

# Heparanase Expression in Primary and Metastatic Pancreatic Cancer<sup>1</sup>

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## Abstract

The human endoglycosidase heparanase (hpa) degrades heparan-sulfate proteoglycans, which constitute prominent components of basement membranes and extracellular matrix. Due to the critical function of hpa in cancer cell invasion and metastasis, we have analyzed the expression of hpa in human primary and metastatic pancreatic cancer as well as in the normal pancreas and in chronic pancreatic inflammation. By real-time quantitative PCR, there was a 7.9- and 30.2-fold increase of hpa mRNA in chronic pancreatitis and pancreatic cancer tissue samples, respectively, in comparison with normal pancreatic tissues. There was a significant correlation between enhanced hpa mRNA expression and shorter postoperative patient survival. hpa mRNA and protein localized in the cancer cells of primary and metastatic pancreatic cancer, with a preferentially higher expression at the primary tumor site. Cultured pancreatic cancer cells transfected with a full-length hpa construct displayed enhanced invasiveness in an invasion chamber assay. These results suggest that hpa overexpression in human pancreatic cancers facilitates cancer cell invasion, thereby enhancing the metastatic potential of the tumors.

## Introduction

Despite its relatively low incidence (9–10 cases/100,000 people), pancreatic cancer is one of the leading causes of cancer-related mortality in Western industrialized countries, with a poor prognosis and an overall 5-year survival of less than 1% (1). Diagnosis is often established at a late tumor stage, precluding curative resection. In addition, conventional oncological strategies often fail due to resistance of pancreatic cancers to chemotherapeutic regimens and the propensity of pancreatic tumors to metastasize. Although it has been reported that pancreatic cancers overexpress a variety of mitogenic growth factors and their receptors (2), harbor proto-oncogene mutations such as k-ras, exhibit alterations in growth-inhibitory pathways such as Smad4 and p16 mutations, and evade apoptosis through p53 mutations (3) and aberrant expression of apoptosis-regulating genes (4, 5), the reasons for the aggressiveness of this cancer are not fully understood.

Tumor cell invasion and subsequent distant spread via blood and lymph vessels and the perineural space are critical steps in pancreatic cancer progression that worsen the prognosis and limit therapeutic options. Cancer cell spread is facilitated via disassembly of the main structural elements of basement membranes and ECM<sup>3</sup> by enzymes produced by the cancer cells (6). HSPGs, proteins substituted with

glycosaminoglycan side chains predominantly of the heparan sulfate type, are among the main components of basement membranes and ECM (7). HSPGs have a physiological role as cofactors in many cellular processes such as cell-cell and cell-matrix interactions and cell invasiveness (7, 8). The heparan sulfate moieties interact with various components of the ECM such as collagen, laminin, and fibronectin and with different attachment sites on plasma membranes, thus playing an essential role in the self-assembly and insolubility of ECM components (9). HSPGs also bind and sequester a variety of bioactive proteins, including growth factors, chemokines, cytokines, and enzymes (10), thereby playing a critical role in malignant transformation, tumor growth, and tumor cell adhesion.

hpa, a recently cloned enzyme secreted by activated platelets and neutrophils, exhibits endoglycosidase (mainly endo- $\beta$ -D-glucuronidase) activity that degrades the heparan sulfate side chains of HSPGs, thus facilitating disassembly of the ECM and enhancing cell invasion. Heparan sulfate side chain degradation also results in substantial release of bioactive proteins bound at these sites, such as cytokines and growth factors (11, 12).

The differential and possibly tumor-specific expression of HSPGs in pancreatic cancer (13, 14), as well as the importance of the degradation of basement membranes and ECM for cancer cell invasion and metastasis, led us to examine the expression of hpa in primary and metastatic pancreatic cancer to further elucidate its role in the invasive and metastatic potential of this tumor.

## Materials and Methods

**Tissue Samples.** Thirty-three pancreatic cancer tissue samples were obtained from 19 male and 14 female patients (median age, 65 years; range, 44–78 years). According to the Union Internationale Contre le Cancer classification, there were 7 stage I, 6 stage II, 16 stage III, and 4 stage IV ductal cell adenocarcinomas. Nine tumors were well differentiated (grade 1), 21 tumors were moderately differentiated (grade 2), and 3 tumors were poorly differentiated (grade 3). Twelve chronic pancreatitis tissue samples were obtained from patients undergoing a pancreatic head resection. Ten normal pancreatic tissue samples were obtained from previously healthy individuals through an organ donor program. Tissue samples were fixed for immunohistochemistry or *in situ* hybridization in 4% formalin solution and embedded in paraffin. Immediately after surgical removal, tissues destined for RNA extraction were snap-frozen in liquid nitrogen and maintained at  $-80^{\circ}\text{C}$  until use. All studies were approved by the Human Subjects Committee of the University of Bern (Bern, Switzerland).

**Real-time Quantitative RT-PCR.** Total RNA was isolated by the single-step guanidinium method (13). After DNase treatment, total RNA was reverse transcribed into cDNA using random hexamers according to the manufacturer's instructions (Roche Diagnostics, Rotkreuz, Switzerland). The real-time quantitative RT-PCR analysis was performed with an automated sequence detection system (Prism 7700 Sequence Detector; PE Applied Biosystems, Weiterstadt, Germany) combined with a dual-label fluorogenic detection system (TaqMan) based on the five-nuclease assay (15) as described previously in detail (14). The following primers were used: (a) hpa, forward primer 5-TCA-CCA-TTG-ACG-CCA-ACC-T-3, reverse primer 5-CTT-TGC-AGA-ACC-CAG-GAG-GAT-3, and probe 5-FAM-CCA-CGG-ACC-CGC-GGT-TCC-T-

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<sup>3</sup> The abbreviations used are: ECM, extracellular matrix; hpa, heparanase; HSPG, heparan sulfate proteoglycan; RT-PCR, reverse transcription-PCR; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; TBS-T, 20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20; FGF, fibroblast growth factor; EGF, epidermal growth factor.

3TAMRA; and (b) 7S, forward primer 5-ACC-ACC-AGG-TTG-CCT-AAG-GA-3, reverse primer 5-CAC-GGG-AGT-TTT-GAC-CTG-CT-3, probe 5FAM-TGA-ACC-GGC-CCA-GGTCGG-AAA-3TAMRA. Concentrations of primers and probes were optimized to 300 nM for hpa forward primer, reverse primer, and probe (FAM/TAMRA). Thermal cycling was initiated with a 2-min incubation at 50°C for uracil *N*-glycosylase reaction, followed by a 10-min reaction at 95°C to activate the AmpliTaq Gold and 45 PCR cycles for hpa and 7S at 95°C for 15 s and 60°C for 1 min. Experiments were performed in duplicate for each data point. Each PCR run included five standards, one template control, and certain experimental sample points. Standard curves for both hpa and 7S were generated using a cDNA synthesized from serial 1:5 dilution of total RNA from one pancreatic cancer sample. The threshold was set at 10 SDs above the mean of the baseline fluorescence emission calculated from cycles 3–20. The point at which the amplification plot crosses this threshold was defined as *C<sub>t</sub>*, which represents the cycle number at this point (15). The relative standard curve was constructed using seven standard *C<sub>t</sub>* values as *Y*-axis values and the log of the input standard RNA amount (copy number). For each experimental sample, the amounts of hpa mRNA and 7S mRNA were determined from the standard curve. The normalized amount of hpa was determined by dividing the amount of hpa mRNA by the amount of 7S mRNA for each sample.

**In Situ Hybridization.** *In situ* hybridization was performed as reported previously (13). Briefly, tissue sections (4 μm) were deparaffinized, rehydrated, and incubated in 0.2 M HCl for 20 min. After washing with 2× SSC, the tissues were treated with proteinase K at a concentration of 25 μg/ml for 15 min at 37°C. After fixation with 4% paraformaldehyde in PBS (5 min) and washing in 2× SSC, the sections were prehybridized for 2 h at 63°C in a buffer containing 50% (v/v) formamide, 4× SSC, 2× Denhardt's reagent, and 250 μg/ml RNA. Hybridization was performed overnight at the same temperature in 50% (v/v) formamide, 4× SSC, 2× Denhardt's reagent, 500 μg/ml RNA, and 10% (v/v) dextran sulfate. The final concentration of the DIG-labeled hpa probes was approximately 500 ng/ml. Excess probe was removed by washing in 2× SSC followed by RNase treatment with 100 units/ml RNase T1 at 37°C for 30 min. Tissues were washed at 65°C in 2× SSC for 10 min and washed three times in 0.2× SSC and 50% formamide for 10 min each. Sections were then incubated with an anti-DIG antibody conjugated with alkaline phosphatase (Roche). For the following color reaction, 5-bromo-4-chloro-3-indolyl phosphatase and nitroblue tetrazolium (Sigma Chemical Co., Buchs, Switzerland) were used.

**Immunohistochemistry.** Paraffin-embedded tissue sections (2–3-μm thick) were subjected to immunostaining using the Envision<sup>+</sup> System, Peroxidase (Dako Diagnostics, Zug, Switzerland). Tissue sections were deparaffinized and rehydrated, and antigen retrieval was performed by pretreatment of the slides in citrate buffer (pH 6) in a microwave oven (8 min at 850 W and another 10 min at 410 W). Thereafter, slides were cooled to room temperature, placed in washing buffer solution [0.05 M Tris-HCl and 0.3 M NaCl containing 0.1% Tween 20 (pH 7.2–7.6)], and subjected to immunostaining. After quenching of endogenous peroxidase activity (Envision<sup>+</sup> System/HRP-step 1), sections were incubated for 1 h at room temperature with a monoclonal anti-hpa antibody (HP-92.4 antibody; InSight Strategy & Marketing Ltd., Rehovot, Israel) directed against the NH<sub>2</sub> terminus region of hpa (16, 17). Afterward, the slides were rinsed with washing buffer and incubated with labeled polymer, peroxidase, and antimouse antibody (Envision<sup>+</sup> System/HRP-step 2) for 30 min at room temperature. Buffered substrate of 3,3'-diaminobenzidine chromogen (Envision<sup>+</sup> System/HRP-step 3) were used for localization of the antibody. Finally, sections were counterstained with Mayer's hematoxylin.

**Western Blot Analysis.** Proteins were extracted in a suspension buffer [10 mM Tris-Cl (pH 7.6) and 100 mM NaCl] containing a protease inhibitor mixture (CompleteTM, mini; Roche Diagnostics, Rotkreuz, Switzerland), subjected to SDS-PAGE, and transferred to nitrocellulose filters by electroblotting. Non-specific binding was blocked by incubating the membranes in 5% nonfat milk in TBS-T. The membranes were incubated with the monoclonal anti-hpa antibody (H-130 antibody; InSight Strategy & Marketing Ltd.) directed against the COOH terminus of the active enzyme (16) for 1 h at room temperature. The membranes were then washed with TBS-T and incubated with a horseradish peroxidase-conjugated (sheep) antimouse IgG (1:3000 dilution; Bio-Rad Laboratories, Hercules, CA), for 1 h at room temperature. Antibody detection was

performed with an enhanced chemiluminescence reaction (Amersham Life Science, Amersham, United Kingdom).

**Cell Culture, Transfection, and Invasion Assays.** ASPC-1, MIA PaCa-2, PANC-1, and T3M4 human pancreatic cancer cells were routinely grown in DMEM (MIA PaCa-2 and PANC-1) or RPMI (ASPC-1 and T3M4) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (complete medium). After reaching 30–50% confluence, T3M4, ASPC-1, PANC-1, and MIA PaCa-2 cells were maintained in serum-free medium and transiently transfected with the respective plasmid using the LipofectAMINE method as reported previously (13). Briefly, 10 μg of the full-length hpa expression vector (16) or 10 μg of the empty control vector were incubated with 50 μl of LipofectAMINE and 500 μl of serum-free medium for 30 min and added to the cells. After a 4-h incubation period, equal volumes of medium containing 20% fetal bovine serum were added for 12 h. After 36 h of subsequent incubation in complete medium, cells were collected for invasion tests and RNA extraction. BioCoat Matrigel Invasion Chambers (Becton Dickinson Labware, Bedford, MA) were used for the invasion tests in accordance with the manufacturer's instructions. Briefly, pancreatic cancer cells ( $2.5 \times 10^4$ ) were suspended in complete medium (500 μl) and immediately placed onto the upper compartment of the plates. Subsequently, the lower compartment was filled with complete medium. After a 22-h incubation period, membranes were fixed in methanol and stained with H&E. Cells on the

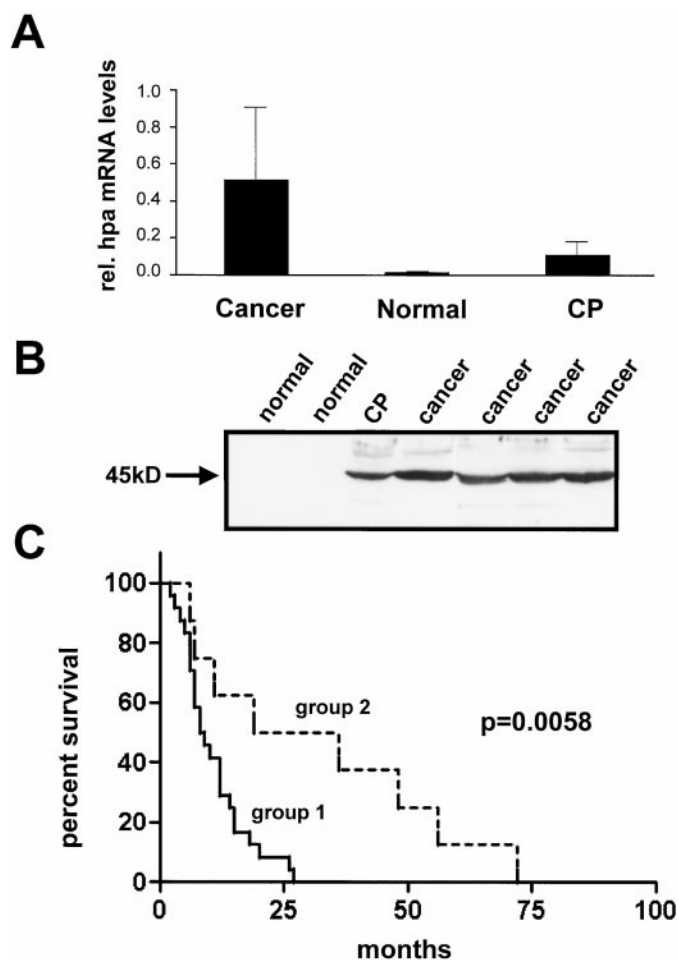


Fig. 1. Expression of hpa in human pancreatic tissues. *A*, real-time quantitative RT-PCR was performed as described in "Material and Methods." Results are expressed as the mean of the ratio of hpa and 7S values for each sample  $\pm$  SD. *B*, Western blot analysis of hpa in the normal pancreatic tissues (*normal*), chronic pancreatitis (*CP*), and pancreatic cancer (*cancer*). The molecular weight of hpa is 45,000. *C*, survival curves. Kaplan-Meier plots of the postoperative survival period were carried out in two groups of patients. In one group, hpa mRNA levels were increased more than 6-fold above that of normal controls (*solid line*). In the other group, hpa mRNA levels were increased less than 6-fold above that of normal controls (*broken line*). Log-rank analysis of the postoperative survival periods indicated that patients in group 1 had a shorter survival ( $P = 0.0058$ ) than those in group 2.

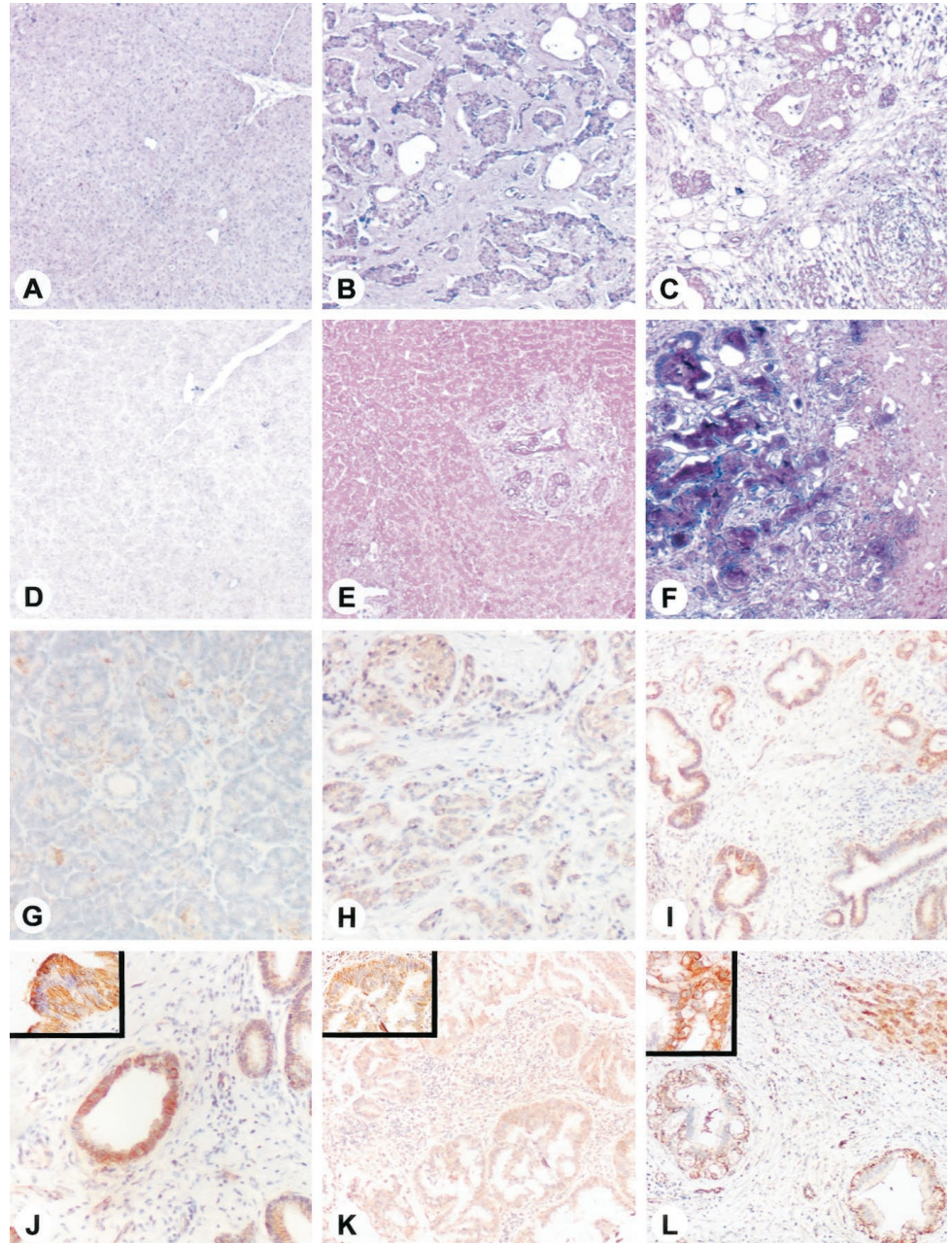


Fig. 2. *In situ* hybridization (A–F) and immunohistochemical analysis (G–L) of hpa expression in human pancreatic tissues. In the normal pancreas, hpa *in situ* hybridization (A) and immunostaining (G) signals were present at low levels. In chronic pancreatitis tissues, hpa immunostaining signals were increased in areas with degenerating acini (H). In the cancer tissues, hpa *in situ* hybridization (B) and immunostaining (I and J) signals were abundantly expressed in the cytoplasm of duct-like cancer cells (J, *inset*), whereas signals in stromal elements adjacent to cancer cells were faint or absent (I and J). Lymph node metastases of pancreatic tumors exhibited moderate hpa *in situ* hybridization (C) and immunostaining (K) signals in the cancer cells (K, *inset*), whereas the surrounding lymphatic tissue did not reveal any hpa *in situ* hybridization or immunostaining signals. Pancreatic cancer liver metastasis exhibited weak-to-moderate hpa *in situ* hybridization (E and F) and immunoreactivity (L) signals in the cancer cells (L, *inset*). Hybridization with sense probes revealed no specific *in situ* hybridization signals (D).

upper surface of the filter were removed carefully with a cotton swab, and the cells that had migrated through the membrane to the lower surface of the filter were counted in nine different fields under a light microscope. Each experiment was performed in triplicate.

**Statistics.** Student's *t* test was used for statistical analysis of the invasion assays. Results are expressed as mean  $\pm$  SE of the mean. The Mann-Whitney *U* test was used to determine the relationship between hpa expression and clinicopathological parameters. Postoperative survival periods were computed by the method of Kaplan-Meier and compared by using the log-rank test.  $P < 0.05$  was taken as the level of significance for all tests.

## Results and Discussion

hpa activity has been identified in a variety of normal tissues and cells in which invasion and intravasation/extravasation are critical components of physiological processes, such as skin fibroblasts, cytotrophoblasts, endothelial cells, platelets, mast cells, neutrophils, macrophages, lymphocytes, and hepatocytes. However, the human

hpa cDNA has only recently been identified and sequenced from human placenta, platelets, and a human hepatoma cell line (16, 18).

In the present study, we evaluated the expression of hpa in normal pancreatic tissues, chronic pancreatitis, and pancreatic cancer tissues. Chronic pancreatitis samples exhibited a mean 7.9-fold increase of hpa mRNA over normal controls ( $P < 0.05$ ), whereas pancreatic cancer exhibited a mean 30.2-fold increase ( $P < 0.05$ ; Fig. 1A). These results were also confirmed by Western blot analysis of normal and pancreatic cancer tissues, revealing a single  $M_r$  45,000 band in all pancreatic cancer specimens (Fig. 1B). For further analysis, pancreatic cancer patients were divided into two groups. Tumors of group 1 patients ( $n = 25$ ) exhibited a  $>6$ -fold increase in hpa mRNA levels compared with the normal controls, whereas tumors of group 2 patients ( $n = 8$ ) displayed a  $<6$ -fold increase in hpa mRNA levels. Interestingly group 1 patients had a significantly shorter postoperative survival time than did group 2 patients (median survival time was 10.5

months in group 1 versus 31.3 months in group 2;  $P < 0.05$ ; Fig. 1C). In contrast, there was no significant relationship between the two groups and tumor stage and grade. Although there was a tendency toward less differentiation in group 1 tumors, this difference did not reach statistical significance, probably due to the fact that the majority of the tumors were moderately differentiated (grade 2; 64%). Furthermore, there was no significant correlation between hpa expression and tumor stage; again, most of the tumors were stage II and III (67%); therefore, the lack of correlation has to be interpreted cautiously. Nonetheless, our observations suggest that hpa contributes to disease progression in pancreatic cancer.

Next, we analyzed hpa mRNA localization in normal and ductal pancreatic cancer tissue specimens as well as lymph node and liver metastatic pancreatic cancer tissue specimens. In normal pancreatic tissues, hpa mRNA expression was below the level of detection by *in situ* hybridization (Fig. 2A). In contrast, primary pancreatic cancers exhibited intense hpa mRNA *in situ* hybridization signals in the cancer cells (Fig. 2B). Surprisingly, metastatic pancreatic cancers in lymph nodes (Fig. 2C) and the liver (Fig. 2, E and F) exhibited weak-to-moderate hpa mRNA signals. Consecutive control sections incubated with the sense hpa mRNA revealed no specific hybridization signal (Fig. 2D). Immunostaining results were comparable to those obtained by *in situ* hybridization. Thus, hpa immunoreactivity was faint in the acini and ductal cells of the normal pancreas (Fig. 2G) and chronic pancreatitis (Fig. 2H) tissues, whereas primary pancreatic cancer exhibited strong immunoreactivity in the cancer cells (Fig. 2, I, J, and J, inset). Moderate hpa immunoreactivity was observed in the lymph node and liver metastatic pancreatic cancer (Fig. 2, K and L) and was localized in the cancer cells (Fig. 2, K, inset and L, inset). Thus, hpa protein expression closely follows patterns of hpa mRNA expression, suggesting that hpa overexpression is regulated mainly at a transcriptional level. Control slides incubated without primary antibody did not reveal any specific signal.

The above-mentioned findings of high hpa expression in primary pancreatic cancers and comparably lower expression in lymph node and liver metastasis suggest that hpa enzymatic activity might be involved in the detachment and escape of the tumor cells from the primary site. ECM and basement membranes are collagen-containing structural barriers that must be breached for cancer cells to invade, intravasate, and subsequently metastasize. Therefore, a sufficient degradative enzymatic capacity is required; this capacity is derived primarily from the cancer cells, but stromal and inflammatory cells contribute as well. Proteolytic enzymes such as matrix metalloproteinases produced by cancer cells are responsible for the degradation of structural components of the ECM (19), and other enzymes such as hpa are required for the degradation of other major components of the ECM and basement membranes, such as HSPGs. Thus, the preferential higher hpa expression in primary pancreatic cancer cells, in comparison with lymph node and liver metastasis, may be attributed to the increased requirement for ECM-degrading enzymes at the primary tumor site to allow cancer cells to metastasize.

hpa activity has been reported in cancer cell lines; interestingly, its levels correlate strongly with the metastatic potential of the cell lines (16, 18, 20–23). B-lymphocytes produce hpa during certain stages of their maturation and lymphoma transformation (24). Ricoveri *et al.* reported that cell extracts and intact cells of metastatic murine and human fibrosarcomas and melanomas degrade heparan sulfate faster than their nonmetastatic counterparts (25). In addition, nonmalignant cells have been shown to have the potential to enhance hpa production and activation, thereby contributing to the metastatic and invasive properties of cancer cells (26). High expression of hpa mRNA and protein has also been reported in human tumors *in vivo*, and this expression is related to the metastatic potential of the tumors (16, 17).

To further investigate the functional significance of hpa expression in pancreatic cancer, the effects of hpa expression on the invasive potential of pancreatic cancer cells were investigated. To this end, pancreatic cancer cells were transiently transfected with an expression vector encoding full-length hpa cDNA or with an empty control vector. Transfection efficiency was determined by real-time quantitative RT-PCR 36 h after transfection. Compared with the control-transfected cells, hpa mRNA levels increased 120- to 10,000-fold in the four pancreatic cancer cell lines (Fig. 3, A and B). Invasion chamber assays revealed a statistically significant increase (range, 1.5–2.3-fold;  $P < 0.05$ ) in the invasiveness of hpa-transfected cells in comparison with the control-transfected cells (Fig. 3C). These results support the hypothesis that hpa contributes to the invasiveness of pancreatic cancer cells.

hpa activity can be blocked with various nonanticoagulant heparin derivatives and related sulfated negatively charged molecules (27,

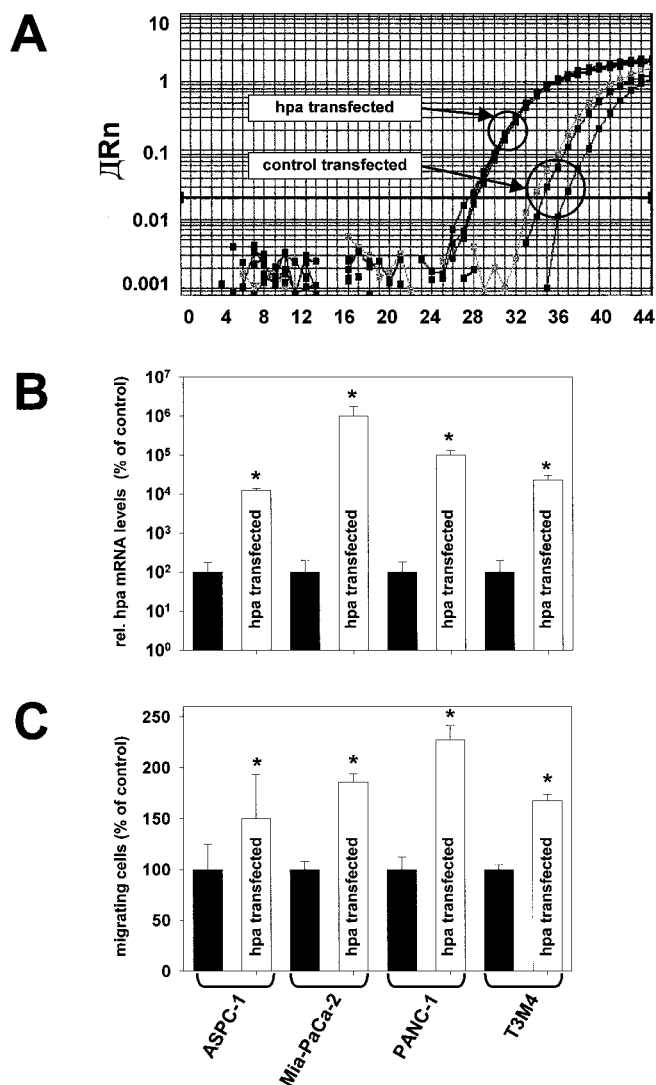


Fig. 3. Effects of hpa on pancreatic cancer cell invasion. To assess the ability of hpa to increase invasiveness of pancreatic cancer cells, assays were performed as described in "Materials and Methods." A and B, pancreatic cancer cells were transiently transfected with an expression vector encoding the full-length hpa cDNA or an empty control vector. Transfection efficiency was determined by real-time quantitative RT-PCR 36 h after transfection (the actual RT-PCR curve is shown for ASPC-1 cells in A). C, pancreatic cancer cells ( $2.5 \times 10^4$ ) were placed onto the upper compartment of BioCoat Matrigel Invasion Chambers plates. Migration across the membrane was assessed after 22 h. The number of cells in nine separate high-power microscopic fields was counted in triplicate wells. Data are expressed as the mean  $\pm$  SE of the mean. Migration of the control cells is set to 100%. \*,  $P < 0.05$ .

28). These and other factors that decrease hpa expression and/or inhibit hpa enzymatic activity in cancer cells significantly reduce their metastatic properties, underscoring the importance of hpa in cancer cell spread.

There is growing evidence that hpa enzymatic activity also plays a cofactorial role in cancer cell adhesion and angiogenesis by releasing various mitogenic, angiogenic, and chemotactic heparin-binding growth factors (*e.g.*, basic fibroblast growth factor, platelet-derived growth factor, and heparin-binding EGF-like growth factor) from their storage sites within the basement membranes (11, 12). Syndecan-1 and glypican-1, two HSPGs, are up-regulated in pancreatic cancer (13, 14) and function as an ECM storage pool for various mitogenic heparin-binding growth factors that are overexpressed in pancreatic cancer [*e.g.*, FGF-2 and heparin-binding EGF-like growth factor (2)]. Enhanced hpa enzymatic activity in a pancreatic cancer mass releases these heparin-binding growth factors into the tumor microenvironment, thereby stimulating pancreatic cancer cell growth and angiogenesis. Moreover, enzymatic degradation of heparan sulfate domains of syndecan-1 and possibly glypican-1 by hpa produces heparin-like heparan sulfate fragments that are potent FGF-2 activators that have the potential to increase the mitogenic and angiogenic activity of FGF-2 (29, 30). Thus, the connection between HSPG overexpression and the activation of various mitogenic growth factors that are up-regulated in pancreatic cancer (2, 31–33) is evident.

In conclusion, we report the enhanced expression of hpa mRNA and protein in primary and metastatic pancreatic cancer and show that enhanced hpa mRNA levels correlate negatively with patient survival, suggesting a role of basement membrane- and ECM-degrading enzymes in tumor microenvironment alterations that facilitate pancreatic cancer cell growth, invasion, and metastasis formation. Therefore, the development of drugs acting as inhibitors or blocking agents of hpa action may add a new therapeutic modality in the future treatment of pancreatic cancer.

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