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Carbonic anhydrase IX antigen differentiates between preneoplastic malignant lesions in non-small cell lung carcinoma

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ABSTRACT: The MaTu interval (MN)/carbonic anhydrase (CA) IX tumour-associated antigen is a protein that is normally expressed in the gut and belongs to the carbonic anhydrase enzyme family (CA IX). It has been detected in tumour cell lines and in some solid tumours including cervical, oesophageal and clear cell renal carcinoma. This study determined MN/CA IX expression in 65 primary non-small cell lung cancer resected with curative intent and in 38 bronchial preneoplastic lesions, carcinoma *in situ* or microinvasive carcinoma as well as in normal bronchial tissue.

The presence of MN/CA IX was detected using immunohistochemistry and Western blot analysis, whenever frozen material was available.

Immunostaining was positive in 52/65 (80%) of the tumour samples. The staining was more often focal than diffuse. The percentage of stained cells in positive tumours was highly variable, ranging 1–85%. The pattern of immunostaining was predominantly cytoplasmic with a membranous reinforcement (87%). The intensity was mainly strong (69%). The presence of the protein in the tumour was confirmed by Western blot analysis in the eight samples tested. All the morphologically normal epithelia, except in close vicinity of tumours in some cases, as well as the preneoplastic bronchial lesions (basal cell hyperplasia, metaplasia and dysplasia) were immunonegative for MN/CA IX expression. In contrast, carcinoma *in situ* and microinvasive epithelioma showed the presence of MN-immunopositive tumoural cells in 5/7 and 4/5 of the samples, respectively.

These data suggest that MN/CA IX is a useful marker for the differentiation between preneoplastic lesions and bronchial non-small cell lung cancer in the lung.
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In industrialized countries, lung cancer is the leading cause of death, and its high mortality is mainly attributed to late diagnosis. The disease develops insidiously, with symptoms that usually occur when the tumour has reached a size or degree of local invasion that compromises curative surgical resection. Recent efforts have been performed to understand the natural history of lung cancer, namely by the study of preneoplastic lesions [1] that can now be better detected by new techniques with high sensitivity such as fluorescence bronchoscopy [2].

The recognition of multiple morphological and biological alterations in the epithelium of patients at a high risk of developing lung cancer has lead to an intensive search for new biomarkers, that would be specifically associated with malignant transformation. The detection of these biomarkers in sputum or biopsies would possibly allow an earlier diagnosis.

Recently, a tumour-associated antigen, called MaTu (MN) or carbonic anhydrase IX (CA IX)), has been described in the highly malignant cervical HeLa cell line [3]. The MN/CA IX antigen is an *N*-glycosylated protein detected as two distinct protein bands of 54 and 58 kDa on Western blots, probably due to the posttranslational processing of a single gene product. The expression of MN in normal tissues seems to be confined to the gastrointestinal tract. It is found in the basolateral surfaces of the gastric mucosal cells and of enterocytes of the small and large intestine. In the small intestine, its expression is particularly intense in the duodenum and jejunum in the crypts. Its expression is weaker in the colon [4]. Thus, the presence of MN appears to correlate with the rapidity of cell proliferation in the normal gut.

MN/CA IX has also been found in various solid tumours developed from tissues that normally do not express it. It was first described in cervical dysplasia, cervical intraepithelial neoplasia (CIN) as well as in invasive squamous and glandular neoplasms of the uterine cervix [5–7]. The MN/CA IX protein has also been detected in preneoplastic lesions, squamous cell carcinomas and adenocarcinomas of the oesophagus [8]. Finally, it is expressed in clear cell carcinomas of the kidney, while it is not detectable in normal kidney and in benign renal lesions [9, 10].

The presence of MN in lung cancer has not been studied to date. The present report is an investigation of MN/CA IX expression in primary non-small cell lung cancer (NSCLC) resected with curative intent, in bronchial preneoplastic lesions and early lung cancer (carcinoma *in situ* (CIS) and microinvasive carcinoma).

Materials and methods

Tissue specimens

Tissue samples were obtained from 65 resected invasive NSCLC and from preneoplastic and early lung cancer lesions as well as normal mucosa obtained by bronchial biopsies in patients at high risk for lung cancer, using fluorescence bronchoscopy. The 65 resected tumours were obtained from the Pathology Department; they included 31 adenocarcinomas, five bronchio-alveolar carcinomas, 24 squamous cell carcinomas, four adenosquamous carcinomas and one undifferentiated large cell carcinoma. Normal bronchial (n=34) and alveolar (n=42) epithelium in close contact to the tumour as well as normal matched lung tissues were also assessed. Preneoplastic and early lung cancer lesions as well as normal mucosa samples were obtained by bronchial biopsies in 36 patients at high risk for lung cancer, using fluorescence bronchoscopy. The samples were chosen as representative of particular morphological preneoplastic alterations, including normal epithelium (n=10), basal cell hyperplasia (n=8), metaplasia (n=10), dysplasia (n=8), CIS (n=7) and micro-invasive tumours (n=5).

All tissues were fixed in 10% neutral buffered formalin within a few hours following the surgical resection. In available cases, fresh tissue was snap-frozen in liquid nitrogen and stored at -80°C until processing. Histology was classified according to the new World Health Organisation (WHO) 1998 classification [11], based on routine haematoxylin-eosin staining, combined with periodic acid Schiff staining and immunohistochemistry when necessary. Bronchial biopsies were classified as normal epithelium, basal cell hyperplasia, metaplasia, dysplasia, CIS or microinvasive epithelioma. Morphological staging was performed blindly by two of the authors (C. Roufosse and P. Vermylen).

Immunohistochemical studies

Immunohistochemistry was performed according to a standard peroxidases technique with the murine monoclonal antibody M75 (kindly provided by J. Pastorek, Institute of Virology, Slovak Republic). Five-micron thick sections of paraffin-embedded tissues were deparaffinized. The endogenous peroxidases were quenched with a solution of 0.3% hydrogen peroxide in methanol for 30 min. The slides were submitted to antigen retrieval in citric acid monohydrate 0.01 M (pH 6.0) consisting of three 5-min of microwave treatments at 800 W [5]. Pre-incubation with blocking serum (bovine serum albumin 1.5% in tris-hydroxymethyl-amino methane (Tris) buffer, pH 7.5) for 20 min enabled the specificity of the immunostaining to be increased by lowering non-specific background according to the method of Liao *et al.* [5]. Contact with the primary monoclonal antibody M75, diluted 1:5,000 in Tris-HCl was performed at room temperature for 60 min, followed by 30 min incubation with a secondary biotinylated anti-mouse immunoglobulin G antibody ("Super Sensitive" kit; Biogenex, San Ramon, CA, USA), before being exposed to horseradish peroxidase-conjugated streptavidine ("Super Sensitive" kit; Biogenex) for 30 min. The slides were then stained with diaminobenzidine tetrahydrochloride (Dako, Carpinteria, CA, USA). They were counterstained with haematoxylin and mounted with permount. All the incubations were performed at room temperature, in humidified chambers. The slides were rinsed twice in Tris buffer for 10 min between each incubation. Positive controls consisted of HeLa cells while negative controls were performed by omitting the primary M75 antibody. Immunostaining was semi-quantitatively scored as the percentage of positive tumour cells in the total field of a single section. The pattern of staining was qualified as cytoplasmic (C), cytoplasmic with a membranous reinforcement (CM) or membranous only (M). The intensity of the staining was recorded as absent, weak or strong.

Western blot analysis of MN expression

Total protein content was extracted from the frozen tissue samples after homogenization on ice in a buffer containing Phenyl methyl sulphonyl fluoride, in 10 mM ethylene diamine tetra-acetic acid (EDTA) (RIPA) buffer solution containing a broad spectrum of protease inhibitors (phosphate-buffered saline (PBS) pH 7.2, 1% Triton X-100, 0.1% sodium deoxycholate and 10 $\mu g \cdot mL^{-1}$ phenylmethylsulfonyl fluoride). Snap-frozen samples of gastric mucosa were used as positive controls. Protein quantification was performed according to the Lowry method using the Biorad protein assay (Biorad Laboratories, Hercules, CA, USA) assay. A 40-µg sample of each protein extract was separated by electrophoresis on a 12% sodium dodecyl sulphate (SDS) (Prosieve 50; Sanvertech, Boechout, the Netherlands) polyacrylamide electrophoresis gel, then transferred to a nitrocellulose membrane (polyvinylidene fluoride (PVDF)) in buffer (Tris 25 mM, glycine 190 mM, methanol 20%). The membrane was blocked for 60 min at room temperature in TBST buffer (Tris-HCl 10 mM, NaCl 150 mM, Tween-20 0.1%) and 5% dry milk. The blot was then incubated overnight at 4°C with the murine monoclonal antibody M75, diluted 1:3,000 in TBST. The membrane was incubated with a secondary peroxidase-bound goat anti-mouse antibody diluted 1:2,500 in TBST (Dako) for 60 min at room temperature. The protein bands were then revealed with a sensitive chemiluminescent detection system (ECL-plus; Amersham, Arlington Heights, IL, USA).

Statistical analysis

Comparisons of the distributions were made by using nonparametric tests (Kruskal-Wallis and Mann-Whitney tests). A p-value <0.05 was considered to be statistically significant.

Results

Tumour specimens included 31 adenocarcinoma, five bronchioloalveolar carcinoma, 24 squamous cell carcinoma, four adenosquamous carcinoma and one undifferentiated NSCLC. Normal bronchial (n=34) and alveolar (n=42) epithelium in close contact to the tumour was assessed as well as normal matched lung tissue. Bronchial biopsies were obtained in 24 patients at high risk for lung cancer and who underwent fluorescence bronchoscopy. The examination was performed for preoperative assessment of a synchronous lung cancer in four patients, or of a synchronous head and neck tumour in three, or for

Table 1. – MN expression in resected non-small cell lung cancer (NSCLC)

	Histological type					
	Adenoc	BAC	Squam	Adenosq	Undiff	Total
Patients n	31	5	24	4	1	65
Percentage of cells immunostained						
Negative (0%)	6	3	1	2	1	13
Positive (1–100%)	25	2	23	2	0	52
1–10%	12	1	9	1	0	23
11–20%	2	0	1	0	0	3
21–40%	3	0	4	0	0	7
41–60%	6	1	4	1	0	12
61–80%	2	0	3	0	0	5
81–100%	0	0	2	0	0	2
Immunostaining pattern						
Cytoplasmic	0	2	2	0	0	4
Membranous	3	0	0	0	0	3
Both	22	0	21	2	0	45
Immunostaining intensity						
Absent	6	3	1	2	1	13
Weak	7	1	8	0	0	16
Strong	18	1	15	2	0	36

Adenoc: adenocarcinoma; BAC: bronchiolo-alveolar carcinoma; Squam: squamous cell carcinoma; Adenosq: adenosquamous carcinoma; Undiff: undifferentiated NSCLC.

screening of lung cancer in nine patients with previous history of lung cancer and in eight with severe tobacco exposure. They included morphologically normal epithelium (n=10), basal cell hyperplasia (n=8), metaplasia (n=10), dysplasia (n=8), CIS (n=7) and microinvasive tumours (n=5).

Immunohistochemical MN antigen expression in invasive tumours

The results are shown in table 1. Immunostaining was positive in 80% of the tumour samples (52/65). The staining (fig. 1) was more often focal than diffuse, the positive cells being isolated or in islands. The percentage of stained cells in positive tumours was highly variable, ranging 1–85% (mean±SD 25±25%). The pattern of immunostaining was predominantly cytoplasmic with a membranous reinforcement (45/52; 87%). The intensity was mainly strong (69%). Considering the percentage of positive cells, intensity and pattern of staining, there were no significant difference between squamous cell and adenocarcinoma histological subtypes (Kruskal-Wallis test; MN frequency, p=0.122; MN intensity, p=0.449; MN staining pattern, p=0.609). There was no correlation with tumour differentiation either (Kruskal-Wallis test; MN frequency, p=0.0792; MN intensity, p=0.225; MN staining pattern, p=0.404).

"Normal" bronchial and alveolar epithelium in close vicinity to the tumours had cells with immunopositive staining in 14/34 bronchial samples (41%) and in 3/42 alveolar samples (7%). All matched normal lung specimens sampled at a distance from the tumour were immunonegative.

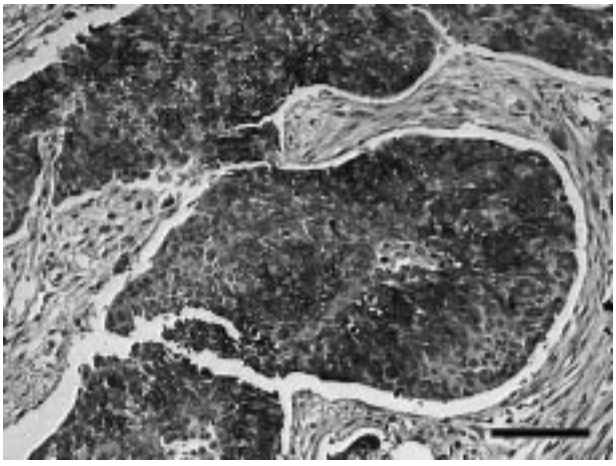

Fig. 1. – Squamous cell carcinoma with a positive immunostaining for MN with a cytoplasmic-membranous pattern. (Internal scale bar=25 μm.)

Western blot analysis of MN expression

A series of eight frozen tumour specimens, with a matched sample of normal lung tissue taken at a distance from the tumour and in the same patient, were examined by Western blot. The eight tumour specimens contained the 54–58 kDa bands, recognized by the murine M75

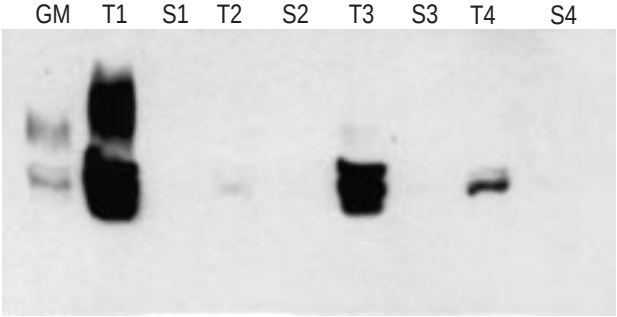

Fig. 2. – Western blot analysis of a squamous cell carcinoma showing the 54–58 kDa bands recognized as MN by the murine M75 monoclonal antibody, gastric mucosa (GM) being used as the positive in four matched samples of tumour (T) and normal tissues (S).

monoclonal antibody, and thus indentifying the presence of MN protein (fig. 2). All the eight control lung tissue specimens were negative.

Immunohistochemical MN antigen expression in bronchial biopsies

The results are shown in table 2. All the morphologically normal epithelia as well as samples of basal cell hyperplasia, metaplasia and dysplasia were immunonegative for MN/CA IX expression. In contrast, CIS and microinvasive epitheliomas showed the presence of MN-immunopositive tumoural cells in 5/7 and 4/5 of the samples, respectively. This immunostaining was predominantly weak and cytoplasmic in CIS (fig. 3) while it was more intense and most often cytomembranous in microinvasive lesions (fig. 4). Western blot analysis of MN expression in CIS was not performed because the size of the lesions was too small to obtain adequate material to perform the test. Figure 5 provides a graphical summary of the main study results.

Discussion

This study suggests that MN/CA IX is a biomarker of potential interest for lung cancer because it appears to be expressed when a bronchial lesion becomes neoplastic. Indeed, immunostaining is negative in normal epithelium (except when it is taken from close vicinity to tumours, in some cases) and in precancerous stages and, in contrast, is positive in a high percentage of CIS, microinvasive epithelioma and various histological subtypes of resected NSCLC.

MN belongs to the family of the CAs. These proteins are zinc metallo-enzymes that catalyse the interconversion of carbonic acid to carbon dioxide, thus participating in a wide variety of biological processes [12, 13]. In addition, the CA domain could possess a catalytically independent function of ligand-binding sites that mediates the signal transduction and influences the cell behaviour. This was proven for the receptor protein tyrosine phosphatase (RPTPb) containing an enzymatically inactive CA domain [14]. When tumour cell line 3T3 from the National Institutes of Health, Bethesda, MD, USA (NIH 3T3) fibroblasts are transfected with a plasmid expressing the MN/CA IX protein, they under-go morphological transformation with all typical features *in vitro*, including increased proliferation and anchorage independence [3]. On the basis of the expression pattern in normal and cancerous tissues, MN/CA IX is considered to play a potential regulatory role in intercellular communication and cell proliferation, and appears to be involved in oncogenesis.

When initially found in HeLa cells, the MN protein was not known to have a CA domain [5]. Its structure has been recently studied by complementary deoxyribonucleic acid and genomic sequence analysis. It consists of an N-terminal proteoglycan-like region, a central portion with an active enzyme centre that has a high sequence identity to members of the CA family, a transmembrane anchor and a C-terminal intracytoplasmic tail. Correspondingly, the MN protein has a weak carbonic anhydrase activity, for which reason it is also called CA IX. The membranous reinforcement described in immunohistochemical stainings is in keeping with the presence of a transmembrane anchor.

In humans, it is physiologically expressed in the epithelial cells of the gut with a high proliferation capacity [4]. In colorectal neoplasms, its expression is intense and is correlated with the presence of Ki-67, a well-established marker of cell proliferation [15]. In addition, the presence of MN protein or messenger ribonucleic acid has been documented in cancer arising from tissue that do not normally express this protein, such as the uterine cervix [5] or the kidney [9], suggesting that it may play an important role in the development of solid tumours.

The MN/CA IX protein is not expressed in the normal lung [9] and the present study performed on normal lung by immunochemistry and Western blot analysis confirms the previously published data. It is expressed in CIS but it has not been found in dysplasia, metaplasia and basal cell hyperplasia. This observation is different from what has been documented in cervical cancer [5]. As already reported, MN is not expressed in the normal cervix. Its expression occurs in the various stages of dysplasia (intraepithelial neoplasia) and it is not specific for cancer-associated human papillomavirus subtypes or of high grade lesions [15]. It is present in 100% of the cases of invasive squamous cell carcinomas [5] and in 65% of those of invasive adenocarcinomas [6].

The lack of MN expression in preneoplastic disease of the lung suggests that it would be a very specific marker of the neoplastic phenotype which may help in the differential diagnosis between dysplasia and CIS/microinvasive carcinoma. Indeed, there is so far no specific marker for this purpose since several genetic alterations appear very early in preneoplastic lesions, such as 3p deletion, K-ras activation, 9p deletion or p53 mutation [1]. The data presented here derived from a relatively small cohort are encouraging enough to warrant a large-scale investigation.

Table 2. – MN expression in preneoplastic and early neoplastic bronchial lesions

	Histological lesion	
	CIS	Microinvasive epithelioma
Patients n	7	5
Percentage of cells immunostained		
Negative (0%)	2	1
Positive (1–100%)	5	4
1–20%	5	3
21–40%	0	1
41–60%	0	0
61–80%	0	0
81–100%	0	0
Immunostaining pattern		
Cytoplasmic	5	1
Membranous	0	0
Both	0	3
Immunostaining intensity		
Absent	2	1
Weak	4	1
Strong	1	3

CIS: carcinoma *in situ*. All the morphologically normal epithelia (n=10) and samples of basal cell hyperplasia (n=8), metaplasia (n=10) and dysplasia (n=8) were negative for MN/CR IX expression.

Resected lung cancer, a more advanced disease than CIS or microinvasive radio-occult squamous cell carcinoma, is not invariably associated with MN expression. In squamous cell carcinoma of the lung, however, MN appears to be particularly interesting since all but one of the 24 cases studied were found to be immunopositive. Nevertheless, a potential bias due to the limited number of patients, most of which had stage I or II disease, must be taken into account.

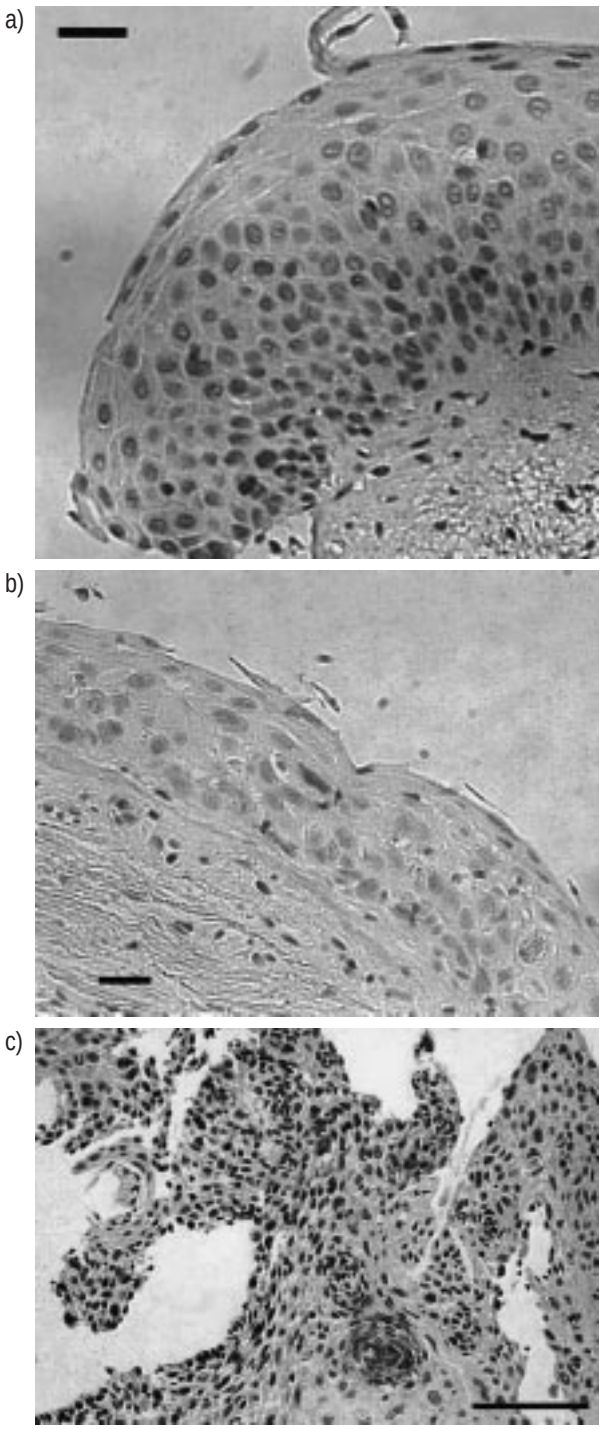

Fig. 3. – Examples of a) metaplasia, b) dysplasia and c) bronchial carcinoma *in situ* (CIS) with immunostaining for MN, only the CIS being positive with a cytoplasmic pattern. (Internal scale bars=50 μm.)

Additional data for locoregionally advanced (stage III) and metastatic (stage IV) tumours are needed. It is indeed possible that MN/CA IX may exert a negative control on cell growth as suggested by a reduction or a loss of its expression in progression of gastric tumour [16], although it may also act as a positive regulator, as observed in other tumours such as colorectal or cervical cancers [5–10, 17, 18].

Usually, studies classify samples as either positive or negative for the presence of a marker. MN/CA IX, at least in primary lung tumours, may present with a very heterogeneous repartition, including areas of dense expression and areas of absent or low expression within the same specimen. In these conditions, this study analysed in a semi-quantitative way the MN/CA IX pattern of expression and, for tumours, the complete section sample. This assessment method may be an explanation for the relatively low frequency of immunopositive cells for the tumour as a whole.

In some of the resected NSCLC samples, MN was shown to be expressed in morphologically normal epithelium in close vicinity of the tumour, whereas it was not the case for the normal tissue at distance. The authors have no clear explanation for this observation. A similar observation has been made in the uterine cervix, and has been exploited on cervical smears, where immunopositivity of even normal appearing cells has been found to be predictive of the presence of intraepithelial or invasive carcinoma [19]. It may be speculated that the expression of the MN gene may be induced by the tumour microenvironment [20]. On the other hand, this expression may also reflect a field of cancerization present in the lung, as suggested in a case report [21] by the widespread presence of single somatic *p53* mutation in the bronchi. If the theory of field carcinogenesis in the respiratory epithelium is correct, MN could be another example of such diffuse event. However, the absence of its expression in preneoplastic lesions, contrary to mutated *p53*, may be not in favour of this hypothesis. Further investigations are necessary to clarify this point.

In conclusion, MN/CA IX appears as a potentially useful marker in thoracic oncology to differentiate between preneoplastic bronchial lung lesions and early lung cancer, whether *in situ* or microinvasive. Expression of MN in bronchial cells of the sputum should be investigated in

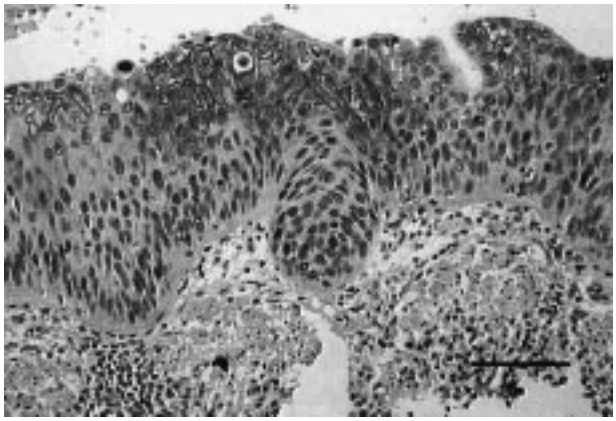

Fig. 4. – Microinvasive squamous cell epithelioma with a strong positive immunostaining for MN with a cytomembranous pattern. (Internal scale bar=25 μm.)

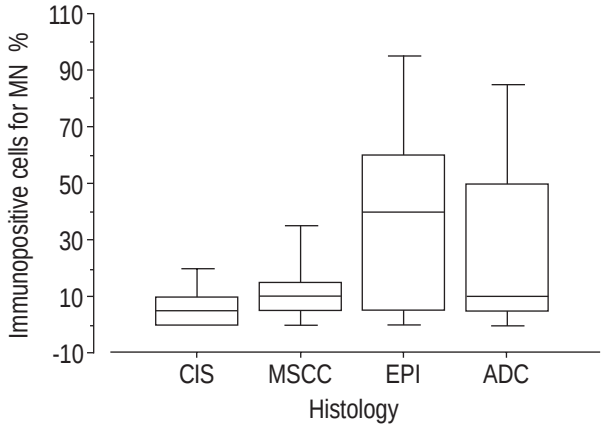

Fig. 5. – Expression of MN (percentage of immunopositive cells) in the main groups studied carcinoma *in situ* (CIS), microinvasive squamous cell carcinoma (MSCC), invasive squamous cell epithelioma (EPI), adenocarcinoma (ADC). Box and whisker plots with median, interquartile range and minimum and maximum values.

order to assess its potential as a new biological tool for lung cancer screening in high-risk populations. On the other hand, MN is often present in resected lung cancer but its expression in more advanced tumours has to be analysed in order to determine its potential prognostic value with respect to the survival of the patients or on the response to treatment.

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