Histol Histopathol (2016) 31: 73-81

**http://www.hh.um.es**

**Histology and Histopathology**

*From Cell Biology to Tissue Engineering*

# Heparanase and heparanase 2 display differently deregulation in neuroendocrine tumors, depending on their differentiation grade

**Beatriz García[1,2,3,*], Olivia García-Suárez[4,*], Iván Fernández-Vega[5], Aitana Vallina[1], Aurora Astudillo[1,6] and Luis M. Quirós[2,3]**

[1]Fundación de Investigación Oftalmológica (FIO), Instituto Oftalmológico Fernández Vega, Oviedo, [2]Instituto Universitario de Oncología de Asturias (IUOPA), Oviedo, [3]Departamento de Biología Funcional, Universidad de Oviedo, Oviedo, [4]Departamento de Morfología y Biología Celular, Universidad de Oviedo, Oviedo, [5]Departamento de Patología, Hospital Universitario de Araba, Álava and [6]Departamento de Patología, Hospital Universitario Central de Asturias, Oviedo, Spain

*Both equally contributed to the study

**Summary.** Heparanase is a glucuronidase that appears upregulated in many human cancers and is involved in cellular invasion and tumor metastasis. Heparanase 2 is a homologue of heparanase that lacks enzymatic activity and displays anti-metastatic features. The aim of this work was to analyze the expression of both molecules in neuroendocrine tumors. We investigated the transcription of heparanases in lung neuroendocrine tumors well- and poorly differentiated using RT-PCR, and the expresion of the proteins by means of immunohistochemistry. The tumors were selected according to different malignancy WHO 2013 grades and were arranged in tissue arrays. The prometastatic enzyme heparanase appeared overexpressed in well- but not in poorly differentiated tumors, irrespective of their location. Moreover, the anti-metastatic heparanase 2 increased its expression in well-differentiated tumors, but strongly decreased in poorly differentiated ones, again independently of anatomic origin. Given the involvement of both molecules in tumor progression, through both their catalytic and non-enzymatic properties, there would seem to be a relationship between the regulation of their expression and the features of the neuroendocrine tumor.

**Key words:** Neuroendocrine tumorm, Proteoglycan, Glycosaminoglycan, Heparanase

*Offprint requests to:* Luis M. Quirós, PhD., Departamento de Biología Funcional, Facultad de Medicina, Julián Clavería s/n. 33006, Oviedo, Spain. e-mail: quirosluis@uniovi.es

DOI: 10.14670/HH-11-650

## Introduction

The neuroendocrine neoplasms (NENs) comprise a heterogeneous group of neoplasms that arise from an endodermal diffuse neuroencocrine cell type, and may originate in many parts of the body, although gastroenteropancreatic and lung tumors account for around 90% of all cases. Although some of the clinical and pathologic features of these tumors are characteristic of the organ, their common origin in a singular cell type means that there are many similarities between NENs in different areas of the the body (Duerr and Chung, 2007).

Neuroendocrine tumors can be classified according to different criteria; such as neoplasm grade, associated secretory symptoms, anatomic location or histology. Classification based on anatomic location has usually divided tumors between foregut, midgut and hindgut (Mougey and Adler, 2007), whereas that based on histological features divides the tumors into well- and poorly differentiated (Rindi et al., 2010; Klimstra et al., 2010). Well-differentiated NENs are either low or intermediate in grade and display a characteristic organoid arrangement of the tumor cells while poorly differentiated NENs display a more sheetlike or diffuse architecture and are considered high-grade in all cases (Mougey and Adler, 2007; Klimstra et al., 2010).

Heparan sulfate proteoglycans (HSPGs) comprise a small group of specific proteins covalently linked to HS glycosaminoglycan (GAG) chains. These glyco-conjugates play diverse roles in cancer, influencing the growth, invasion and metastatic properties of cancerous cells (Raman and Kuberan, 2010; García-Suárez et al.,

2013). The altered expression of certain genes related to HSPGs has been described in various NENs, including changes in the expression of genes encoding PG core proteins or GAG biosynthetic enzymes (Dilley et al., 2005; García-Suárez et al., 2014).

Heparanase (HPSE) is an endo-β-D-glucuronidase that cleaves specific linkages in the structure of the HS, yielding fragments which are able to contain biological activity. HPSE expression is induced in all major types of human cancer, and is often associated with reduced patient survival and increased tumor metastasis (Arvatz et al., 2011). Several spliced variants of HPSE have been described, one of which, T5, is particularly interesting because it has been associated with cell proliferation and tumor formation (Arvatz et al., 2011).

Heparanase 2 (HPSE2) is a homologue of HPSE that lacks HS-degrading activity, although it is able to interact with HS with high affinity (Arvatz et al., 2011). HPSE2 is capable of associating with HPSE and thereby possibly modulating its enzymatic activity and signalling properties, resulting in an anti-metastatic function being proposed for it (Levy-Adam et al., 2010b; Arvatz et al., 2011).

In this paper, we investigate the expression of the 2 genes encoding heparanases in NENs of differing malignancy grade. Alterations in transcript levels were analyzed by RT-PCR using cDNA samples obtained from lung tumor samples. The detection and quantification of proteins was carried out by immunohistochemistry using a tissue matrix which included NENs from various organs. Tumors were grouped histologically into well- and poorly differentiated and expression levels were also analyzed with regard to tumor stage and grade. The aim of the work was to analyze the expression levels of these two proteins, both strongly involved in tumor progression and metastasis, in NENs from different anatomic origins and with varying tumoral features.

## Materials and methods

### *Materials*

The following materials were purchased from the manufacturers indicated: RNeasy Kit and RNase-Free DNase from Qiagen (Hilden, Germany); High-Capacity cDNA Reverse Transcription Kit and PowerSYBR Green PCR Master Mix from Applied Biosystems (Foster City, CA); GenElute PCR clean-up kit and 3-3' diaminobenzidine from Sigma-Aldrich (St. Louis, MO); EnVision™ Flex/HRP and Envision FLEX target retrieval solution of high pH from Dako (Glostrup, Denmark);. All other chemicals were obtained from commercial sources and were of analytical grade.

The following antibodies were used in this study: Goat Anti-heparanase 1 polyclonal antibody (L-19) was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Rabbit anti-heparanase-2 polyclonal antibody from GeneTex (Atlanta, GA). Anti-rabbit (sc-2004) and anti-goat (sc-2005) secondary antibodies were also from Santa Cruz Biotechnology (Santa Cruz, CA).

### *Patients and samples*

Samples from 39 patients with gastroenteropancreatic (GEP) and pulmonary (L)-NENs were collected at the Biobank of the Hospital Universitario Central de Asturias between February 2000 and October 2009 following approval from the same institute's review board on ethical procedures, and after receiving informed consent from each patient for research on the samples. The diagnosis of neuroendocrine tumor was based on morphologic criteria according to the most up-to-date World Health Organization grading classification (2010). The characteristics of the patients studied (age, gender, toxic habits) and the clinicopathological features of their tumors (WHO classification, tumor site, size, differentiation, stage, mitotic index, proliferation index, vascular invasion, lymph node status, necrosis and presence of metastasis) were recorded. Tissues obtained from biopsies were fixed in 10% formaldehyde and paraffin embedded then cut into 4μm slices, mounted on treated slides and stained with hematoxylin-eosin (H&E). The neuroendocrine phenotype was confirmed by chromogranin A and synaptophysin immunohistochemical staining and normal tissue present in each tissue section was used as a reference.

### *Tissue microarray construction*

Representative tumor regions were identified and selected in each sample for a tissue microarray containing three tissue cores (diameter 1.5 mm) from each of 39 GEP- and L-NENs. The core diameter was 1.5 mm. After 5 minutes at 60°C the TMA blocks were cut into 4 μm thick sections in preparation for the immunohistochemical analysis.

### *Total RNA isolation and cDNA synthesis*

To obtain the RNA, tissue fragments of between 20 and 30 mg in weight were homogenized using a polytron PT 2100 (Kinematica Inc; Bohemia, NY), and RNA was isolated using the RNeasy kit, following the manufacturer's specifications. To ensure removal of residual contaminating DNA, samples were subjected to treatment with RNase-free DNase during the purification process itself. The concentration of RNA obtained was determined spectrophotometrically by measuring absorbance using a Picodrop Microliter UV/Vis pectrophotometer (Picodrop Limited, UK). The samples were divided into aliquots of 10 $\mu$l and used for reverse transcription reactions or stored at -20°C until further use.

cDNA synthesis was carried out using the High Capacity cDNA Transcription Kit following the manufacturer's specifications. The reactions were performed using a thermocycler iCycler IQ (BioRad;

Hercules, CA), using 2 $\mu$g of RNA as starting material. The reaction products were cleaned using the PCR Clean-Up GenElute kit following the manufacturer's instructions. Finally, the aliquots containing the cDNA were diluted 1:20 with water and used for qRT-PCR assays or stored at -20°C until use.

### *qRT-PCR reactions*

In all cases, specific oligonucleotides were designed on different exons or exon junctions, using the program Primer 3. (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The size of the amplicon was situated in all cases between 70 and 150 base pairs, ensuring wherever possible that the Tm was above 77°C. The theoretical Tm for each amplicon was determined using the program Biomath (http://www.promega.com/biomath/calc11.).

The primer sequences were: *HPSE* (Gene ID 10855) forward 5' ATGCTCAGTTGCTCCTGGAC 3', reverse 5' CTCCTAACTGCGACCCATTG 3'; *HPSE* splice variant T5 forward 5' TGCTACTCCGAGAACAC TACCA 3', reverse 5' CAACTTCCGTAGGACTTG CTAGA 3'; *HPSE2* (Gene ID 60495) forward 5' CACCCTGATGTTATGCTGGAG 3', reverse 5' TCCAGAGCAATCAGCAAAGTTA 3'.

At least four repetitions of all the qRT-PCR reactions were carried out in a final volume of 10 $\mu$l, according to the manufacturer's specifications, using 1 $\mu$l of the cDNA dilution as template, with 2 $\mu$l of primer pair mix (200 nM final concentration) and 5 $\mu$l of SYBR Green mix all assembled in 96 well microtiter plates. The plates were sealed with optical film and centrifuged at 2500 rpm for 5 min before being placed in a Real-Time ABI Prism Detection System device (Applied Biosystems; Foster City, CA) using the following cycling conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s followed by 60°C for 60 s. Following the thermal cycling and data collection steps, amplimer products were analyzed using a melt curve program (95°C for 1 min, 55°C for 1 min, then increasing by 0.5°C per cycle for 80 cycles of 10 s each). For each amplification the presence of a single peak with a Tm corresponding to that previously calculated was verified. Actin was included on each plate as a control gene to compare run variation and to normalize individual gene expression.

### *Data analysis*

To calculate the efficiencies of amplification for each gene we used the program LinRegPCR (http://www.gene-quantification.de/download.html), using the best correlation coefficient (considering a minimum of 3 points within the window of linearity) and establishing the average of all positive amplifications. The expression values of the genes of interest were calculated as $(1+\text{efficiency})^{-\Delta Ct}$ (relative to actin as the housekeeping gene).

### *Inmunohistochemistry*

Paraffin embedded tissue sections were treated with xylene to render them diaphanous (the paraffin being removed later by passing it through decreasing alcohol concentrations until water was reached). Rehydrated sections were rinsed in phosphate buffered saline (PBS) containing 1% tween-20. For detection of heparanase 2 sections were heated in high pH Envision FLEX target retrieval solution at 65°C for 20 min and then incubated for 20 min at room temperature in the same solution. For detection of heparanase the final step was omitted.

Endogenous peroxidase activity (3% $H_2O_2$) and non-specific binding (33% fetal calf serum) were blocked and the sections were incubated overnight at 4°C with primary antibodies using a 1:100 dilution. As secondary antibodies we used labelled polymer-HRP ready to use from DAKO and 3-3' diaminobenzidine was employed as a chromogen. Finally, samples were counterstained with hematoxylin, dehydrated and mounted in Entellan® (Merck, Germany). The sections were studied and photographed (x20 and x40 objective) under a light microscope (Nikon - Eclipse 80i).

### *Immunohistochemistry assessment*

The protein expression levels were evaluated by two independent observers (and a third one in the case of disagreement) considering the immunohistochemical signal intensity on a scale of 0-3.

### *Statistical analysis*

All analyses were performed using the Statistics for Windows program (Statsoft Inc; Tulsa, OK). Median values between two samples were compared using the Mann-Whitney U test and between multiple samples by the Kruskal-Walis test. Correlations were assessed by Spearman's correlation coefficient. $p<0.05$ was accepted as significant.

## Results

### *Analysis of differential gene expression*

The aim of this work was to investigate the differential expression of the 2 genes encoding heparanases in NENs. Initially we used qRT-PCR to perform a quantitative analysis of the differential transcription of the coding genes using 8 samples of lung NENs. Poorly differentiated large cell NENs were analyzed, and compared to well-differentiated typical carcinoids. The samples corresponded to patients of both sexes aged between 49 and 84 years. Determination of the expression of the heparanases in NENs from different locations was performed by immunohistochemistry using a tissue array on samples of both tumor and healthy tissue from 39 patients (Table 1).

*Differential expression of heparanase*

Analysis of the transcription levels of the HPSE gene, both in well- and poorly differentiated L-NENs, by means of qRT-PCR did not show any significant differences between them. However, amplification of the T5 mRNA isoform was able to detect its presence at levels around 5% of those observed for the wild type, and in addition it displayed a statistically significant overexpression (approximately 3-fold) in poorly differentiated tumors (p=0.007, Mann-Whitney test) (Fig. 1).

Changes in the expression of HPSE in NENs as evaluated by immunohistochemistry using tissue arrays showed some level of staining in healthy tissues, which increased significantly in well-differentiated tumors, but decreased in poorly differentiated NENs (Fig. 2). Semiquantitative analysis of the expression levels showed an overall alteration in HPSE expression in NENs (p=0.002, Kruskal-Wallis test). Compared with healthy tissue, HPSE appeared overexpressed in well- (p=0.001), but not in poorly differentiated NENs (p=0.25), irrespective of the location of the tumor (Fig. 3).

We also analyzed the expression levels of the protein when grouping tumors according to their location. Comparisons between cases from foregut, midgut and hindgut did not show significant differences between groups for either well- or poorly differentiated tumors. When the values of the two tumor groups are compared, the difference approaches statistical significance (p=0.07), mainly due to the reduction in HPSE in tumors from the hindgut compared to healthy tissue (p=0.09), in contrast to the difference observed in tumors from the other locations (p=0.44), which suggests that significance would probably have been reached if a larger number of hindgut samples had been considered.

Analysis of HPSE levels in NENs relative to tumor grade showed significant increased expression between healthy and grade 1 samples (p=0.001), although this then began to decline, the decrease between stages 1 and 3 being statistically significant (p=0.01) (Fig. 4A).

As regards stage, significant differences in expression were also found (p=0.0022, Kruskal-Wallis test); in all cases the highest levels of HPSE were

**Table 1.** Patiet demographics, histologic grade and stage. D: well-differentiated; PF. porly differentiated.

| Patient no. | Age | Sex | Location | Grade | Stage | Differentiation |
|---|---|---|---|---|---|---|
| 1 | 81 | F | ileum | G3 | III | PD |
| 2 | 40 | F | pancreas | G3 | IV | PD |
| 3 | 76 | M | esophagus | G3 | III | PD |
| 4 | 44 | M | colon | G1 | III | WD |
| 5 | 66 | M | ileum | G1 | III | WD |
| 6 | 76 | M | stomach | G3 | III | PD |
| 7 | 66 | M | ileum | G1 | III | WD |
| 8 | 49 | M | liver | G1 | II | WD |
| 9 | 63 | M | appendix | G1 | I | WD |
| 10 | 40 | F | pancreas | G2 | IV | WD |
| 11 | 48 | M | pancreas | G1 | I | WD |
| 12 | 61 | M | colon | G1 | II | WD |
| 13 | 73 | F | colon | G1 | II | WD |
| 14 | 60 | F | ileum | G1 | IV | WD |
| 15 | 62 | M | appendix | G1 | I | WD |
| 16 | 65 | M | pancreas | G3 | III | PD |
| 17 | 60 | M | colon | G3 | III | PD |
| 18 | 62 | F | jejunum | G1 | III | WD |
| 19 | 71 | M | ileum | G1 | III | WD |
| 20 | 69 | M | stomach | G3 | III | PD |
| 21 | 74 | M | stomach | G3 | II | PD |
| 22 | 52 | M | jejunum | G3 | III | PD |
| 23 | 55 | M | ileum | G1 | II | WD |
| 24 | 70 | M | pancreas | G1 | II | WD |
| 25 | 70 | M | colon | G3 | IV | PD |
| 26 | 77 | M | stomach | G3 | IV | PD |
| 27 | 64 | F | ileum | G1 | I | WD |
| 28 | 42 | M | colon | G2 | IV | WD |
| 29 | 64 | F | colon | G3 | IV | PD |
| 30 | 87 | F | colon | G2 | I | WD |
| 31 | 71 | F | pancreas | G1 | I | WD |
| 32 | 66 | M | lung | G3 | II | PD |
| 33 | 49 | F | lung | G3 | III | PD |
| 34 | 67 | M | lung | G2 | I | WD |
| 35 | 65 | M | lung | G3 | III | PD |
| 36 | 63 | M | lung | G1 | I | WD |
| 37 | 51 | F | lung | G1 | I | WD |
| 38 | 84 | M | lung | G1 | I | WD |
| 39 | 49 | M | lung | G1 | I | WD |

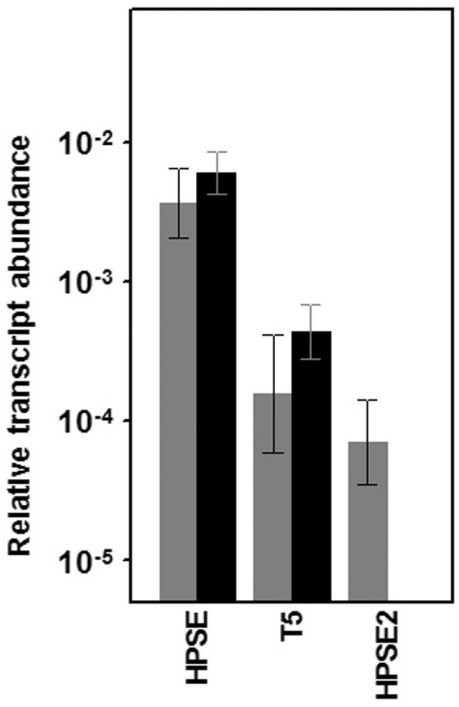


**Fig. 1.** Differential transcription of heparanases in lung NENs. Relative abundance for well differentiated (gray bars) and poorly differentiated (black bars) tumors are plotted on a log scale for each gene assayed and the spreads represent the standard deviations. Values on the Y axis are represented on a logarithmic scale.

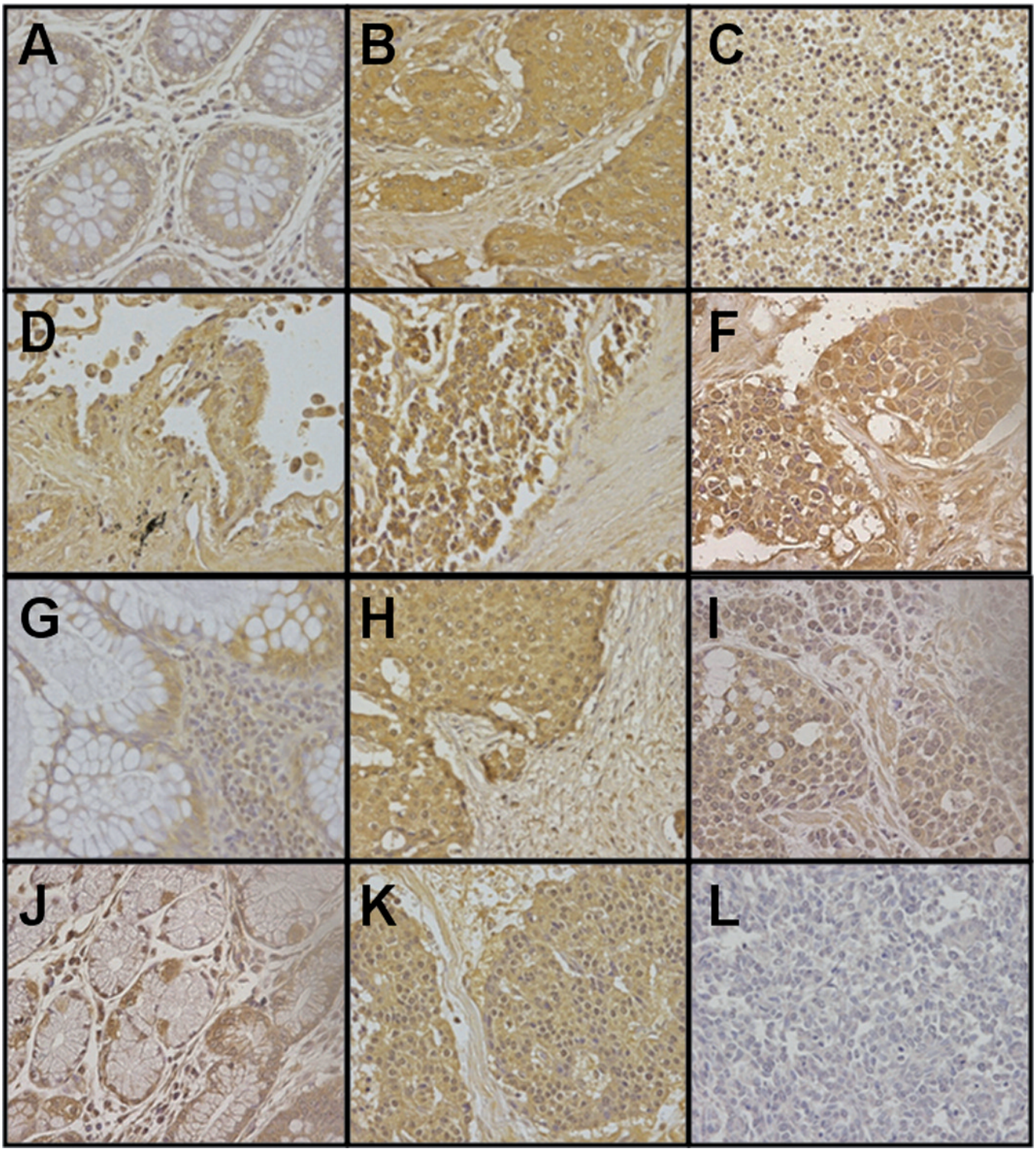


**Fig. 2.** Immunohistochemical staining of HPSE in NENs. **A, D, G, J.** Healthy tissue from colon **(A)**, lung **(D)**, small intestine **(G)** and gastric transitional mucosa **(J). B, E, H, K.** Well differentiated NENs from colon **(B)**, lung **(E)**, small intestine **(H)** and pancreas **(K). C, F, I, L.** Poorly differentiated NENs from colon **(C),** lung **(F)**, stomach **(I)** and pancreas **(L)**. x 200.

detected in stage 2, decreasing in later stages (Fig. 4B).

The above results evaluated only the complete and catalytically active form of the enzyme, since the antibody used recognizes a peptide mapping near the C-terminus of HPSE, which is absent in the T5 isoform.

### *Differential expression of heparanase 2*

We analyzed the levels of transcription of *HPSE2* in L-NENs using primers designed to detect wild-type transcripts (HPSE2c). The presence of mRNA was only detected in well-differentiated tumors (Fig. 1). HPSE2 protein expression was analyzed by immunohistochemistry, and while its expression was detected in healthy tissues, the magnitude of expression was higher in well-differentiated tumors. In contrast, its expression severely decreased in poorly differentiated NENs (Fig. 5). Semiquantitative analysis performed on healthy tissues and on tumors showed a very similar pattern of expression regardless of tumor location (Fig. 3). The differences were more marked when comparing well- with poorly differentiated tumors (p=0.01, Mann-Whitney test), but they were also significant for the increase in expression found between healthy tissue and well-differentiated tumors (p=0.05, Mann-Whitney test) (Fig. 3).

Analysis of expression according to tumor grade resulted in a significant increase in grade 1 compared to healthy tissue (p=0.003, Mann-Whitney test), and a subsequent gradual decrease in the later stages (p=0.04 when comparing grades 1 and 3, Mann-Whitney test) (Fig. 6A). With respect to tumor stage, differences in the data were not clearly defined and although there seems to be an increase in expression in stages 2 and 3 and a subsequent progressive decrease in more advanced stages, they did not reach statistical significance (p=0.1, Kruskal-Wallis test) (Fig. 6C). Moreover, statistical analysis of the expression levels could not determine the existence of any correlation between the expression levels of HPSE and HPSE2 (r Spearman 0.22, p=0.18).

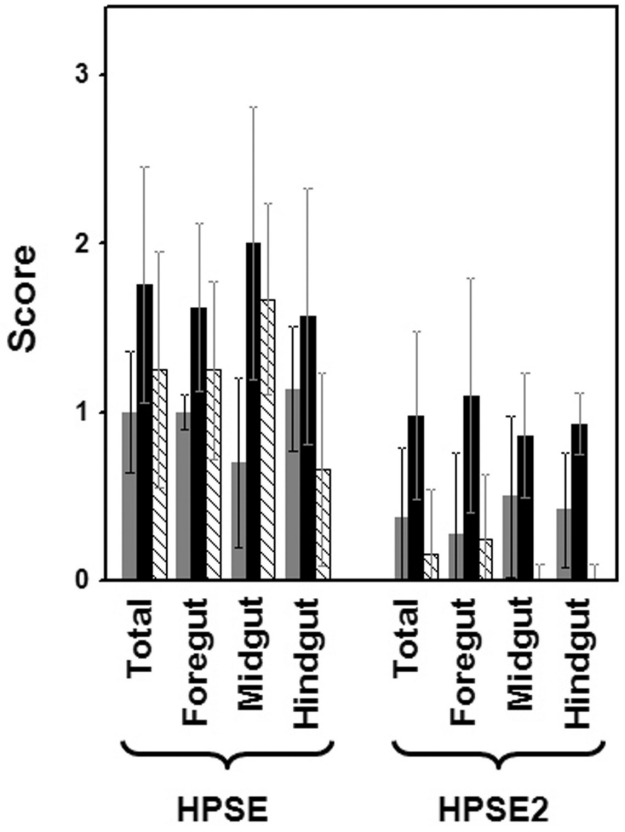


**Fig. 3.** Differential expression of heparanases in NENs. Tumors were considered both altogether and divided into groups acccording to their anatomical location, and expression levels determined by immunochemical staining. A semiquantitative scale from zero detection to +++ was used, indicated on the Y axis as values 0 to 3. Gray bars: normal tissues; black bars: well differentiated NENs; mixed bars: poorly differentiated NENs.

## Discussion

NENs arise from neuroendocrine cells, which are distributed throughout the body, meaning that, in contrast to other tumor types, which are defined by their organic origin, they can be generated in many parts of the organism. Although some characteristics of NENs are specific to their location, their common origin in a specific cell type means that certain attributes are shared by these neoplasms wherever they are in the body (Duerr and Chung, 2007).

HPSE is an endo-β-glycuronidase that has been described as playing a decisive role in cellular invasion and tumor metastasis (Arvatz et al., 2011). The analysis of HPSE mRNA levels in well- and poorly differentiated lung tumors showed no significant differences between them.

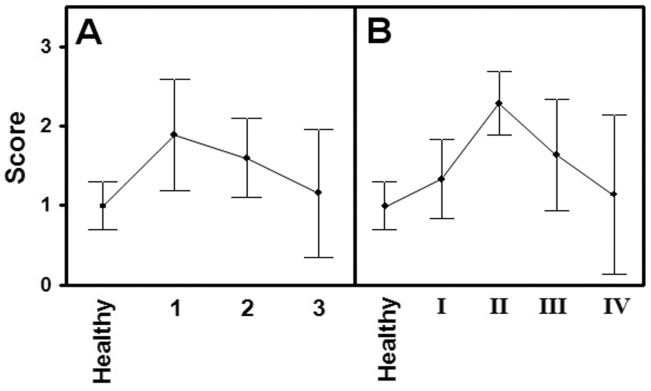


**Fig. 4.** Quantification of immunochemical staining of HPSE. Relative to tumor grade **(A)** and relative to tumor stage **(B)**. A semiquantitative scale from zero detection to +++ was used, indicated on the Y axis as values 0 to 3. Vertical bars represent standard deviation.

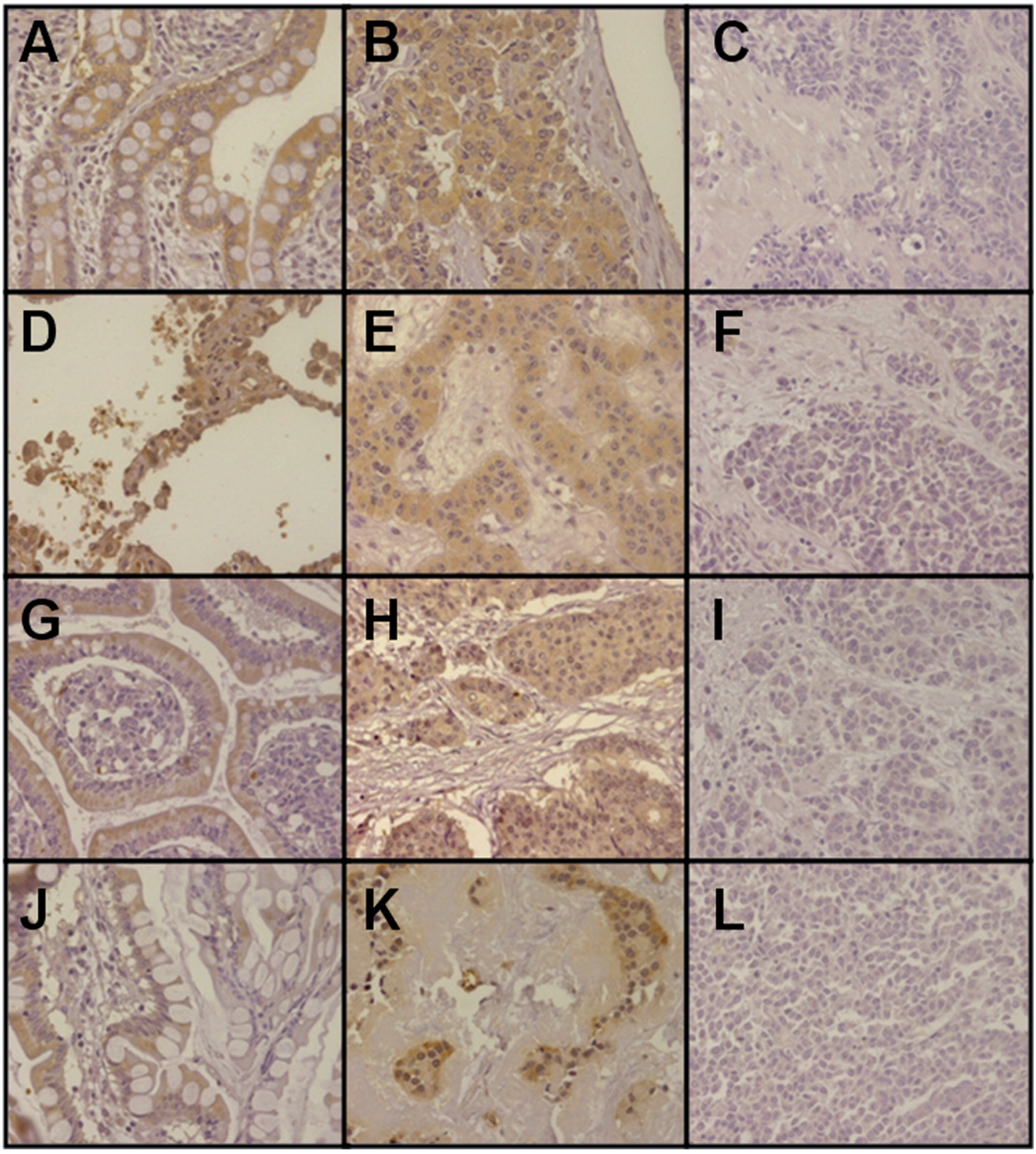


**Fig. 5.** Immunohistochemical staining of HPSE2 in NENs. **A, D, G, J.** Healthy tissue from colon **(A)**, lung **(D)**, small intestine **(G)** and gastric transitional mucosa **(J). B, E, H, K.** Well differentiated NENs from colon **(B)**, lung **(E)**, small intestine **(H)** and pancreas **(K)**. **C, F, I, L.** Poorly differentiated NENs from colon **(C)**, lung **(F)**, stomach **(I)** and pancreas **(L)**. x 200.

When analysis was carried out at the protein level, HPSE appeared overexpressed in well- but, interestingly, not in poorly differentiated NENs relative to healthy tissues, due principally to the decrease in this protein in tumors located in the hindgut. The increment in HPSE in well-differentiated NENs accords with numerous previous reports describing that HPSE expression is induced in all major types of human cancer (Ilan et al., 2006; Arvatz et al., 2011; Hunter et al., 2013). Interestingly, in adenocarcinomas arising in similar locations of the colon and rectum, HPSE has been involved in both tumor initiation and progression, and a gradual increase in its expression from severe dysplasia through well-differentiated to poorly differentiated colon carcinoma has been reported (Friedmann et al., 2000; Doviner et al., 2006; Hermano et al., 2012). However, there are studies that describe the deregulation of HPSE in certain cases, such as acute myeloid leukaemia cells (Eshel et al., 2005), and hepatocellular carcinoma, where genetic alteration and reduction in HPSE expression are associated with tumor progression and poor prognosis, suggesting that HPSE may behave as a tumor suppressor (Huang et al., 2012).

T5 is a functional human splice variant of HPSE, in which 144 bp of intron 5 are joined with exon 4, resulting in an enzymatically inactive protein (Barash et al., 2010). Its transcription was detected in L-NENs at levels of around 5% that of HPSE, and it displayed a statistically significant, approximately 3-fold overexpression in poorly differentiated relative to well-differentiated tumors. T5 levels have been described as affecting tumor cell proliferation and been associated with tumor formation (Barash et al., 2010; Arvatz et al., 2011), and this splice variant posseses clinical relevance since it has been described as up-regulated in some circumstances, such as renal cell carcinoma (Barash et al., 2010; Arvatz et al., 2011). Since the analysis of differential transcription was carried out exclusively on samples of lung, the question of determining whether this isoform is also expressed in other NENs remains to be addressed, and whether its expression is influenced or not by the organ in which the tumor develops.

HPSE2 is a homologue of HPSE devoid of enzymatic activity and for which antimetastatic features have been proposed (Levy-Adam et al., 2010b; Arvatz et al., 2011). Wild-type HPSE2 transcripts were detected in well-differentiated L-NENs but not in poorly differentiated ones. In the same way, immunohistochemical analysis detected the presence of the protein in well-differentiated NENs and, to a lesser extent, in healthy tissue, but its levels sharply decreased in poorly differentiated tumors. HPSE2 showed a very similar pattern of expression regardless of tumor location. Moreover, its expression was related to tumor grade, i.e. higher in grade 1 tumors compared to healthy tissue and progressively declining in later stages. Alterations in the expression levels of this protein have been previously described in some neoplasies: it appears upregulated in head and neck tumors, and is associated with reduced lymph node metastasis (Levy-Adam et al., 2010b); in invasive squamous neoplasia of the cervix a progressive increase in expression according to the severity of the lesion has been described (Marques et al., 2012); increased expression has also been described in colorectal and ovarian carcinomas (Peretti et al., 2008; De Moura et al., 2009); in breast cancer, however, HPSE2 showed a strong downregulation affecting both metastatic and non metastatic tumors (Fernández-Vega et al., 2013). HPSE2 has been described as possesing pro-adhesive properties (Levy-Adam et al., 2010a), it may inhibit HPSE catalytic activity by competing for the HS substrate (Levy-Adam et al., 2010b), and it is also capable of associating physically with HPSE and thus possibly modulates its enzymatic activity and signalling properties (Levy-Adam et al., 2010b).

In summary, both heparanases undergo alterations in their expressions in NENs. In well-differentiated tumors the pro-invasive and prometastatic enzyme HPSE is overexpressed, as has also been described in all major types of human cancer and, concurrently, HPSE2 is also overexpressed. Conversely, levels of both proteins decline in poorly differentiated tumors, although the decrease in HPSE2 is more pronounced, which could be related to its anti-metastatic features, based on the modulation of the enzymatic and non-enzymatic activities of HPSE. The pattern of expression of both proteins is significantly dependent on the grade and stage of the tumor, which could confer an interesting role in relation to diagnosis.

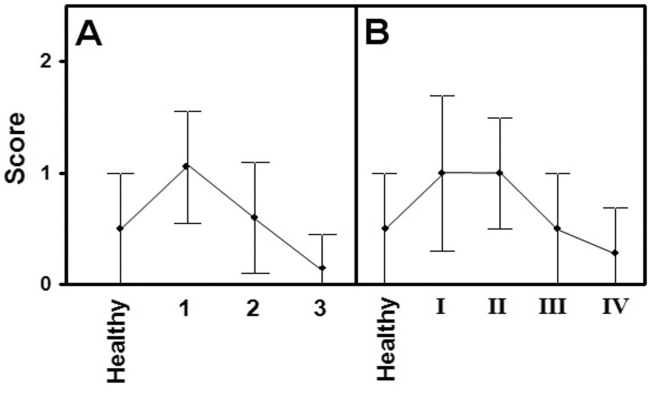


**Fig. 6.** Quantification of immunochemical staining of HPSE2: relative to tumor grade **(A)** and relative to tumor stage **(B)**. A semiquantitative scale from zero detection to +++ was used, indicated on the Y axis as values 0 to 3. Vertical bars represent standard deviation.

*Acknowledgements.* The University Institute of Oncology of Asturias is supported by Obra Social Cajastur, Asturias, Spain.

## References

Arvatz G., Shafat I., Levy-Adam F., Ilan N. and Vlodavsky I. (2011). The

heparanase system and tumor metastasis, is heparanase the seed and soil?. Cancer Metast. Rev. 30, 253-268.

Barash U., Cohen-Kaplan V., Arvatz G., Gingis-Velitski S., Levy-Adam F., Nativ O., Shemesh R., Ayalon-Sofer M., Ilan N. and Vlodavsky I. (2010). A novel human heparanase splice variant, T5, endowed with protumorigenic characteristics. FASEB J. 24, 1239-1248.

De Moura J.P. Jr, Nicolau S.M., Stávale J.N., Da Silva Pinhal M.A., de Matos L.L., Baracat E.C. and De Lima G.R. (2009). Heparanase-2 expression in normal ovarian epithelium and in benign and malignant ovarian tumors. Int. J. Gynecol. Cancer 19, 1494-1500.

Dilley W.G., Kalyanaraman S., Verma S., Cobb J.P., Laramie J.M. and Lairmore T.C. (2005). Global gene expression in neuroendocrine tumors from patients with the MEN1 syndrome. Mol. Cancer. 4, 9.

Doviner V., Maly B., Kaplan V., Gingis-Velitski S., Ilan N., Vlodavsky I. and Sherman Y. (2006). Spatial and temporal heparanase expression in colon mucosa throughout the adenoma-carcinoma sequence. Mod. Pathol. 19, 878-888.

Duerr E.M. and Chung D.C. (2007). Molecular genetics of neuroendocrine tumors. Best Pract. Res. Clin. Endocrinol. Metab. 21, 1-14.

Eshel R., Ben-Zaken O., Vainas O., Nadir Y., Minucci S., Polliack A., Naparstek E., Vlodavsky I. and Katz B.Z. (2005). Leukomogenic factors downregulate heparanase expression in acute myeloid leukemia cells. Biochem. Biophys. Res. Commun. 335, 1115-1122.

Fernández-Vega I., García O., Crespo A., Castañón S., Menéndez P., Astudillo A. and Quirós L.M. (2013). Specific genes involved in synthesis and editing of heparan sulphate proteoglycans show altered expression patterns in breast cancer. BMC Cancer. 13, 24.

Friedmann Y., Vlodavsky I., Aingorn H., Aviv A., Peretz T., Pecker I. and Pappo O. (2000). Expression of heparanase in normal, dysplastic, and neoplastic human colonic mucosa and stroma. Evidence for its role in colonic tumorigenesis. Am. J. Pathol. 157, 1167-1175.

García-Suárez O., Fernández-Vega I. and Quirós L.M. (2013). Multiple alterations of heparan sulphate in cancer. OA Cancer 1, 1.

García-Suárez O., García B., Fernández-Vega I., Astudillo A. and Quiros L.M. (2014). Neuroendocrine tumors show altered expresión of chondroitin sulfate, glypican 1, glypican 5, and syndecan 2 depending on their differentiation grade. Front. Oncol. 4, 15.

Hermano E., Lerner I. and Elkin M. (2012). Heparanase enzyme in chronic inflammatory bowel disease and colon cancer. Cell. Mol. Life Sci. 69, 2501-2513.

Huang G.L., Li B.K., Zhang M.Y., Wei R.R., Yuan Y.F., Shi M., Chen X.Q., Huang L., Zhang H.Z., Liu W., Huang B.J., Li H., Zheng X.F., Luo X.R. and Wang H.Y. (2012). Allele loss and down-regulation of heparanase gene are associated with the progression and poor prognosis of hepatocellular carcinoma. PLoS One 7, e44061.

Hunter K.E., Palermo C., Kester J.C., Simpson K., Li J.P., Tang L.H., Klimstra D.S., Vlodavsky I. and Joyce J.A. (2013). Heparanase promotes lymphangiogenesis and tumor invasion in pancreatic neuroendocrine tumors. Oncogene 33, 1799-1808.

Ilan N., Elkin M. and Vlodavsky I. (2006). Regulation, function and clinical significance of heparanase in cancer metastasis and angiogenesis. Int. J. Biochem. Cell. Biol. 38, 2018-2039.

Klimstra D.S., Modlin I.R., Coppola D., Lloyd R.V., and Suster S. (2010). The pathologic classification of neuroendocrine tumors. Pancreas 39, 707-712.

Levy-Adam F., Ilan N. and Vlodavsky I. (2010a). Tumorigenic and adhesive properties of heparanase. Semin. Cancer Biol. 20, 153-160.

Levy-Adam F., Feld S., Cohen-Kaplan V., Shteingauz A., Gross M., Arvatz G., Naroditsky I., Ilan N., Doweck I. and Vlodavsky I. (2010b). Heparanase 2 interacts with heparan sulfate with high affinity and inhibits heparanase activity. J. Biol. Chem. 285, 28010-28019.

Marques R.M., Focchi G.R., Theodoro T.R., Castelo A., Pinhal M.A. and Nicolau S.M. (2012). The immunoexpression of heparanase 2 in normal epithelium, intraepithelial, and invasive squamous neoplasia of the cervix. J. Low Genit. Tract. Dis. Jul; 16, 256-262.

Mougey A.M., and Adler D.C. (2007). Neuroendocrine tumors, review and clinical update. Hosp. Physician 51, 12-20.

Peretti T., Waisberg J., Mader A.M., de Matos L.L., da Costa R.B., Conceição G.M., Lopes A.C., Nader H.B. and Pinhal M.A. (2008). Heparanase-2, syndecan-1, and extracellular matrix remodeling in colorectal carcinoma. Eur. J. Gastroenterol. Hepatol. 20, 756-765.

Raman K. and Kuberan B. (2010). Chemical Tumor Biology of heparan sulphate proteoglycans. Curr. Chem. Biol. 1, 20-31.

Rindi G., Arnold R. and Bosman F.T. (2010). Nomenclature and classification of neuroendocrine neoplasms of the digestive system. In: WHO classification of tumours of the digestive system. 4th ed. Bosman T.F., Carneiro F., Hruban R.H., Theise N.D. (eds). International Agency for Research on cancer (IARC), Lyon. pp 13-14.

Accepted July 31, 2015