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# Heparanase expression and localization in different types of human lung cancer☆

Teresa Cristina Fernandes dos Santos [a,1], Angélica Maciel Gomes [b,1], Marcos Eduardo Machado Paschoal [c], Mariana Paranhos Stelling [b], Vivian Mary Barral Dodd Rumjanek [d], Alyson do Rosário Junior [e], Paulo Marcos Valiante [f], Kalil Madi [f], Heitor Siffert Pereira de Souza [e], Mauro Sergio Gonçalves Pavão [b,**], Morgana Teixeira Lima Castelo-Branco [a,*]

[a] Laboratório de Imunologia Celular, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
[b] Laboratório de Bioquímica e Biologia Celular de Glicoconjugados, Instituto de Bioquímica Médica Leopoldo de Meis, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
[c] Instituto de Doenças do Tórax, Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
[d] Laboratório de Imunologia Tumoral, Instituto de Bioquímica Médica Leopoldo de Meis, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
[e] Laboratório Multidisciplinar de Pesquisa, Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
[f] Serviço de Anatomia Patológica, Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

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ABSTRACT

*Background:* Heparanase is the only known mammalian glycosidase capable of cleaving heparan sulfate chains. The expression of this enzyme has been associated with tumor development because of its ability to degrade extracellular matrix and promote cell invasion.
*Methods:* We analyzed heparanase expression in lung cancer samples to understand lung tumor progression and malignancy. Of the samples from 37 patients, there were 14 adenocarcinomas, 13 squamous cell carcinomas, 5 large cell carcinomas, and 5 small cell carcinomas. Immunohistochemistry was performed to ascertain the expression and localization of heparanase.
*Results:* All of the tumor types expressed heparanase, which was predominantly localized within the cytoplasm and nucleus. Significant enzyme expression was also observed in cells within the tumor microenvironment, such as fibroblasts, epithelial cells, and inflammatory cells. Adenocarcinomas exhibited the strongest heparanase staining intensity and the most widespread heparanase distribution. Squamous cell carcinomas, large cell carcinomas, and small cell carcinomas had a similar subcellular distribution of heparanase to adenocarcinomas but the distribution was less widespread. Heparanase expression tended to correlate with tumor node metastasis (TNM) staging in non-small cell lung carcinoma.
*Conclusion:* In this study, we showed that heparanase was localized to the cytoplasm and nucleus of tumor cells and to cells within the microenvironment in different types of lung cancer. This enzyme exhibited a differential distribution based on the type of lung tumor.
*General significance:* Elucidating the heparanase expression patterns in different types of lung cancer increased our understanding of the crucial role of heparanase in lung cancer biology. This article is part of a Special Issue entitled Matrix-mediated cell behaviour and properties.



## 1. Introduction

Lung cancer is the leading cause of cancer-related death worldwide [1]. This multifactorial disease can be organized into two groups: non-small cell lung cancer (approximately 85% of all lung cancers) and small cell lung cancer (approximately 15%) [2]. Despite recent advances in drug discovery and early detection, lung cancer continues to be associated with a poor prognosis and low survival rates. Therefore, continued research to increase our understanding of the biological features of this disease is strongly encouraged.

Tumor growth occurs in a complex microenvironment composed of stromal cells and extracellular matrix (ECM) [3]. Heparan sulfate

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* Correspondence to: M. T. L. Castelo-Branco, ICB, UFRJ, Av. Carlos Chagas Filho, 373, CCS, Bloco B, sala 19, Ilha do Fundão, CEP 21941-599, Rio de Janeiro, RJ, Brazil. Tel.: +55 21 25626428.
** Correspondence to: M. S. G. Pavão, HUCFF, UFRJ, Rua Prof. Rodolpho P. Rocco, 255, 4º andar, sala 4A-08, CEP 21941-913, Rio de Janeiro, RJ, Brazil. Tel: +55 21 2562 2093.
*E-mail addresses:* mpavao@hucff.ufrj.br (M.S.G. Pavão), morganalima@ufrj.br (M.T.L. Castelo-Branco).

[1] Both authors contributed equally to this work.

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proteoglycans (HSPGs), located in the ECM and on the cell surface, are composed of a core protein with covalently attached heparan sulfate (HS) chains. HSPGs regulate several aspects of cell physiology and ECM organization. Indeed, HSPGs sequester bioactive molecules, assemble ECM proteins, regulate growth factor-induced cell signaling and mediate cellular interactions with components of the tumor microenvironment [4]. Thus, the balance of HS levels is expected to control cell behavior and might represent a tipping point between health and disease.

Heparanase is the only known mammalian glycosidase capable of cleaving heparan sulfate chains, and consequently it regulates HS function in the ECM and on the cell surface. Heparanase is expressed in different cell types, but there is preferential expression in tumor cells [5]. Indeed, heparanase expression is upregulated in all the human carcinomas that have been studied [6]. Moreover, heparanase activity has been associated with high metastasis rates and poor prognosis in several cancer subsets [7]. This association can be explained by the direct cleavage of HS chains in the ECM and basement membrane by heparanase, which enables cell invasion and dissemination. Additionally, heparanase activity profoundly increases tumor metastasis and vascular density and reduces the postoperative survival rate. Therefore, these data suggest that heparanase is a strong candidate target for cancer treatment.

In this study, we analyzed lung tumor samples of four subtypes: adenocarcinoma, squamous carcinoma, large cell carcinoma, and small cell lung carcinoma. We determined that heparanase was expressed in 90% of the samples. Heparanase staining was detected in several cellular compartments, such as the plasma membrane, nucleus, and cytoplasm, as well as in the extracellular milieu. Regarding tissue distribution, heparanase was expressed in tumor cells as well as in certain epithelial cells and macrophages. Finally, we report the heparanase distribution profile in small cell lung carcinoma.

## 2. Materials and methods

### 2.1. Patients

A retrospective survey was conducted with a local population consisting of patients attending the Federal University of Rio de Janeiro Hospital Medical School that were diagnosed with non-small cell lung cancer (NSCLC) or small cell lung cancer (SCLC). The protocol was approved by the Ethics in Research Committee of the Clementino Fraga Filho University Hospital of the Federal University of Rio de Janeiro.

### 2.2. Histological analysis

Samples of normal or tumor pulmonary tissue were obtained surgically or via biopsy. The specimens were fixed in 40 g/l formaldehyde saline and embedded in paraffin for the histological analysis. Formalin-fixed 5-μm-thick lung sections were stained with hematoxylin and eosin to determine the tumor type. All of the histological analyses were performed by three independent pathologists. For the immunohistochemical study, 5-μm-thick sections were mounted on poly-L-lysine-coated slides.

### 2.3. Immunohistochemistry

Paraffin sections were cut and placed on slides that had been pretreated with poly-lysine; these slides were subsequently deparaffinized with xylene and were used to characterize heparanase expression using the indirect immunoperoxidase technique. Immunohistochemical staining was performed applying the rabbit polyclonal antibody HPA1 H-80 (catalog number sc-25825, Santa Cruz Biotechnology, Dallas, TX, USA) to paraffin-embedded sections following antigen retrieval. A total of 37 samples were analyzed, including 14 adenocarcinoma samples, 13 squamous cell carcinoma samples, 5 large cell carcinoma samples, and 5 small cell carcinoma samples. Subsequently, the tissue sample slides were immersed in 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity. After being rinsed in phosphate-buffered saline (PBS) containing 0.5% Tween 20 for 10 min, the tissue sections were incubated with non-immune horse serum for 30 min and then with the respective monoclonal antibody in a humidified chamber overnight at 4 °C. Sections from each sample were incubated with mouse monoclonal IgG1 (concentration-matched) (Dako A/S, Glostrup, Denmark) to serve as the negative controls. After a 10-min wash with PBS, all of the tissue sections were incubated for 30 min with the LSAB+ system HRP kit reagents (Dako, Glostrup, Denmark). After washing with PBS, all the sections were developed using a solution containing hydrogen peroxide and diaminobenzidine. The preparations were lightly counterstained with Harris's hematoxylin, dehydrated, and mounted in Permount (Fisher Scientific, Pittsburgh, PA, USA). Heparanase-immunostained lung sections were analyzed by three independent pathologists who were blinded to the clinical data, including the tumor node metastasis (TNM) classification and the smoking history. The pathologists quantified the heparanase staining in the tumor samples using the following scores: 0, no staining; 1, weak staining; and 2, moderate or strong staining. The pathologists also examined the heparanase-positive tumor stroma cells to determine their identity based on morphology.

### 2.4. Immunofluorescence and confocal laser scanning microscopy

In a double indirect immunofluorescence study, sections were incubated overnight at 4 °C with blocking buffer (2.5% bovine serum albumin (BSA), 2.0% skim milk, and 8.0% fetal bovine serum (FBS)) with shaking. The slides were rinsed once with PBS and 0.05% Tween 20 and then incubated with the primary antibodies diluted in PBS. The tissue sections were incubated with the rabbit anti-human polyclonal HPA1 H-80 antibody (catalog number sc-25825, Santa Cruz Biotechnology, USA) and the mouse monoclonal anti-human CD68 fluorescein isothiocyanate (FITC) antibody or the mouse monoclonal anti-human CD3 FITC antibody (both from Dako Glostrup, Denmark) overnight at 4 °C. After incubation, the slides were rinsed three times and were incubated with the Alexa Fluor® 546-conjugated anti-rabbit IgG antibody (Molecular Probes, Eugene, OR) for 1 h at room temperature. Sections

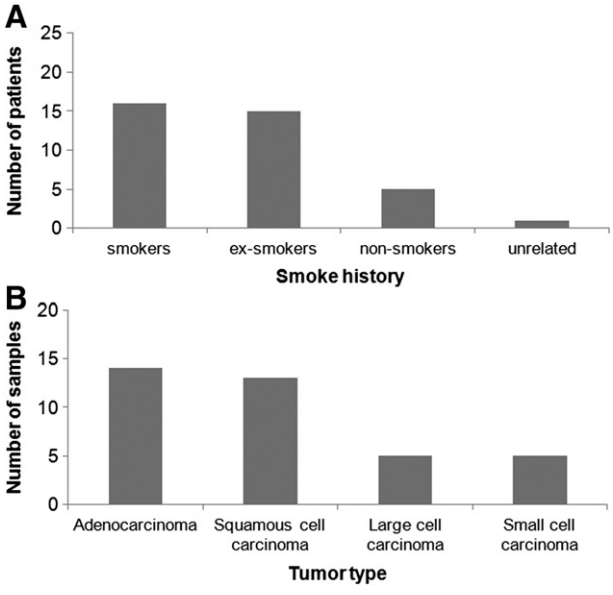


**Fig. 1.** Sample data. Patient information: (A) smoking history and (B) tumor type.

from each sample were incubated with either PBS alone or the secondary antibody alone to serve as negative isotype controls. The slides were air-dried, fixed for 5 min in a 1% paraformaldehyde solution, and mounted in an antifading medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Labs, Inc., Burlingame, CA, USA). Protein expression and localization were observed with a Leica TCS-SP5 AOBS confocal laser scanning microscope (Leica, Heidelberg, Germany), and representative images of each sample were captured.

### 2.5. Statistical analysis

The statistical analyses were performed using GraphPad Prism. The contingency test was used to determine the statistical significance.

## 3. Results

### 3.1. Patient and tumor sample characteristics

To evaluate heparanase expression in lung carcinoma, we performed immunohistochemistry on tumor samples from lung cancer patients. Fig. 1 presents the patient data regarding smoking history and tumor type. Most of the patients were male (21 males and 16 females), with an average age of 63 years (median age, 64.5 years) and a history of smoking (Fig. 1A). The samples represented four different tumor types: adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and small cell carcinoma (Fig. 1B). In terms of tobacco use, the majority of the adenocarcinoma patients were nonsmokers, the former smokers predominantly presented with adenocarcinoma or squamous carcinoma, and most of the current smokers had squamous carcinoma. The positive and negative controls for the heparanase antibody were prepared prior to sample processing. Supplementary Fig. 1 shows images of heparanase staining of third trimester placental tissue as the positive control [8] and of negative controls for each tumor type.

### 3.2. Heparanase distribution in the adenocarcinoma samples

All 14 adenocarcinoma samples were positive for heparanase. In these samples, this glycosidase was detected in different parts of the lung sections, including tumor cells, fibroblasts, and immune cells, as

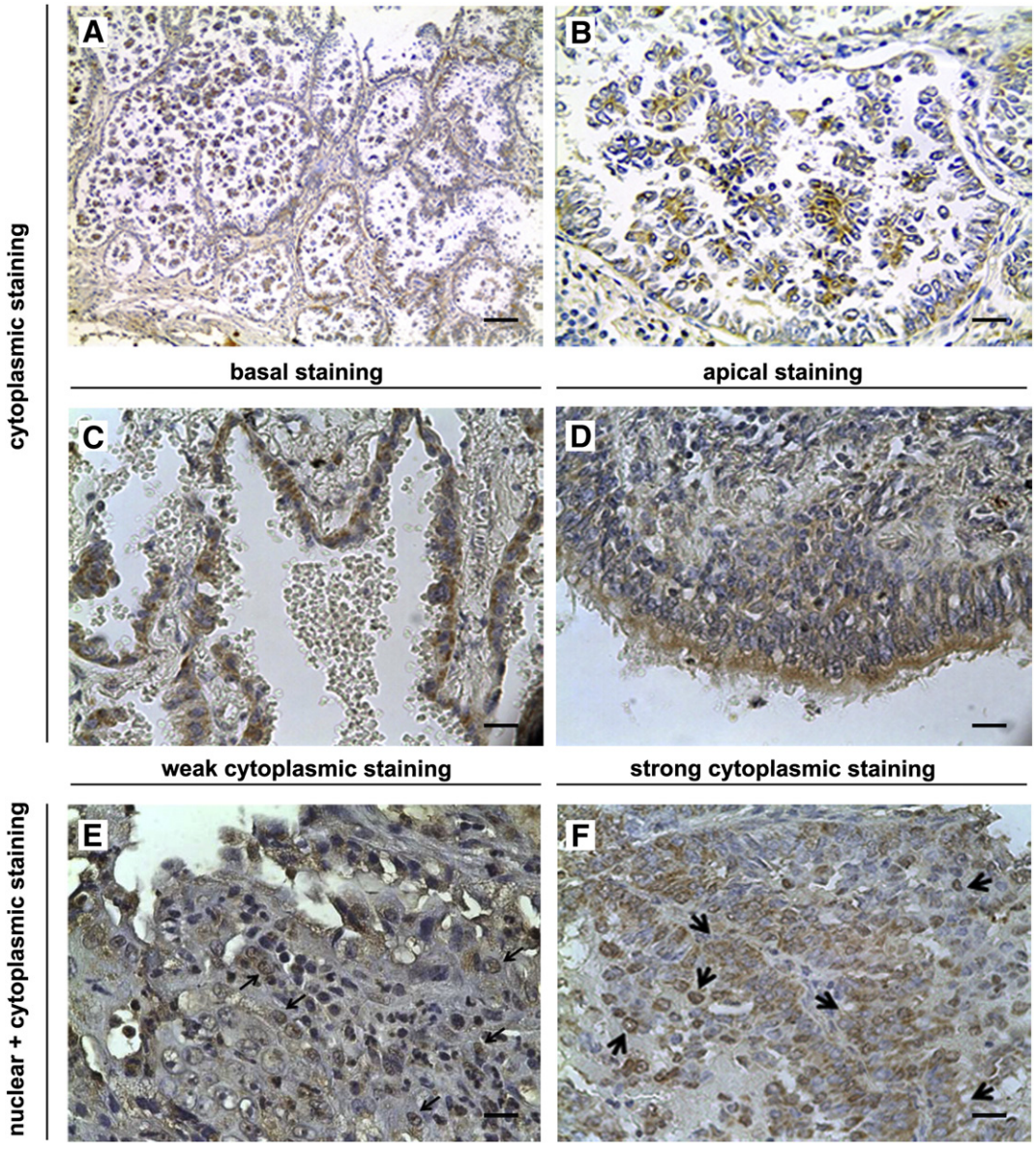


**Fig. 2.** Cytoplasmic heparanase staining patterns in adenocarcinoma. Examples of heparanase expression: (A and B) diffuse staining, (C) basal staining, and (D) apical staining. Heparanase expression that was exclusive to the cytoplasm or nucleus was (E) weak or (F) strong. Scale bars, (A) 80 μm and (B–F) 20 μm.

well as in different cellular compartments, such as the basal and apical membranes, the cytoplasm, and the nucleus.

Fig. 2 illustrates heparanase staining in different areas within the cytoplasm, such as areas of diffuse staining (Fig. 2A and B) or localized expression on the basal (Fig. 2C) or apical (Fig. 2D) surfaces. In certain samples, we observed nuclear heparanase expression as well as cells with mild (Fig. 2E) or more prominent (Fig. 2F) cytoplasmic heparanase staining. Membrane staining was observed in a reticular pattern (Fig. 3A) and in an apical pattern (Fig. 3B).

In addition to the staining in different locations within the tumor cells, we also detected heparanase expression in tumor cells at different stages of differentiation. Fig. 4 presents examples of heparanase-positive tumor cells with ciliated structures (Fig. 4A) or in a disorganized stage (Fig. 4B and C).

The tumor microenvironment also contained heparanase-positive cells, including potential fibroblasts (Fig. 5A and B), inflammatory cells (Fig. 5C and D), and macrophages. These results (Fig. 5E and F) indicated that heparanase was expressed by both tumor cells and cells in the tumor microenvironment.

### 3.3. Heparanase staining in squamous cell carcinoma

Squamous cell carcinoma samples presented a different staining pattern than the lung adenocarcinoma samples. Heparanase expression was detected in different parts of the tumor environment, similar to the adenocarcinoma samples. However, of the 13 analyzed samples, 3 were negative for heparanase, which could be because of a lack of heparanase expression in the tumor or because there was no heparanase expression in the specific part of the tumor biopsy that was analyzed.

Fig. 6 illustrates different patterns of heparanase staining, such as focal staining (Fig. 6A and B), dotted staining (Fig. 6C and D), and nuclear staining (Fig. 6E and F). In addition, heparanase staining was associated with the differentiation stage of the tumor cells. For instance,

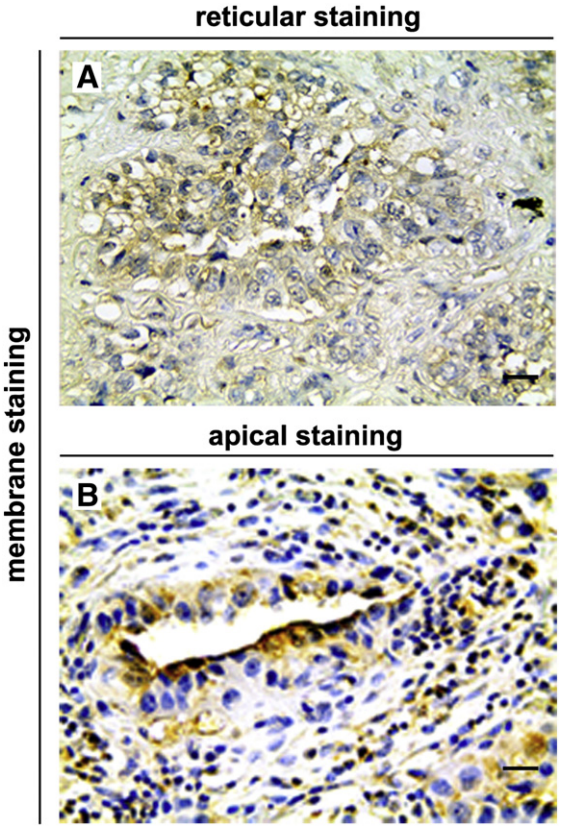


**Fig. 3.** Cell membrane heparanase staining patterns in adenocarcinoma. Examples of heparanase expression: (A) reticular pattern and (B) apical pattern. Scale bars, 20 μm.

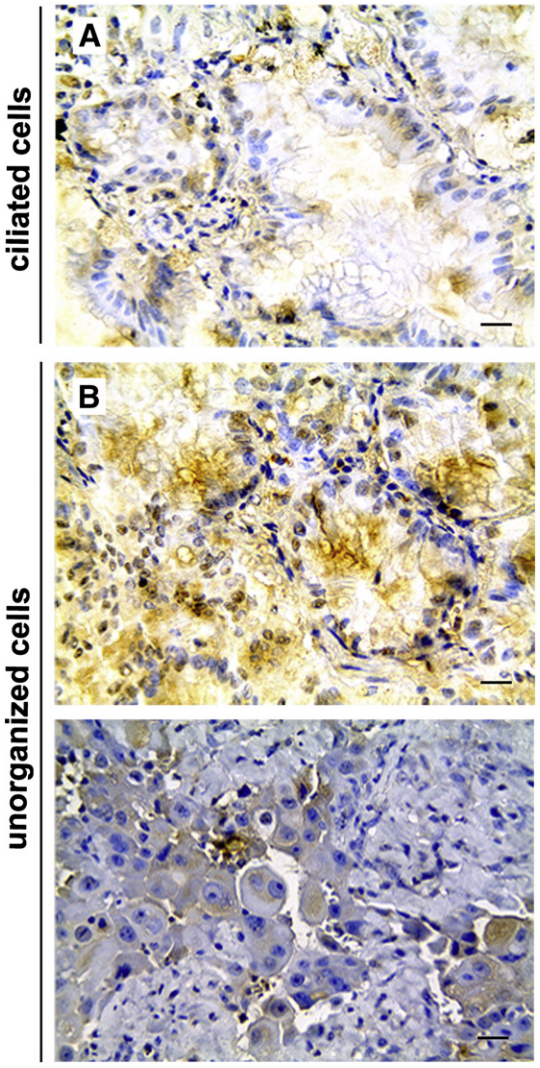


**Fig. 4.** Differential heparanase expression in more versus less differentiated tumor cells. Heparanase staining in (A) ciliated cells and (B and C) more undifferentiated, unorganized cells. Scale bars, 20 μm.

more differentiated cells exhibited weak staining (Fig. 7A), whereas less differentiated cells were more strongly positive (Fig. 7B).

The tumor microenvironment was also positive for heparanase. Specifically, we detected heparanase expression in non-tumor cells (Fig. 8A) and in cells with the morphology of inflammatory cells, such as macrophages (Fig. 8D) or others (Fig. 8B, C and E).

### 3.4. Heparanase distribution in large cell carcinoma

Of the five large cell carcinoma samples, only one was negative for heparanase expression. Heparanase was detected within the tumor and in the stroma. The prevalence of nuclear heparanase staining was higher (Fig. 9A and B) in the large cell carcinoma samples than in the adenocarcinoma and squamous cell carcinoma samples. In addition to the nuclear staining, cytoplasmic staining within the tumor cells was also detected (Fig. 10). The heparanase staining in fibroblast-like cells and inflammatory cells was similar to that in the adenocarcinoma and squamous cell carcinoma samples (Fig. 11).

### 3.5. Heparanase staining in small cell carcinoma samples

Small cell lung carcinoma is a malignant disease associated with poor prognosis and a high rate of early metastasis [9,10]. This type of

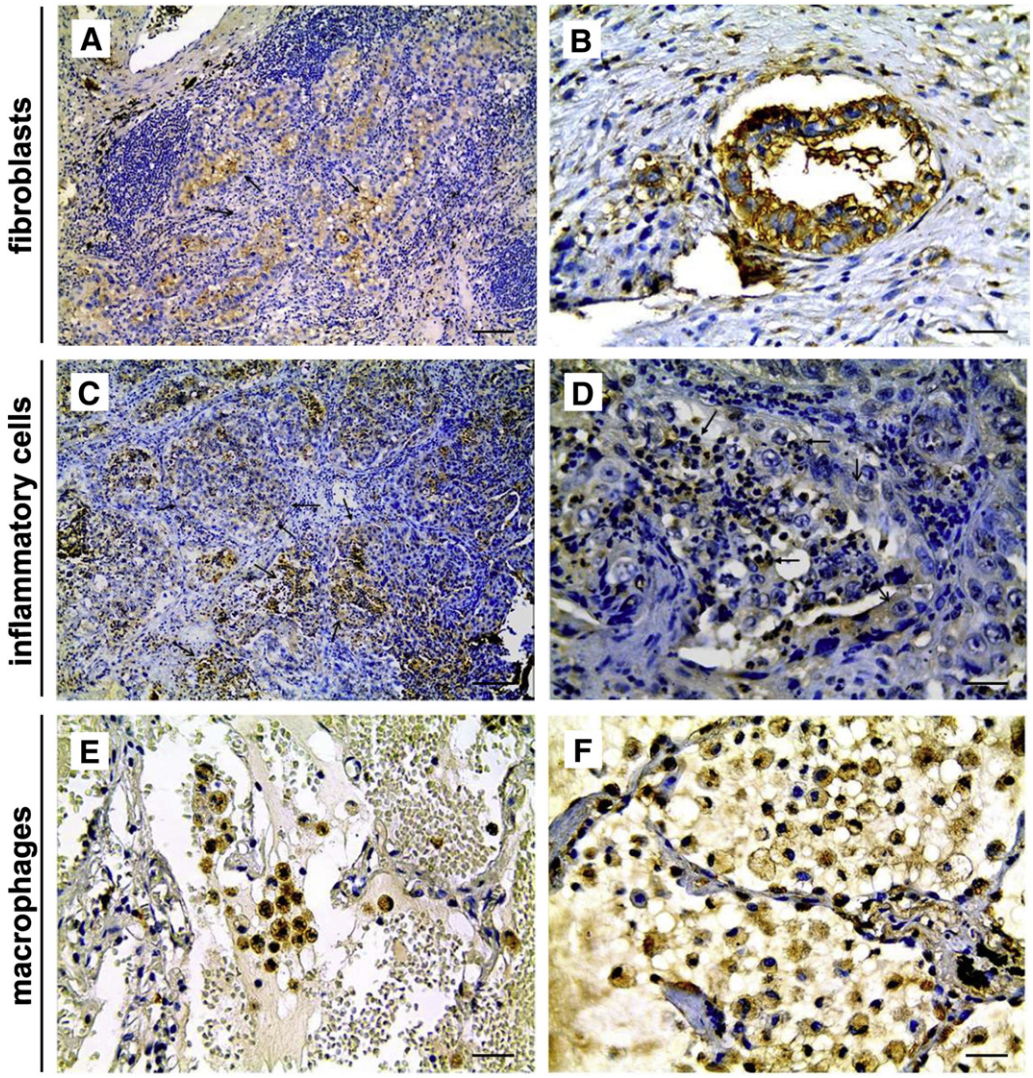


**Fig. 5.** Heparanase expression in the adenocarcinoma microenvironment. Heparanase was detected in (A and B) fibroblasts and (C and D) inflammatory cells, specifically in (E and F) macrophages. Scale bars, (A and C) 80 μm and (B, D–F) 20 μm.

lung cancer is typically not treated with surgery [11] because surgery does not significantly increase patient survival. Usually, metastasis has already occurred at the time of cancer diagnosis. Therefore, small cell lung carcinoma samples are rare, which makes it difficult to study a large number of samples. Of the small cell carcinoma samples that were analyzed, only one was negative for heparanase. Heparanase staining was not found in the nucleus; heparanase was only expressed in the cytoplasm (Fig. 12A), where it exhibited a diffuse expression pattern. Nevertheless, we cannot eliminate the possibility of nuclear heparanase staining in this type of tumor because only a few samples were analyzed. Similar to the other lung tumor types, the microenvironment was positive for heparanase, specifically in cells with a similar morphology to epithelial and inflammatory cells (Fig. 12B).

### 3.6. Heparanase colocalized with macrophages and lymphocytes

The heparanase expression results obtained by immunofluorescence with confocal microscopy confirmed the indirect immunoperoxidase assay results. Representative images from the colocalization studies in non-small cell lung cancer are presented in Fig. 13. Heparanase (red) colocalized with T cells (CD3, green, Fig. 13A) and macrophages (CD68, green, Fig. 13B), as evidenced by double immunofluorescence by confocal microscopy.

### 3.7. Evaluation of heparanase staining

We also analyzed heparanase expression in normal lung tissue (collected distant from the tumor lesions). These samples did not show any heparanase staining. We quantified the heparanase staining intensity in the different types of lung cancer (Table 1). We observed moderate-strong heparanase staining in 64% of the adenocarcinoma samples, whereas 61% of squamous cell carcinoma samples had weak staining. The heparanase staining intensity in the large cell carcinoma and small cell carcinoma samples was similar, with 60% of the samples showing weak-moderate staining. A contingency test was utilized to verify a correlation between the heparanase staining intensity and tumor type. Despite the absence of significance ($p = 0.20$), we observed a trend of strong heparanase staining in adenocarcinoma samples.

### 3.8. Nuclear heparanase tended to correlate with metastasis

The data presented here revealed a trend towards predominantly cytoplasmic heparanase expression in tumor cells. Nevertheless, the tumor microenvironment was also well represented (Fig. 14). Nuclear staining within the tumor cells was the least prevalent pattern detected among all the tumor types, especially in small cell carcinoma, which was completely negative for nuclear staining. A contingency test was

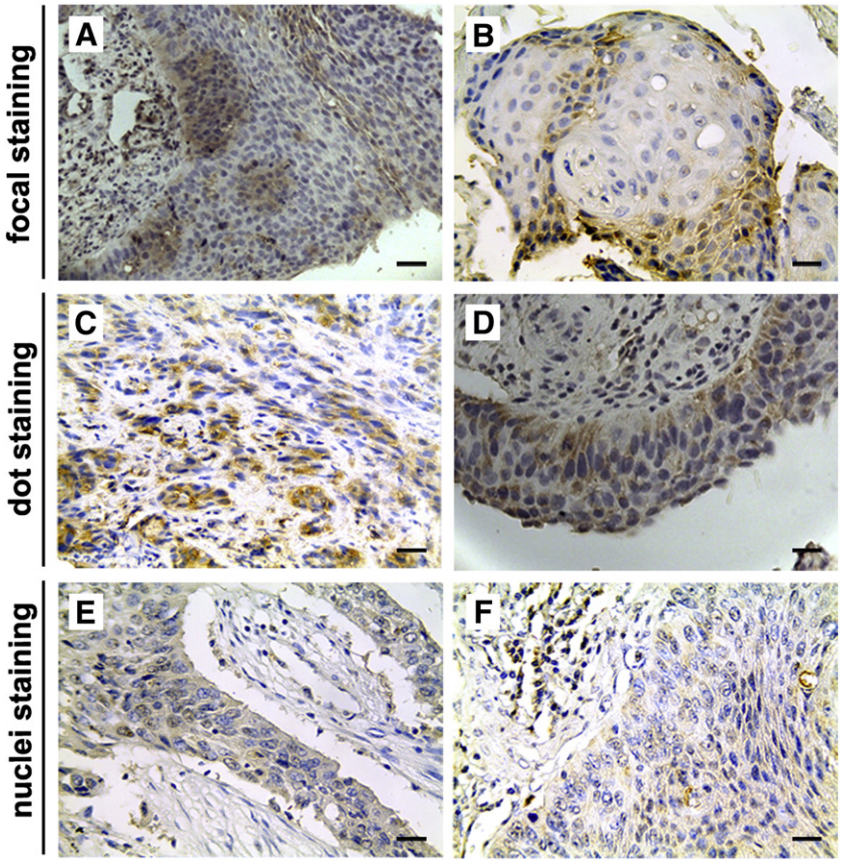


**Fig. 6.** Heparanase expression patterns in squamous cell carcinoma. Examples of heparanase expression: (A and B) focal staining pattern, (C and D) dotted staining pattern, and (E and F) nuclear staining. Scale bars, 20 μm.

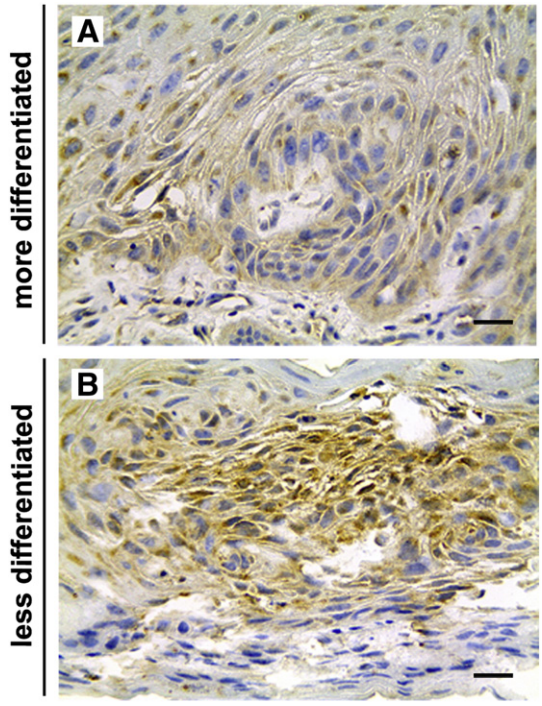


**Fig. 7.** Heparanase expression in squamous cell carcinoma was associated with tumor cell differentiation. Examples of heparanase staining in (A) more differentiated cells and (B) less differentiated cells. Scale bars, 20 μm.

utilized to verify the correlation between heparanase localization and metastasis. Although the data were not significant ($p = 0.40$), we observed a trend towards a correlation between nuclear heparanase expression and metastasis (Fig. 14). In analyzing the four groups together, 48% of the samples were negative for nuclear heparanase and positive for metastasis, and 37% of the samples were positive for nuclear heparanase and negative for metastasis. Only a small subset (15%) was positive for both nuclear heparanase and metastasis.

### *3.9. Heparanase expression tended to correlate with TNM staging in non-small cell lung cancer*

Regarding tumor node metastasis (TNM) staging, the adenocarcinomas and squamous carcinomas were predominantly grade IV, followed by grade IB, and the large cell carcinomas were primarily grade IB (Fig. 15). Heparanase expression was associated with grade IB and IV adenocarcinomas, with grade IV squamous carcinoma and with grade IA, IB, and IIIB large cell carcinomas. These data suggested that heparanase expression was associated with disease aggressiveness and with the development of primary tumors. Although the data did not quite reach statistical significance ($p = 0.06$), most likely because of the small number of patients, the results suggested that heparanase expression was associated with a more advanced and aggressive type of disease. The statistical analyses demonstrated that when the heparanase-positive non-small cell lung carcinoma and small cell lung carcinoma samples were considered as two separate groups, there was a significant correlation between heparanase expression and TNM stage ($p = 0.05$). This result suggested that heparanase expression in

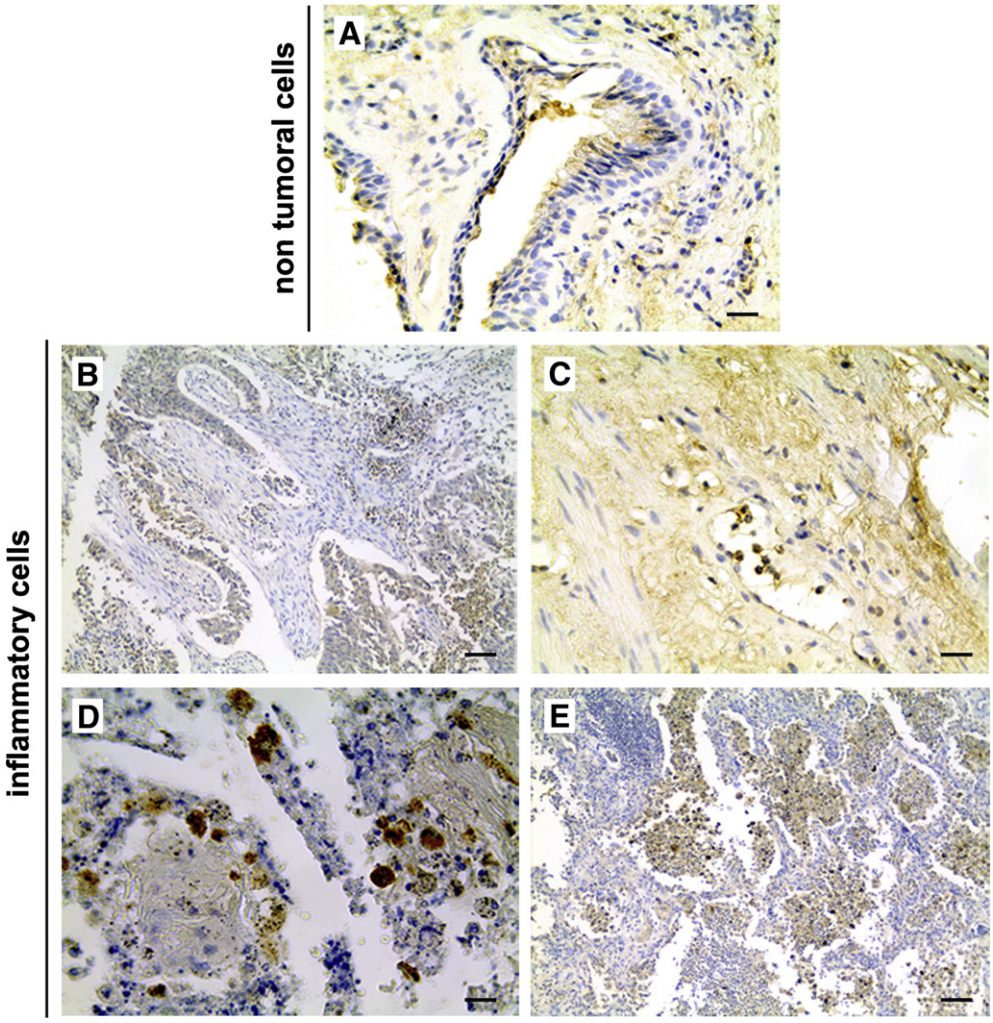


**Fig. 8.** Heparanase expression in the squamous cell carcinoma microenvironment. Examples of heparanase expression in (A) non-tumor cells and (B–E) inflammatory cells. Scale bars, (A, C and D) 20 μm and (B and D) 80 μm.

non-small cell lung carcinoma might play a role in tumor development and be associated with the highest TNM stage. Small cell lung carcinoma is not classified using TNM staging; instead, it is characterized as local/limited or extensive. Among the 5 small cell lung carcinoma samples that were analyzed, we observed heparanase expression in 3 cases of local disease and 1 of extensive disease.

In conclusion, these data improve our understanding of the role of heparanase in tumor progression. Furthermore, this is the first report on heparanase expression in small cell lung cancer.

## 4. Discussion

Lung cancer is the leading cause of cancer-related death worldwide. Despite major advances in treatment, including in chemotherapy, radiotherapy, and surgery, patient prognosis remains poor. Metastasis, local relapse, rapid tumor cell dissemination, advanced disease, and resistance to adjuvant therapy are the main reasons for the poor prognosis. Heparanase is an attractive molecule because it is the only known mammalian glycosidase able to control tissue HS levels. Indeed, balanced HS

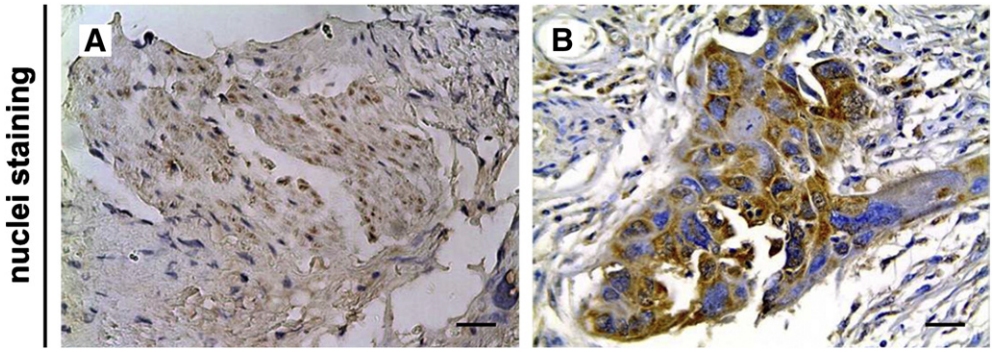


**Fig. 9.** Nuclear heparanase expression in large cell carcinoma. (A and B) Examples of heparanase expression in the nuclei of tumor cells. Scale bars, 20 μm.

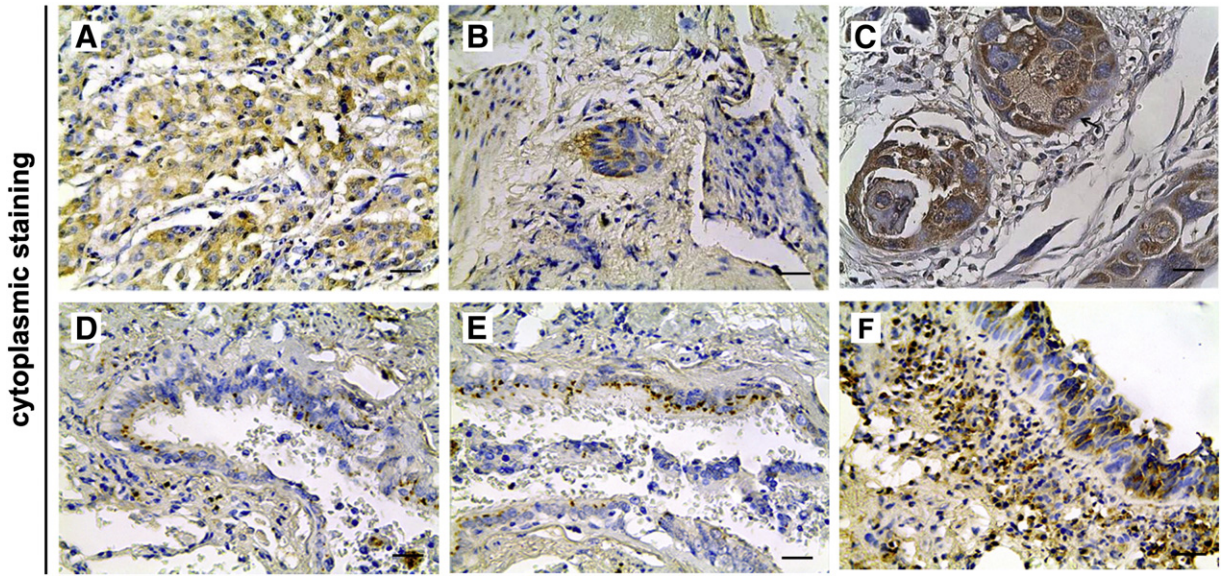


**Fig. 10.** Cytoplasmic heparanase expression in large cell carcinoma. (A–F) Examples of cytoplasmic heparanase staining. Scale bars, 20 μm.

levels are crucial for cell physiology, and altering these levels can result in tumor progression [4]. Thus, HS-based therapeutic targeting in cancer has been considered and discussed in previous reviews [12,13]. Here, we examined heparanase expression in biopsy specimens derived from 37 lung carcinoma patients using immunohistochemistry. Heparanase upregulation, estimated by immunostaining, was observed in the majority (90%) of the lung carcinoma specimens. This finding is in agreement with the heparanase overexpression found in many other human cancers, such as breast, melanoma, and head and neck cancers [7,14,15]. We observed heparanase staining in the cytoplasm and nuclei of the tumor cells. Again, this result corresponded with data from the literature [16]. Previous studies have shown that nuclear HS plays a role in regulating gene expression, inhibiting DNA topoisomerase I activity, and stabilizing the mitotic machinery [17,18]. In addition, it has already been shown that heparanase regulates syndecan-1 levels in the nucleus [19]. Thus, we suggest that nuclear heparanase might modulate the

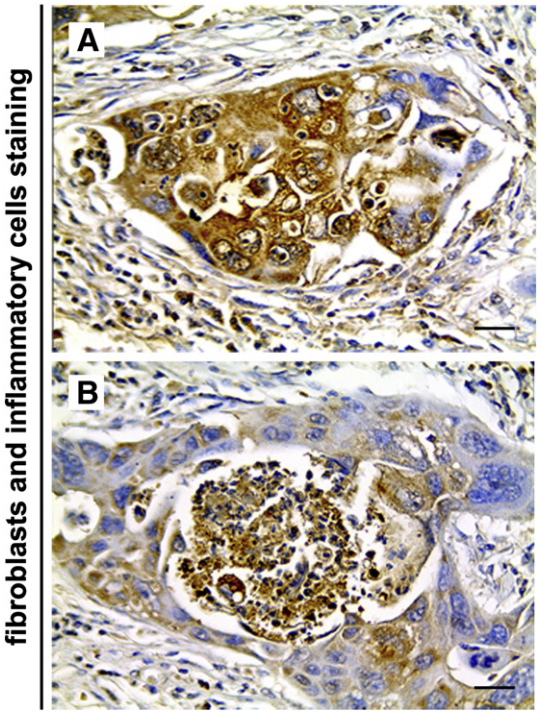


**Fig. 11.** Heparanase expression in the large cell carcinoma microenvironment. (A and B) fibroblasts and inflammatory cells were positive for heparanase. Scale bars, 20 μm.

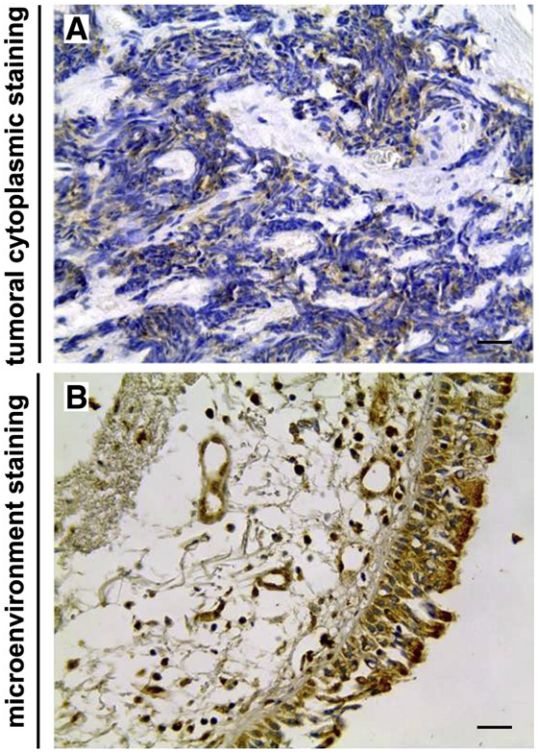


**Fig. 12.** Cytoplasmic heparanase expression in small cell carcinoma cells. Examples of heparanase staining in (A) the cytoplasm of tumor cells and (B) cells within the tumor microenvironment. Scale bars, 20 μm.

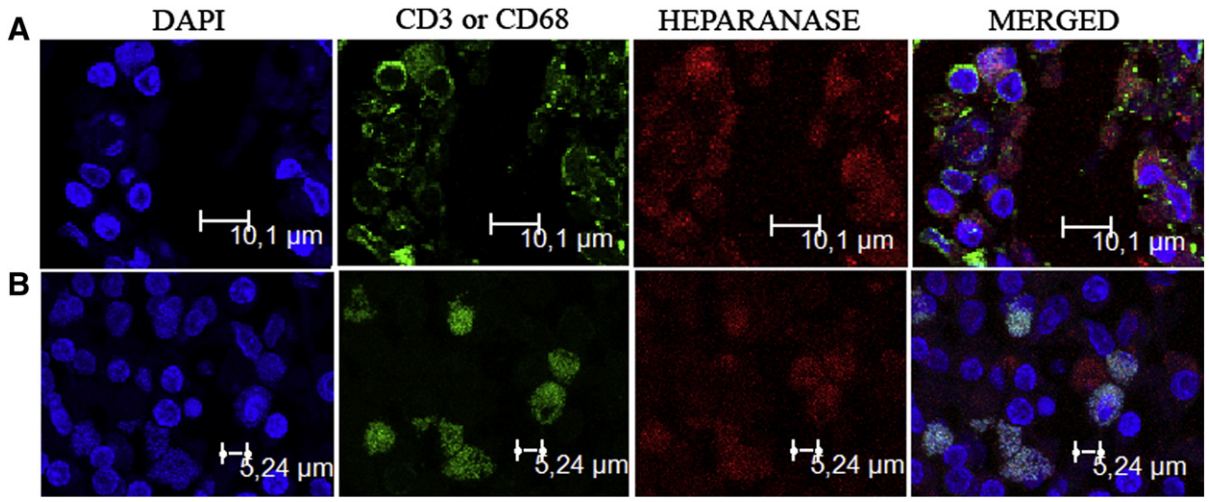


**Fig. 13.** Heparanase colocalized with macrophages and lymphocytes. Representative images from the colocalization studies in non-small cell lung cancer. Heparanase (red) colocalized with T cells (CD3, green, Fig. 13A) and macrophages (CD68, green, Fig. 13B).

transcription of several genes that affect tumor progression. In the squamous carcinoma samples, we found a subset of tumor cells with a dotted heparanase expression pattern in the cytoplasm. We also observed cell surface staining for heparanase in the analyzed samples. Cell surface staining has been associated with tumor angiogenesis and metastasis [20]. After being secreted, heparanase interacts with receptors or HSPG on the plasma membrane to become internalized and activated [21]. We speculate that these cells comprise a subgroup of cells with aggressive and invasive features.

The four subtypes of lung carcinomas that were analyzed presented samples with heparanase staining in normal epithelial cells and in the hyperplastic epithelium. We did not identify any previous studies showing heparanase expression in the lung epithelium, but heparanase expression has been reported in other types of epithelial cells [22].

Cancer-associated fibroblasts are essential components of the tumor microenvironment that play a critical role in tumor progression. These cells are an important source of extracellular matrix-degrading proteases and growth factors, which promote tumor growth and extracellular matrix remodeling [23]. We observed that certain fibroblasts within the lung carcinoma samples expressed heparanase. We speculate that heparanase from fibroblasts might regulate the tumor microenvironment through HS cleavage and consequently enable invasion and the release of growth factors from extracellular matrix depots.

Several cell types are present in the tumor microenvironment, including immune cells. Accumulating evidence suggests that tumor-associated macrophages (TAMs) actively promote aspects of tumor initiation, growth, and progression [24]. Our analyses suggested that lung cancer TAMs express heparanase. In addition, other inflammatory-like cells exhibited cytoplasmic heparanase expression.

Small cell lung carcinoma has been described as a highly malignant tumor [9,10]. Heparanase expression in small cell lung carcinoma is reported for the first time in this study; despite the small number of samples, we observed trends that were similar to those for the other tumor types. Nuclear staining was not observed in the available small cell lung carcinoma samples. This finding corresponds with the knowledge that aggressiveness is inversely correlated with the nuclear localization of heparanase [16].

**Table 1**
Heparanase staining intensity in lung cancer samples.

| | Percentage of patients (n) | | |
|---|---|---|---|
| | Heparanase staining intensity | | |
| Tumor type | 0 | 1 | 2 |
| Adenocarcinoma | 7.1 (1) | 28.6 (4) | 64.3 (9) |
| Squamous cell carcinoma | 23.1 (3) | 61.5 (8) | 15.4 (2) |
| Large cell carcinoma | 20.0 (1) | 60.0 (3) | 20.0 (1) |
| Small cell carcinoma | 20.0 (1) | 60.0 (3) | 20.0 (1) |

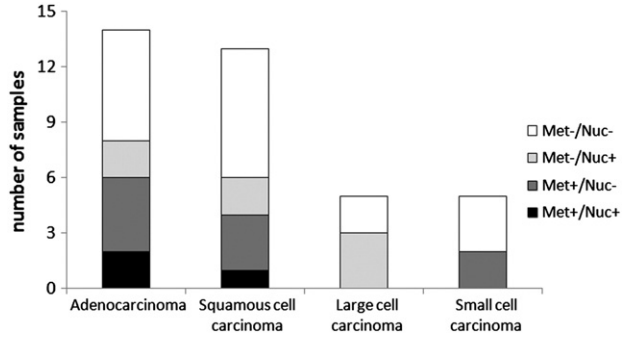


**Fig. 14.** Relationship between nuclear heparanase and metastasis. Samples were classified as metastasis-negative and nuclear heparanase-negative (Met −/Nuc −, white bars), metastasis-negative and nuclear heparanase-positive (Met −/Nuc +, light gray bars), metastasis-positive and nuclear heparanase-negative (Met +/Nuc −, dark gray bars), or metastasis-positive and nuclear heparanase-positive (Met +/Nuc +, black bars).

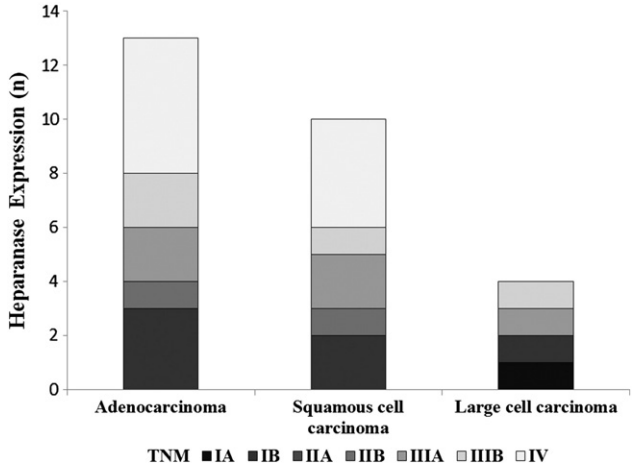


**Fig. 15.** Heparanase expression and TNM stage. The number of non-small cell lung carcinoma samples stratified by tumor node metastasis (TNM) staging and heparanase expression. Black bar, dark gray bars and light gray bars indicate TNM.

In conclusion, we described heparanase localization in the tumor microenvironment, thereby providing novel information about and insights into lung cancer. Therefore, we hypothesize that heparanase constitutes a novel potential marker and/or therapeutic target for lung carcinoma.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.04.010.

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