene [18]. In human NSCLC tissues a mutation in the sequence of the CMP-NeuAc hydroxylase gene, similar to that in normal tissues, have been demonstrated [12]. These results suggest that the preferential aberrant expression of the NeuGc acid in human malignant tissues could be more related with its incorporation from dietary sources due to the altered and more accelerated metabolism of neoplastic cells [19-21]. However, an alternative pathway to the NeuGc synthesis from other intermediates of cellular metabolism in some human tumors has been suggested [22].

In the present work, it was firstly demonstrated the tissue reactivity of P3 mAb in a serie of NSCLC using FFPE samples. The P3 mAb is an IgM that react with NeuGcGM3, other NeuGc-containing gangliosides and sulfated molecules [7]. Almost all lung tumors showed a positive staining with this mAb. The immunorecognition of P3 mAb in both frozen and FFPE tissues, including SCLC [23] and NSCLC [24], have been previously published. Nevertheless, our investigation used 36 patients, which is a larger sample size compared with that in previous reports [23,24]. In this way, our results confirmed the expression of NeuGc residues in NSCLC tissues. Moreover, in a similar study, the expression of NeuGcGM3 using TLC (thin layer chromatographic) immunostaining method and GMR8 mAb has been demonstrated in NSCLC using this kind of samples. GMR8 mAb is an IgM that react with an epitope shared by more than one of the NeuGc-containing gangliosides [25]. In line with this, at least other NeuGc-containing ganglioside, GD1a (NeuGc), was recognized by GMR8 mAb in NSCLC tissues [12].

For these reasons, in this paper it was also used the 14F7 mAb, a highly specific IgG1 against NeuGcGM3. This mAb is able to distinguish between the N-acetyl and N-glycolyl variants of GM3 and not react with other NeuGc-containing gangliosides [8]. The tissue reactivity of 14F7 mAb was detected in 93.3% of NSCLC samples. Our results are in line with those previously published [10,11] and confirmed the reactivity of 14F7 mAb in lung carcinomas using FFPE tissues. Interestingly, the staining with 14F7 mAb was comparable to that detected with P3 mAb. Similar results were obtained by *Scursoni et al.* in a preliminary study in Wilm´s tumors [26].

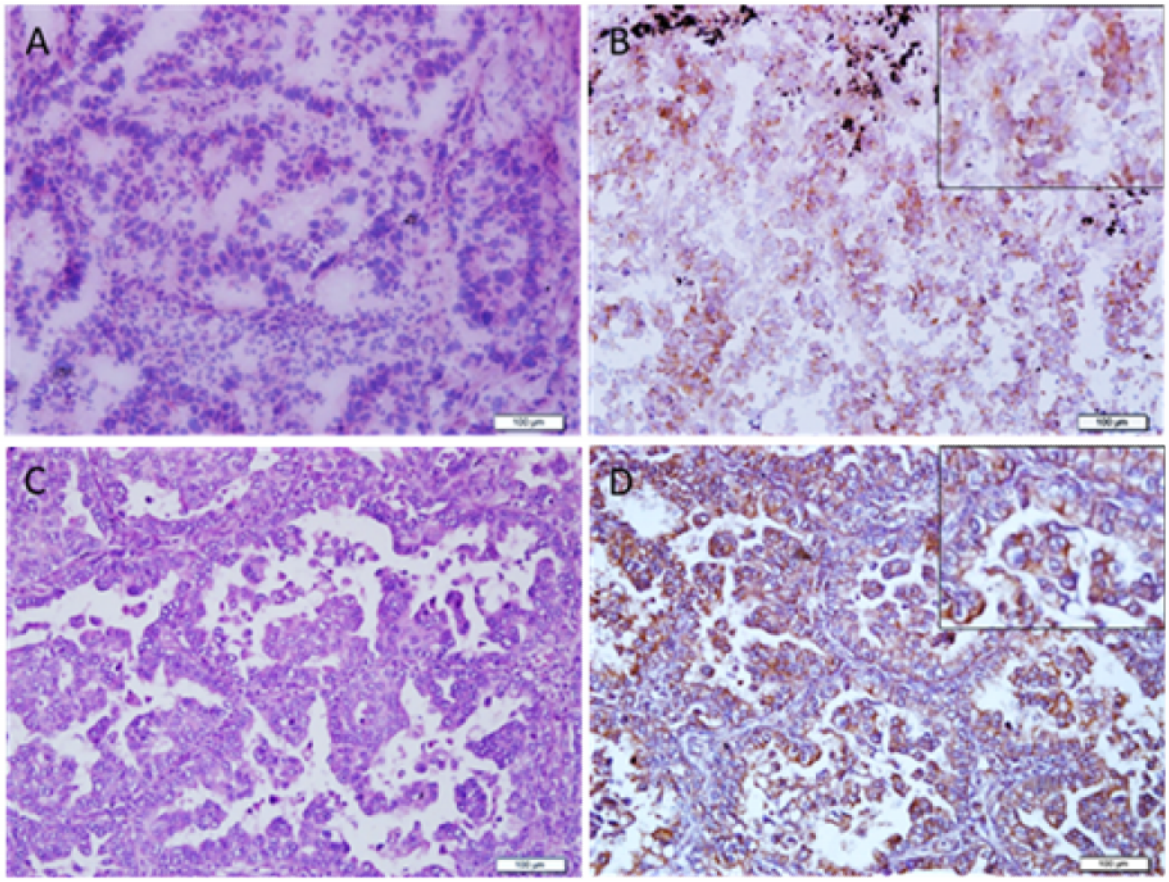

Fig. 3. Microphotographs of frozen and FFPE non-small cell lung carcinoma sections. A: Hematoxilin and eosin staining. Note: intense immunoreactivity of 14F7 mAb (Brown color) in frozen (B) as well as in its FFPE counterpart (D). The recognition of 14F7 was mainly located in the plasmatic membrane of malignant cells (inset on the upper-right corner, 400x magnification). Black color: anthracotic pigment. Counterstaining with Mayer's Hematoxylin (Blue color). White bar=100µm.

The pattern of staining with 14F7 mAb was not only restricted to the plasmatic membrane of malignant cells. The recognition of 14F7 was finely granular as it was previously described [11], but it was localized in both cell membrane and cytoplasm of malignant epithelial cells. In NSCLC samples, a predominance of the cytoplasmic staining with 14F7 mAb were previously evidenced [10]. Similarity, in Wilm´s tumors a membrane pattern and cytoplasmic diffusion of NeuGcGM3 was previously observed using the same mAb [26]. In addition, a nuclear staining with both P3 and 14F7 mAbs was also detected. In a previous study, *Van cruisen et al* published an additional nuclear staining of 14F7 in 15% of the NSCLC samples [10]. These changes could be related with a weak dissolving and/or a distorting in the localization of the antigenic determinant recognized by 14F7 mAb after the fixation and processing of tissues for routine histology. It is known, that gangliosides are partially or completely extracted from the tissues after ethanol and absolute methanol treatment [13].

Consequently, in the present study it was assessed the staining of 14F7 mAb in frozen tissues after 4% paraformaldehyde fixation. The expression of NeuGcGM3 by mean of 14F7 mAb reactivity was extensively observed in lung carcinoma sections, whereas it was not detected in normal lung tissues [8,11,27]. Nevertheless, *Carr et al.* reported no staining with 14F7 mAb in both

NSCLC and SCLC using frozen tissues and avidin-biotin complex (ABC) method [8]. The reason for this discrepancy might be that the labeled streptavidin biotin (LSAB) detection system, used in the present study, provides even more sensitivity than either the standard peroxidase-antiperoxidase (PAP) or avidin-biotin complex (ABC) systems [28]. It has been also suggested that LSAB is about 5 to 10 times more sensitive than standard ABC method [29]. Therefore, our results confirm the expression of NeuGcGM3 ganglioside in lung carcinomas.

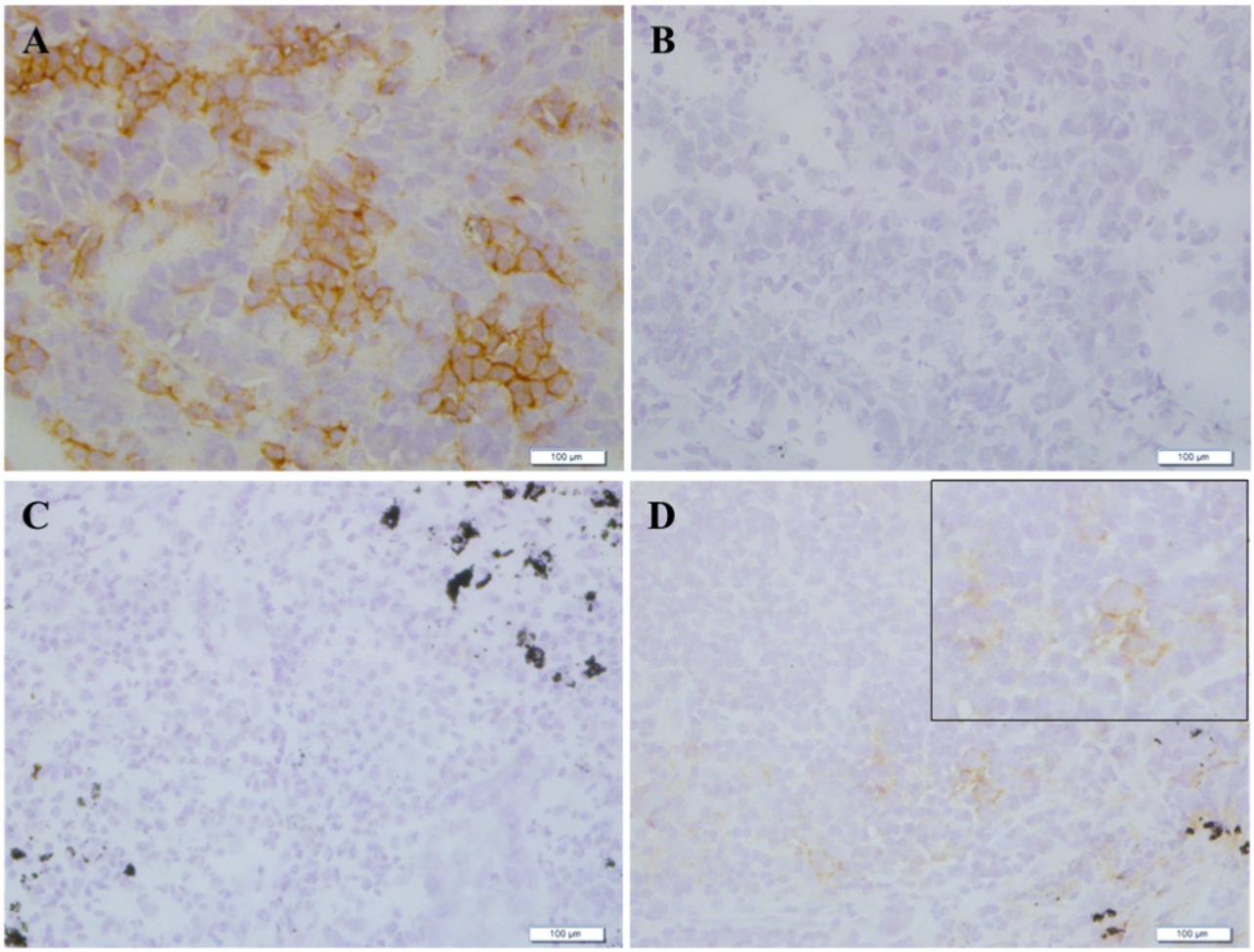

Fig. 4. Microphotographs of the 14F7 mAb reactivity in NSCLC sections after different chemical extraction protocols. An intense, homogeneous and finely granular reactivity of 14F7 mAb was observed in tissues treated with methanol after 4% neutral-buffered formaldehyde fixation (A) (Brown color). Observe: the complete removal of N-Glycolyl GM3 ganglioside from ethanol (B) and chloroform/methanol (C) treated tissues before formaldehyde fixation. The reactivity of 14F7 mAb was significantly but not completely eliminated with chloroform/methanol treatment after 4% NBF fixation (D) (inset on the upper-right corner, 400x magnification). Black color: anthracotic pigment. Counterstaining with Mayer's Hematoxylin (Blue color). White bar=100µm.

The pattern of reaction of 14F7 mAb in frozen tissues was finely granular and homogeneous and was mainly located in the plasmatic membrane of malignant cells. Similar pattern was described in breast carcinoma and cutaneous melanoma [8]. The ability of 14F7 mAb labelled with 99mTc to recognize breast tumors *in vivo* by the

radioimmunoscintigrafic technique confirmed the expression of NeuGcGM3 in the cell surface [9].

Additionally, in our study just a lightly cytoplasmatic staining was also detected in frozen tissues. The intracellular movement of glycosphingolipids and especially of gangliosides within the different subcellular compartments has been reported [30,31]. In addition, some authors have suggested the transit of free Neu5Gc to endosomal/lysosomal system via pinocytosis, as well as its transportation to Golgi apparatus and into the cytosolic compartment, where NeuGc could be incorporated to newly synthesized glycoconjugates [20]. These results could explain the cytoplasmatic staining observed with the 14F7.

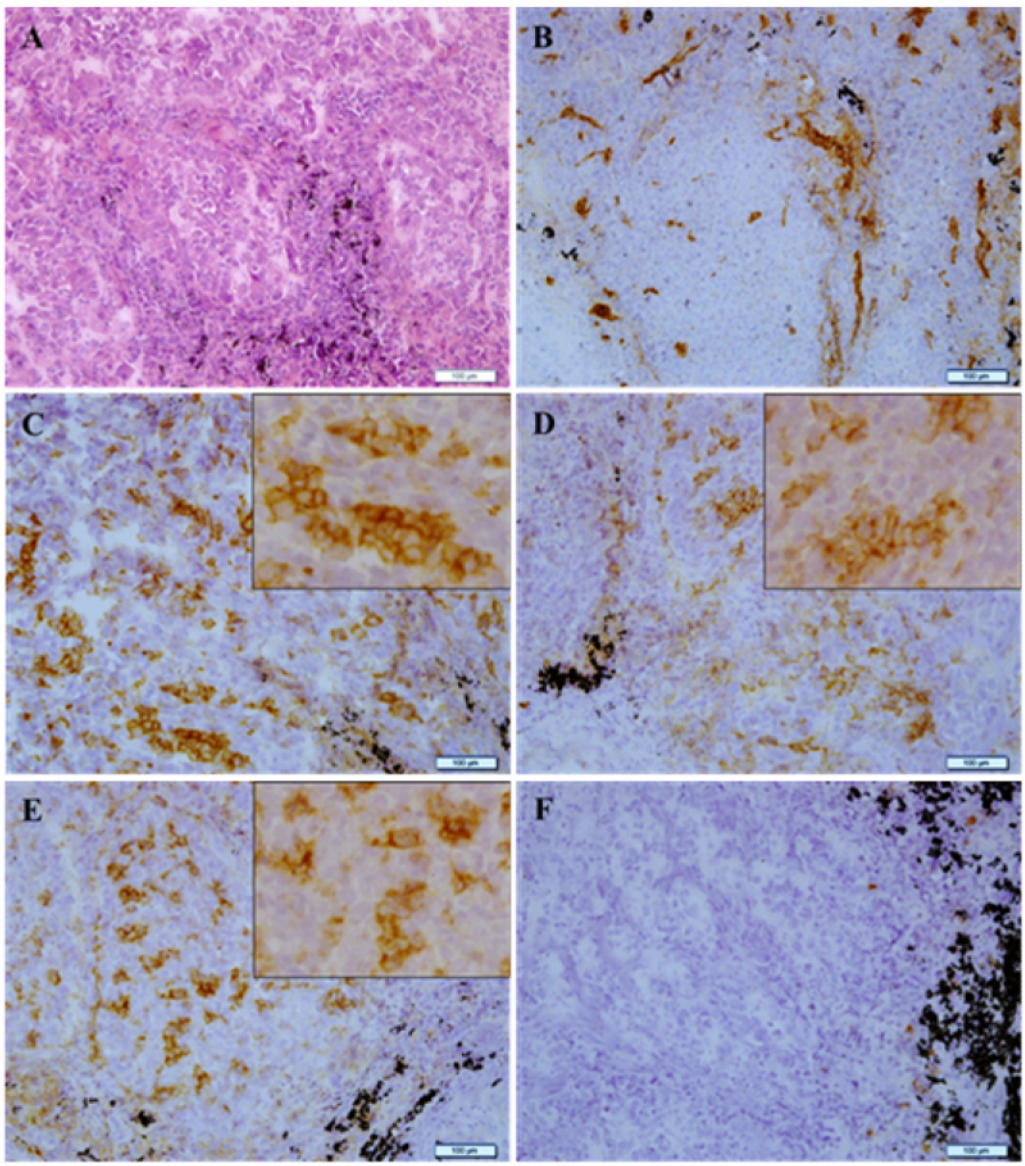

Fig. 5. Microphotographs of frozen non-small cell lung carcinoma sections. A: Hematoxilin and eosin staining. B: The reactivity of the anti-FVIII antibody permitted to confirm the antigenic preservation of the tissues. Note: the intense immunoreactivity of murine (B), chimeric (C) and humanized (D) versions of 14F7 mAb (Brown color). The recognition of 14F7 was mainly located in the plasmatic membrane of malignant cells (inset on the upper-right corner, 400x magnification). Black color: anthracotic pigment. F: negative control. Observe the absence of immunostaining. Counterstaining with Mayer's Hematoxylin (Blue color). White bar=100µm.

On the other hand, therapy with murine-derived mAbs has been limited by their tendency to develop human anti-mouse antibodies (HAMA) response, the relatively short half-life of the mAbs and the limited ability of murine Fc to activate human immune mechanisms [32]. For that reason, both chimeric and humanized versions of 14F7 mAb have been developed in our center. In the present study, both a chimeric and the humanized versions of 14F7 mAb exhibited a similar pattern of immunostaining as compared with the murine counterpart. In previous reports, the similar ability of both the 14F7 murine and the recombinant mAbs to directly kill the NeuGcGM3-positive murine myeloma cells as well as, lymphocytic leukemia cells without participation of complement have been demonstrated [14,15,33]. Taken together, these evidences suggest the potential use of the 14F7 recombinant mAbs for the passive immunotherapy of lung carcinomas expressing NeuGcGM3.

Finally, the treatment with ethanol, methanol and chloroform/methanol before 4% NBF fixation significantly removed NeuGcGM3 ganglioside from frozen NSCLC sections, consistent with the solubility of the glycolipid antigens [13]. Interestingly, only chloroform/methanol treatment extracted NeuGcGM3 from frozen tissues after 4% NBF fixation as well as in FFPE samples. In previous reports, some authors have suggested that the routine tissue processing do not extract or damage the antigenic carbohydrate determinants of gangliosides [34]. It was also demonstrated that formalin fixation prevent the damage and/or extraction of the antigenic determinant recognized by 14F7 mAb, permitting its detection in this kind of samples [35]. In line with this, a correlation between the immunoreactivity of 14F7 mAb in frozen tissues as compared with the FFPE counterparts was observed despite the changes in the pattern of reaction. Recently, the expression of NeuGcGM3 in formalin-fixed and paraffin-embedded NSCLC tissues using GMR8 mAb by immunohistochemical and TLC immunostaining methods has been published [12]. In this sense, 14F7 mAb becomes a useful tool to identify tumors that could be sensitive to anti-NeuGcGM3 specific therapies.

Although, the use of frozen samples is desirable for any determination of gangliosides, as it is known, this kind of samples is often difficult to obtain. Nevertheless, FFPE tissues are routinely performed for the preparation of samples for pathological purposes. In this way, our data and those related with the extraction of NeuGcGM3 from FFPE blocks could support the continuous evaluation of NeuGcGM3 expression using this type of samples. However, the further evaluation of the chemical nature of the antigenic determinant recognized by 14F7 mAb and the standardization of an immunohistochemical method are required.

In summary, we report the tissue expression of NeuGcGM3 in lung tumors by mean of two different mAbs (P3 and 14F7). It was also reported for the first time the tissue expression of NeuGcGM3 in NSCLC frozen samples, supported by the chemical extraction with organic solvents. We also showed a preliminary comparison of the staining pattern of the chimeric and one humanized variants of this antibody with their murine version, in the same system. Our data and those from the pre-clinical studies using chimeric and/or humanized 14F7 mAb are in agreement with their potential use for the passive immunotherapy of lung carcinoma expressing NeuGcGM3. Additionally, we reported a correlation between the immunoreactivity of the 14F7 mAb in frozen tissues as compared with the FFPE counterparts. These results could support the use of FFPE tissues for the selection of patients for the immunotherapy of lung

carcinoma expressing NeuGcGM3. A phase III multinational clinical trial in NSCLC patient using Vaxira® (Racotumumab,1E10 mAb), an anti-idiotypic molecular vaccine that mimicry NeuGcGM3, had been planned. Simultaneously, a comparative cross-reactivity study on normal human tissues using the 14F7 recombinant mAbs is ongoing in our center.

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