# Inverse Correlation between Heparan Sulfate Composition and Heparanase-1 Gene Expression in Thyroid Papillary Carcinomas: A Potential Role in Tumor Metastasis

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## ABSTRACT

***Purpose:*** **Heparanase-1 (HPR1) is an endoglycosidase that degrades the side chains of heparan sulfate proteoglycan (HSPG), a key component in cell surfaces, the extracellular matrix (ECM), and the basement membrane (BM). The purpose of this study was to evaluate HPR1 expression in thyroid neoplasms and its effect in degrading the HSPG substrates in the ECM and BM and to determine its role in thyroid tumor metastasis.**

***Experimental Design*: HPR1 mRNA expression was analyzed by using *in situ* hybridization with a digoxigenin-labeled antisense RNA probe on paraffin-embedded tumor sections and reverse transcription-PCR (RT-PCR) in fresh tumor tissues. HPR1 protein expression was analyzed by using immunohistochemical staining with an anti-HPR1 rabbit antiserum and immunofluorescence (IF) with an anti-HPR1 monoclonal antibody. The effect of HPR1 expression in thyroid neoplasms was analyzed by examining the presence and integrity of the HSPG substrates in the ECM and BM using IF staining with a specific monoclonal antibody against heparan sulfate. The relationship of HPR1 expression in papillary thyroid carcinomas (PTCs) with various clincopathological parameters was analyzed statistically. The role of HPR1 in thyroid tumor metastasis was further examined by comparing HPR1 levels in 10 thyroid tumor cell lines to their invasive and metastatic potential.**

***Results: In situ*** **hybridization analysis of 81 tumor samples (62 papillary carcinomas and 19 follicular adenomas) revealed that HPR1 was expressed at a much higher frequency in PTCs than in follicular adenomas ($P < 0.05$). RT-PCR analyses of fresh tumor tissues revealed that HPR1 mRNA could be detected in primary and metastatic thyroid papillary carcinomas. HPR1 expression was confirmed at the protein level by immunohistochemical staining and IF stainings. IF analysis of HSPG revealed that HS was deposited abundantly in the BM of normal thyroid follicles and benign follicular adenomas but was absent in most thyroid papillary carcinomas. A lack of heparan sulfate in PTCs inversely correlated with HPR1 expression. Clinicopathological data analyses revealed that PTCs with local and distant metastases scored HPR1 positive at a significantly higher frequency than nonmetastatic thyroid cancers ($P = 0.02$). To further explore the role of HPR1 in tumor metastases, we characterized HPR1 expression in 10 thyroid tumor cell lines using RT-PCR and Western blot and measured HPR1 enzymatic activity using a novel ELISA. HPR1 was differentially expressed in different types of cell lines; overexpression of HPR1 in two tumor cell lines led to a dramatic increase of their invasive potential *in vitro* in an artificial BM.**

***Conclusions:*** **Our study suggests that HPR1 expressed in papillary carcinomas is functional and that HPR1 expression is associated with thyroid tumor malignancy and may significantly contribute to thyroid tumor metastases.**

## INTRODUCTION

Thyroid cancer is the most common endocrine neoplasm in the United States. Papillary carcinoma accounts for ~80% of thyroid malignancies (1). Papillary carcinomas usually metastasize via the lymphatic channels and generally have a favorable prognosis (2, 3). Follicular carcinomas are usually well differentiated tumors but differ from papillary carcinomas in their growth pattern and their greater propensity to invade blood vessels. Medullary carcinomas are neuroendocrine neoplasms derived from the parafollicular cells or C cells. Sporadic and familial medullary carcinomas tend to be more aggressive than papillary and follicular carcinomas, with a propensity to metastasize via the bloodstream. Anaplastic carcinomas are poorly differentiated or undifferentiated and are the most malignant thyroid neoplasm with an almost invariably fatal outcome (4). In the past decade, molecular and genetic studies of thyroid neoplasms have demonstrated that the tyrosine kinase encoding *RET/PTC* rearrangements and *RAS* mutation frequently occur in thyroid tumors. Although progress is being made, the molecular

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mechanisms of thyroid tumor invasion and metastasis remain poorly defined.

HPRs[8] are endoglycosidases that specifically degrade the side chains of HSPGs, one of the main components in cell surfaces, BMs, and ECMs (5–7). There are at least two *HPR* genes. HPR1 was first purified to homogeneity from human platelets (8–10). The cDNA of the *HPR1* gene and its promoter region have been cloned and sequenced recently (11–17). Transfection and stable expression of the human *HPR1* gene in murine nonmetastatic T lymphoma cells led to the conversion of these cells from a nonmetastatic to a metastatic phenotype (15). Recent clinical studies demonstrated that HPR1 expression is elevated in hepatocellular (18), colon (19), pancreatic (20, 21), and breast (22) cancers. HPR1 expression correlates with a short survival length of pancreatic cancer patients (20, 21) and with the increased invasive and metastatic potential in hepatocellular and breast carcinomas (18, 22).

A second *HPR* gene, *HPR2*, shares an overall 35% identity in the coding region with *HPR1* (23). However, *HPR2* may not be involved in tumor metastases. First, all three *HPR2* gene products (480, 534, and 592 amino acids) derived from different splicing of *HPR2* mRNA lack a signal peptide, and they are expressed as either membrane-bound or intracellular proteins (30). Second, *HPR2* expression is low or undetectable in colon, lung, ovarian, and prostate tissues and is not increased in colon, lung, ovarian, and prostate cancer xenografts (23). Third, the enzymatic activity of *HPR2* has not been detected, and it is unclear whether *HPR2* is capable of degrading the HSPG substrate.

Our present study sought to examine the relationship between HPR1 expression and the metastatic potential of thyroid tumors as well as their clinicopathological parameters. Our results show that: (*a*) HPR1 expression was associated with malignant thyroid neoplasms; (*b*) HPR1 expression correlated with the lack of HS in the BM of PTC; (*c*) HPR1 was expressed at a higher frequency in metastatic papillary carcinomas than nonmetastatic tumors; and (*d*) overexpression of HPR1 in a thyroid tumor cell line dramatically increased its invasive potential. Taken together, these results suggest that HPR1 may have an important role in promoting thyroid tumor metastases.

## MATERIALS AND METHODS

**Tumor Specimens and Patient Information.** Paraffin-embedded tumor blocks from thyroidectomy specimens of patients with thyroid neoplasms were retrieved for the analysis of *HPR1* gene expression after approval by the Institutional Review Board of Rush Presbyterian St. Luke's Medical Center. A total of 93 specimens, all with adequate clinical and pathological information, were studied. These included 19 follicular adenomas, 62 papillary carcinomas, 3 follicular carcinomas, 4 medullary carcinomas, and 5 Hürthle cell neoplasms. The presence of metastases and chronic thyroiditis or Hashimoto's thyroiditis were determined by reviewing the patients' clinical charts, pathology reports, and subsequent courses. Patients were staged using the Tumor-Node-Metastasis system and classified according to the presence of extrathyroidal extension, cervical nodes, and distant metastases. Fresh tumor tissues were obtained from the operating room and immediately homogenized in TRIzol (Life Technologies, Inc.). Total RNA was extracted following the manufacturer's instructions and used in RT-PCR for the analysis of *HRP1* mRNA expression as described below.

**Cell Lines.** Ten thyroid tumor cell lines were used in this study. One follicular adenoma (KAK-1), one papillary carcinoma (KAT-10), and three anaplastic carcinoma (KAT-4, KAT-18, and SW1736) cell lines were originally established in laboratory of Dr. K. Ain and have been described previously (24, 25). The other five thyroid tumor cell lines, including one papillary (NPA87), two follicular (WRO82 and MRO87), and two anaplastic carcinomas (ARO81 and DRO90), were kindly provided by Dr. Guy J. F. Juillard (University of California at Los Angeles, Los Angeles, CA). All thyroid tumor cell lines were grown in complete RPMI 1640 containing 10% fetal bovine serum. HT1080 cells, a human fibrosarcoma cell line, were purchased from American Type Culture Collection (Manassas, VA) and grown in Eagle's MEM containing 10% fetal bovine serum.

**HPR1 cDNA Cloning.** Total cellular RNA was extracted from platelets of a healthy donor by using TRIzol (Life Technologies, Inc.), following the manufacturer's instructions, and quantitated by OD260 absorbance. The first chain was synthesized by reverse transcriptase with 2 μg of total RNA as template and oligo(dT) as primer. Human HPR1 cDNA was PCR amplified with pfu of DNA polymerase and two primers. The forward primer flanking the sequence from position 87–110 (5′-TATCGGTACCAGGTGAGCCAAGATGCTGC-3′) contains a *Kpn*I site. The reverse primer complementary to the HPR1 cDNA sequence from position 1707–1731 (5′-ACGGTCGAATTCTCAGATGCAAGCAGCAACTTTGGC-3′) contains an *Eco*RI cleavage site (15). PCR product was digested with *Kpn*I and *Eco*RI and subsequently cloned into the pcDNA3 expression vector. The resultant plasmid was designated as pcDNA/HPR1.

**ISH.** The antisense probe of the *HPR1* gene was labeled with digoxigenin by *in vitro* transcription with SP6 RNA polymerase and 2 μg of *Hind*III-digested pcDNA/HPR DNA fragment. The digoxigenen-labeled HPR1 sense probe was also prepared by *in vitro* transcription with T7 RNA polymerase (Roche Diagnostics Corp., Indianapolis, IN) and a PCR fragment as template (amplified from a pcDNA vector containing a *Bam*HI-*Eco*RI fragment of HPR1 cDNA, T7 primer, and a primer complementary to HPR1 cDNA from 965 to 993). Paraffin-embedded tissue sections were dewaxed with xylene and rehydrated by serial concentrations of ethanol, then pretreated with 0.2 N HCl for 30 min, followed by proteinase K digestion (15 μg/ml) for 30 min at 37°C. Sections were preincubated with prehybridization buffer (50:50, formadehyde:8× SSC buffer) for 30 min at 37°C and then hybridized at 42°C overnight with the HPR1 antisense probe (10 ng in 100 μl of hybridization buffer/section) or sense probe as negative control. Slides were

[8] The abbreviations used are: HPR, heparanase; BM, basement membrane; ECM, extracellular matrix; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PTC, Papillary thyroid carcinoma; RT-PCR, reverse transcription-PCR; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; ISH, *in situ* hybridization; IHC, immunohistochemistry; IF, immunofluorescence; mAb, monoclonal antibody; FGF, fibroblast growth factor; HRG, histidine-rich glycoprotein.

washed with prewarmed 2×, 1×, 0.1× SSC solution. Tissue sections were then blocked in blocking solution containing 2% normal sheep serum. Sheep antidigoxigenin (diluted 1:500; 100 μl; Roche Diagnostics Corp.) was added to each section and incubated for 2 h at room temperature, followed by color development with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate substrate according to the manufacturer's instructions (Roche Diagnostics Corp.).

**RT-PCR.** Total cellular RNA was isolated from fresh tissues and cell lines with TRIzol (Life Technologies, Inc.) and quantitated by UV absorption. After reverse transcription of 500 ng of total RNA with oligo(dT) priming, the resulting single-stranded cDNA was amplified using TaqDNA polymerase (Life Technologies, Inc.). Oligonucleotides HPR-3 (5′-TTCGATCCCAAGAA-GGAATCAAC-3′) and HPR-4 (5′-GTAGTGATGCCATGTAACTGAATC-3′) were used for amplifying a 587-bp HPR1 DNA fragment. Oligonucleotides 5′-TGAAGGTCGGAGTCAACGGATTTGGTC-3′ and 5′-ATGGACTGTGGTCATGAGTCCTTCCACG-3′ were used to amplify a 527-bp GAPDH DNA fragment. The PCR reaction was set with an initial denaturation of 2 min at 94°C and subsequent denaturation for 45 s at 94°C, annealing for 45 s at 55°C, and extension for 1 min at 72°C. Thirty to 40 cycles were used to amplify the PCR product.

**IHC.** IHC analysis of HPR1 expression in the sections of thyroid neoplasms was conducted using an anti-HPR1 rabbit antiserum following the protocol in our previous study (22).

**IF.** A subset of 35 samples examined by ISH were also assessed for HPR1 expression by IF using an anti-HPR1 mAb antibody as described previously (11). The sections of tissue specimens were dewaxed and then fixed with 1% paraformaldehyde. The slides were incubated with an anti-HPR1 mAb (clone 3-12; working concentration, 50 μg/ml) at room temperature for 30 min. The same concentration of normal mouse IgM was used as a negative control to stain the same slides. The slides were washed three times with PBS, followed by a goat antimouse IgM conjugated with FITC (1:50; ICN Biomedicals, Aurora, OH), then washed and incubated with rabbit antigoat IgG conjugated with FITC (1:50; ICN Biomedicals). The slides were sealed with 50% glycerin in PBS containing antifade reagent 1,4-diazabicyclo[2.2.2]octane (25 mg/ml) and 4,6-diamidino-2-phenylindole (0.5 mg/ml; Sigma Chemical Co., St. Louis, MO). HPR1 expression was examined under a fluorescence microscope. The pictures were taken by exposing Fuji 400 film for 10 s with ×200 amplification under a microscope. HS composition was detected similarly, except an anti-HS mAb (clone HepSS; Seikagaku Corp., Tokyo, Japan) was used at a concentration of 5 μg/ml. The pictures were taken with a digital camera attached in a Nikon Eclipse TE200 fluorescence microscope (Chiu Technicals Corp.).

**Western Blot Analysis.** Thyroid tumor cell lines grown in 6-well plates were lysed in Triton X-100 lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptinin, and 1 mM phenylmethylsulfonyl fluoride]. Cell lysates were incubated on ice for 30 min and then spun down at 15,000 rpm for 15 min at 4°C. The protein concentrations were quantitated by a Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein (see Fig. 6*B*, 50 μg/lane, and Fig. 7*B*, 2 μg/lane) were loaded onto a 10% polyacrylamide-SDS gel. After electrophoreses, the proteins were transferred onto a nitrocellulose membrane. Because the anti-HPR1 mAb (clone 3-12) does not react with the denatured HPR1 transferred on the nitrocellulose membrane, HPR1 protein was detected by a rabbit anti-HPR1 antiserum (kindly provided by Dr. R. L. Heinrikson, Pharmacia & Upjohn, Kalamazoo, MI), followed by horseradish peroxidase-conjugated goat antirabbit IgG and SuperSignal Western Pico enhanced chemiluminoscence substrate (Pierce Chemical Co., Rockford, IL). This antibody was raised by immunizing a rabbit with a peptide containing the amino acid residues from 273 to 290 of HPR1 protein and does not cross-react with HPR2 because no homology is found between this peptide and any isoforms of the HPR2 amino acid sequence.

**HPR1 Activity Assay.** HPR1 enzymatic activity in 10 thyroid tumor cell lines was quantitated by a novel ELISA. The cell lines were grown in T-25-$cm^2$ flasks. When the cell monolayers reached 90% confluence, the cells was detached by using the Cell Dissociation Solution (Sigma Chemical Co.) and washed twice with PBS. The cells were lysed in 100 μl of HPR1 assay buffer [0.1 M sodium acetate (pH 5.0), 0.1 mg/ml BSA, 0.01% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin] and then frozen and thawed for three cycles. The cell lysates were spun down at 15,000 rpm for 30 min, and the supernatants were collected. Protein concentrations were quantitated by a Bio-Rad Protein Assay kit. Matrigel, an artificial BM that contains abundant HSGP, was dissolved in ice-cold PBS/carbonate-buffered saline (pH 9.6, 50:50) at a concentration of 20 μg/ml and used to coat ELISA plates (25 μl/well) at 4°C overnight. The plates were then washed three times with PBS containing 0.05% Tween 20 and then blocked with 5% BSA in PBS at room temperature for 1 h. The cell lysates were serially diluted at 1:5 in HPR1 assay buffer, and 25 μl of diluted cell lysates were added to each well and incubated at 37°C overnight. Anti-HS-specific mAb (1:1000, diluted in PBS containing 5% BSA) was added and incubated at room temperature for 1 h. After washing, horseradish peroxidase-conjugated goat antimouse IgM antibody (1:2000, diluted in PBS with 5% BSA) was added and incubated at room temperature for 1 h, followed by the addition of 50 μl of 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid substrate. The OD405 absorbance was read in an ELISA plate reader (Bio-Rad).

**Transfection and Establishment of Stable Transfectant Cell Lines.** HT1080, a human fibrosarcoma cell line, and SW1736, a follicular thyroid carcinoma cell line, were seeded in 35-mm dishes. On 70% confluence, the cells were transfected with pcDNA3 or pcDNA3-HPR1 plasmid DNA with the FuGENE6 transfection reagent (Roche Diagnostics Corp.), following the manufacturer's instructions. After incubation at 37°C for 24 h, the cells were split at a ratio of 1:15 and grown in selection medium containing G418 (Life Technologies, Inc.) at a concentration of 1 mg/ml. After 1–2 weeks, the clones were visible; clones of each transfectant cell line were transferred to a 6-well plate and screened for HPR1 expression using Western blot. Three *HPR1*-positive clones were pooled and analyzed further for HPR1 activity using a novel ELISA method as described above.

**Cell Motility and Chemoinvasion Assays.** The assays were conducted essentially as described by Koliopanos *et al.* (21). Briefly, the monolayers of tumor cells grown in T-25-$cm^2$ flasks were detached by using the Cell Dissociation Buffer (Sigma Chemical Co.) and washed twice with the serum-free medium containing 0.1% BSA. The cells ($2 \times 10^4$/well) were seeded in the top chamber of the 24-well Transwell inserts, which were either uncoated or precoated with Matrigel (50 or 100 μg/well; Roche Diagnostics Corp.). The Transwell inserts were placed in a 24-well companion plate filled with 0.75 ml of conditioned medium collected from the supernatant of the same cell line. After incubation for 24 h, the cells in the inner side of top chamber were removed by wiping with cotton swabs. The cells that migrated through Matrigel and the filter insert to the opposite surface were stained with a Diff-Quik kit (Mercedes Medical, Sarasota, FL). The membrane was sliced out and then mounted onto a hemacytometer and sealed with the mounting media. The cells in five square fields were counted under a light microscope.

**Statistical Analysis.** Correlation between *HPR1* gene expression and the histological diagnosis or clinicopathological parameters of the thyroid neoplasms was determined by a $\chi^2$ test or Fisher's exact test.

## RESULTS

**Detection of *HPR1* Gene Expression in Thyroid Neoplasms.** We first conducted RT-PCR to examine the expression of HPR1 mRNA in thyroid neoplasms. Total cellular RNA was extracted from three primary papillary thyroid carcinomas and two metastases and used as the template in the reverse transcription reaction, followed by PCR. As shown in Fig. 1, a 587-bp HPR1 DNA fragment was amplified from all three primary and two lymph node metastases. It should be noted that the primers used in the PCR reaction were located in different exons of the *HPR1* gene, thus excluding the possibility that the PCR product was amplified from the contaminated genomic DNA template. The gene of GAPDH, which was included as an internal control, was equally amplified in all five samples and produced a 527-kb fragment.

We next analyzed *HPR1* gene expression in 93 archival thyroid tumor tissues by using ISH with a digoxigenin-labeled HPR1 antisense RNA probe. The dark brown to purple substrate precipitate indicates the HPR1-positive staining in the cytoplasm. The signals were evaluated by two pathologists experienced in thyroid cancer pathology. Hybridization signals were scored relative to the background signal on a consecutive section hybridized with the sense probe. As shown in Fig. 2, HPR1 mRNA signals were present in the cytoplasm of the cancer cells in the infiltrating nests in a papillary carcinoma specimen (Fig. 2*A*; ×200 amplification) and in a typical PTC (Fig. 2*C*, *red arrows*; ×400 amplification). However, HPR1 signals were absent in the neighboring normal follicles (Fig. 2*A*, *blue arrow*) and in a follicular adenoma (Fig. 2*B*, *blue arrow*). HPR1 signals were not present in most stromal cells. Infiltrating mononuclear cells, particularly in the thyroid neoplasms associated with chronic thyroiditis or Hashimoto's thyroiditis, expressed HPR1 signals (data not shown). A sense probe used as control did not show any signal in HPR1-positive papillary carcinomas (data not shown).

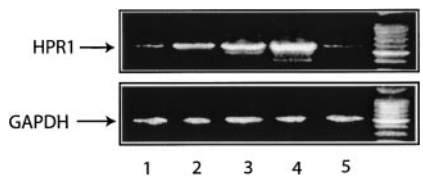


*Fig. 1* RT-PCR of HPR1 expression in fresh tumor tissues. Five specimens of papillary carcinomas (100 mg/sample) were homogenized in 1 ml of TRIzol. Total cellular RNA was isolated using TRIzol (Life Technologies, Inc.). RT-PCR reactions were conducted as described in "Material and Methods." Thirty-five and 42 PCR cycles were used to amplify GAPDH and HPR1 DNA fragments, respectively. The PCR product was separated on a 1.5% agarose gel and visualized by ethidium bromide staining under a UV transluminator.

**IHC and IF Analyses of HPR1 Expression.** A previous study by Dempsey *et al.* (11) showed that a 0.9-kb transcript of the *HPR1* gene, which may represent an alternative nonsense splicing form, is abundantly present in a variety of tissues. This raises the possibility that HPR1 mRNA in tumor tissues detected by ISH and RT-PCR may not be necessarily translated into a full-length, functional HPR1 enzyme. To address this possibility, we first examined HPR1 protein expression in thyroid tissues using IHC staining with a rabbit anti-HPR1 antiserum. As shown in Fig. 2*D*, the dark brown signal of HPR1 expression was present in the cytoplasm of the tumor cells but not in the neighboring normal follicular cells in a specimen used in Fig. 2*A*. HPR1 expression was strongly present in a typical PTC (Fig. 2*F*) but not in a follicular adenoma (Fig. 2*E*). A normal rabbit serum used as a negative control did not show any signal (data now shown).

The specificity of HPR1 expression detected by ISH and IHC was further confirmed by IF staining with an anti-HPR1-specific mAb (clone 3-12). As shown in Fig. 3, HPR1 expression was found in the cytoplasm of papillary (Fig. 3*C*) and medullary carcinoma (Fig. 3*E*) cells; HPR1 was not detected in the epithelial cells of normal thyroid follicles (Fig. 3*A*) and in follicular adenoma cells (Fig. 3*B*) but was detected on the cell membrane of an embryonal adenoma (Fig. 3*D*). A serial section of the tumor sample used in Fig. 3*C* was stained with the same concentration of mouse IgM as a negative control (Fig. 3*F*). Infiltrating immune cells, particularly in the tumor samples with chronic thyroiditis or Hashimoto's disease, also expressed HPR1 (data not shown). HPR1 expression was also found in the endothelial cells of microvessels in some tumor samples (data not shown).

We then tested whether HPR expression detected by ISH and IF staining corroborated each other. Fifteen follicular adenomas and 20 papillary carcinomas were examined for HPR1 expression using assays. As shown in Table 1, 3 follicular adenomas and 12 papillary carcinomas scored HPR1 positive by both ISH and IF analyses, whereas 8 of 11 follicular adenomas and 4 of 7 papillary carcinomas scored HPR1 negative with both the ISH assay and IF staining. However, 3 of 11 follicular adenomas and 3 of 7 papillary carcinomas that scored HPR1 negative in the ISH assay were HPR1 positive when analyzed by the IF staining. Only one of four follicular adenomas and 1 of 13 papillary carcinomas that scored HPR1 positive in the ISH assay

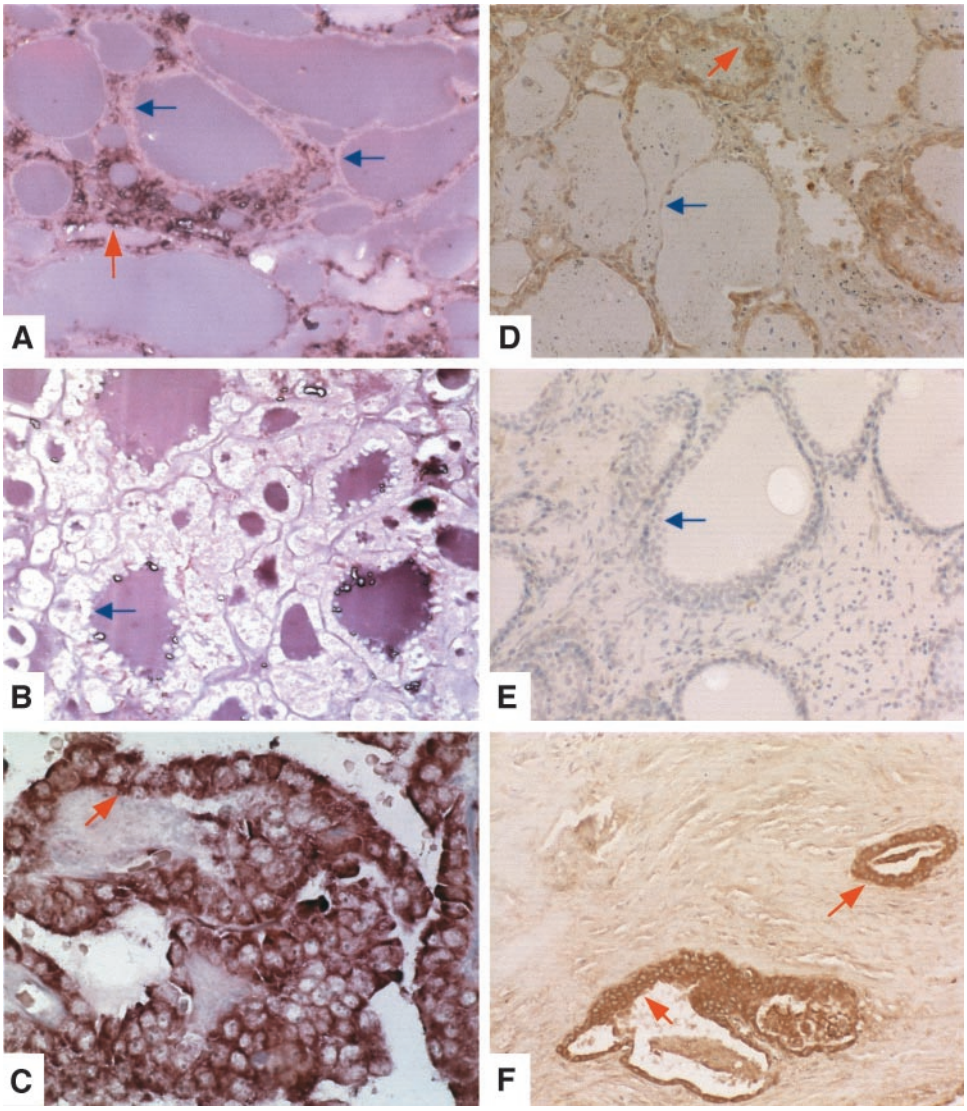


*Fig. 2* *A–C*, ISH analysis of *HPR1* gene expression. Sections of the paraffin-embedded blocks from human thyroidectomy specimens were processed for hybridization with a digoxigenin-labeled human HPR1 antisense probe, followed by alkaline phosphatase-conjugated sheep anti-digoxigenin antibody. The color development was done by the nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate substrate, followed by counterstaining with FastRed. A, HPR1 mRNA signals were absent in normal follicullar cells (*blue arrow*) but were strongly present in the neighboring papillary carcinoma cells (*red arrow*; magnification, ×200). *B*, no signal in a HPR1-negative follicular adenoma (magnification, ×200). *C*, strong HPR1 signal in a PTC (magnification, ×200). *D–F*, IHC analysis of HPR1 expression. Sections of the paraffin-embedded blocks from human thyroidectomy specimens were dewaxed and rehydrated. IHC staining of HPR1 expression was conducted using an anti-HPR1 rabbit antiserum as described previously (22). *D*, HPR1 expression was detected by IHC in the cytoplasm of tumor cells (*red arrow*) but not in the neighboring normal thyroid follicles (*blue arrow*). *E*, no signal was present in a follicular thyroid adenoma. *F*, strong HPR1 expression was seen in the cytoplasm and membrane of the tumor cells in a PTC.

were HPR1 negative when analyzed by the IF staining. These results suggest that HPR1 mRNA expression detected by ISH in the majority of the samples is consistent with HPR1 protein expression detected by IF staining, with an overall concordance rate at 77%. There is a subtle discrepancy in HPR1 expression using these two different methods. In particular, three of seven papillary carcinomas that did not exhibit HPR1 mRNA expression as detected by ISH were HPR1 positive when analyzed by IF. The discrepancy may be attributable to the difference in the relative stability of HPR1 mRNA and protein or caused by the relative sensitivity of the detection methods used. Indeed, three of four HPR1-negative follicular adenomas and three of four HPR1-negative papillary carcinomas detected by ISH was also positive or weakly positive when analyzed using IHC. Alternatively, HPR1 protein detected by IF and IHC in some tumor specimens may be attributable to the deposition of HPR1 secreted by platelets (11).

**HS Content in the BM of Thyroid Tissues.** A previous study by Dempsey *et al.* (11) demonstrated that the presence of active platelet HPR in acutely rejected xenografts results in the loss of HS content in the wall and lumen of the blood vessels. To test whether HPR1 expression in thyroid neoplasms results in HS degradation in the BM, we conducted IF staining with a HS-specific mAb to monitor the HS content. As shown in Fig. 4, normal thyroid follicles (Fig. 4*A*), a HPR1-negative benign thyroid follicular adenoma (Fig. 4*B*), and a HPR1-negative papillary carcinoma (Fig. 4*C*) displayed abundant HS in the BM surrounding the thyroid follicles and papillae. An HPR1-positive PTC (Fig. 4*D*) did not display any HS in the BM of its papillae. HS staining in the BM was broken or interrupted in the follicles of some thyroid adenomas, which were still scored as positive. Mouse IgM used as a negative control in the PTC specimens containing normal thyroid follicles did not give any fluorescence signal (data not shown). A total of 15 follicular adenomas and 37 papillary carcinomas were analyzed for HS content in the BM. Eight of 15 (53%) follicular adenomas had HS staining in the BM (Table 2). However, HS staining was only present in the BM in 10 of 46 (22%) papillary carcinomas.

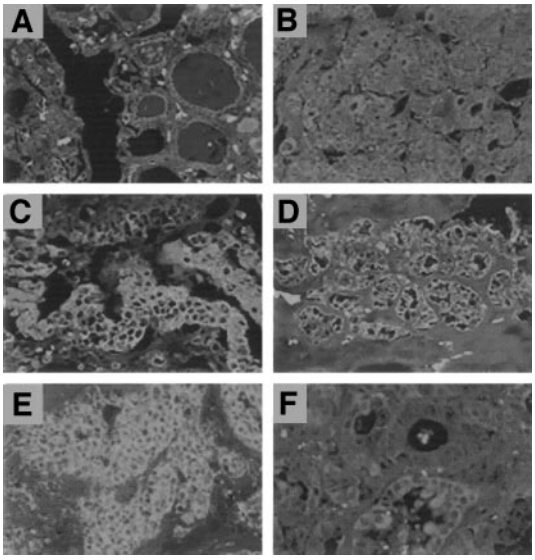


*Fig. 3* Detection of HPR1 expression in thyroid tumors by IF staining. The sections of human specimens derived from a thyroid follicular adenoma (*B*), a papillary carcinoma (*C*), medullary carcinoma (*D*), and an embryonal adenoma (*E*) were dewaxed and then fixed with 1% paraformaldehyde. A section of the normal thyroid follicles adjacent to a thyroid papillary carcinoma tissue was included as a negative control (*A*). The slides were incubated with anti-HPR1 mAb (clone 3-12; working concentration, 50 μg/ml) at room temperature for 30 min. The same concentration of normal mouse IgM was used as a negative control to stain a consecutive section used in *C* (*F*). The slides were washed three times with PBS, followed by FITC-conjugated goat antimouse IgM. The slides were then washed and incubated with FITC-conjugated rabbit antigoat IgG. The slides were sealed and examined for HPR1 expression under a confocal fluorescence microscope.

HS staining could be observed in normal thyroid follicles adjoining both benign and malignant tumor specimens. These results show that HS was differentially deposited in the BM of benign follicular adenomas *versus* malignant papillary carcinomas ($P = 0.045$).

We next tested whether lack of HS content in the BM of thyroid neoplasms correlated with HPR1 expression. As shown in Table 2, 34 of the 37 HPR1-positive papillary carcinomas lacked HS, whereas 7 of the 9 HPR1-negative papillary carcinomas exhibited strong HS staining in the BM. Statistical analysis revealed that HPR1 expression correlated with the lack of HS staining in PTCs ($P < 0.001$), suggesting that HPR1 expression in papillary cancer is responsible for the loss of HS. Among benign thyroid follicular adenomas, HS was detected in six of the eight HPR1-negative follicular adenomas but only in two of the seven HPR1-positive follicular adenomas. However, statistical analysis did not reveal a correlation between HPR1 expression and the lack of HS in benign thyroid tumors ($P = 0.2$), suggesting that HPR1 expressed in thyroid follicular adenomas may not be fully active or quantitatively sufficient to degrade HSPG in the BM.

**HPR1 Expression and its Relationship with the Clinicopathological Parameters of PTCs.** We analyzed HPR1 expression in 93 thyroid neoplastic tissues using ISH staining. HPR1 was present in 48 of 62 papillary carcinomas, 3 of 4 medullary carcinomas, and 3 of 3 follicular carcinomas. In contrast, HPR1 was only present in 5 of 19 thyroid follicular adenomas and 2 of 5 Hürthle adenomas (Table 3). Statistical analyses revealed that HPR1 was preferentially expressed in PTCs compared with follicular adenomas ($P < 0.001$).

Clinicopathological analysis revealed that 27 of the 48 HPR1-positive PTCs (56%) were metastatic, whereas only 3 of the 14 HPR1-negative thyroid neoplasms were metastatic (21%; $P = 0.02$; Table 3). HPR1 expression did not correlate with patient age, gender, or tumor stage (Table 3). The correlation between HPR1 expression and the metastatic potential of Hürthle cell neoplasms and follicular and medullary carcinomas was not analyzed because their numbers were limited.

**RT-PCR Analysis of *HPR1* Gene Expression in Neoplastic Thyroid Cell Lines.** We analyzed HPR1 expression in a panel of thyroid tumor cell lines to determine whether HPR1 was preferentially expressed in certain types of thyroid neoplasms and whether HPR1 expression correlated with their metastatic potential. RT-PCR analysis revealed that HPR1 was undetectable in one follicular adenoma (KAK-1) and three anaplastic (KAT-4, KAT-18, and SW1736) carcinoma cell lines after 40 cycles of PCR amplification (Fig. 5). However, a HPR1 cDNA fragment was amplified with 40 cycles but not with 30 cycles in one follicular (WRO82) and two anaplastic (ARO81 and DRO90) carcinoma cell lines, indicating that HPR1 was expressed at low levels in these three tumor cell lines. HPR1 was expressed at high levels in one follicular (MRO87) and one papillary (NPA87) carcinoma cell line, because the 587-bp fragment of HPR1 cDNA could be readily amplified by a 30-cycle PCR reaction. These results show that HPR1 mRNA was differentially expressed in some types of thyroid neoplastic cell lines.

**Western Blot Analysis of HPR1 Expression in Thyroid Tumor Cell Lines.** To test whether HPR1 protein was made, we next conducted Western blot to analyze HPR1 protein expression in these thyroid tumor cell lines. As shown in Fig. 6*A*, HPR1 was expressed as a predominantly *M*r 50,000 mature enzyme at various levels in several thyroid tumor cell lines. KAT-4, KAT-10, NPA87, and MRO87 cell lines expressed HPR1 at high levels, whereas KAK-1 cells expressed HPR1 at a moderate level. The other five cell lines (KAT-18, ARO81, DRO90, WRO82, and SW1736) expressed HPR1 at very low or undetectable levels. Taken together, these results show that HPR1 protein and mRNA were highly expressed in KAT10, MRO87, and NPA87 cell lines but not expressed in SW1736 and ARO90 cell lines. KAK-1 and KAT-4 cell lines did not express detectable HPR1 mRNA but expressed significant

*Table 1* Comparison of HPR1 expression in thyroid neoplasms detected by ISH and IF

| | Number | HPR1 positive (IF) | Concordance |
|---|---|---|---|
| Follicular adenoma | | | |
| HPR1 positive (ISH) | 4 | 3 | 75% |
| HPR1 negative (ISH) | 11 | 3 | 83% |
| Papillary carcinoma | | | |
| HPR1 positive (ISH) | 13 | 12 | 92% |
| HPR1 negative (ISH) | 7 | 3 | 57% |

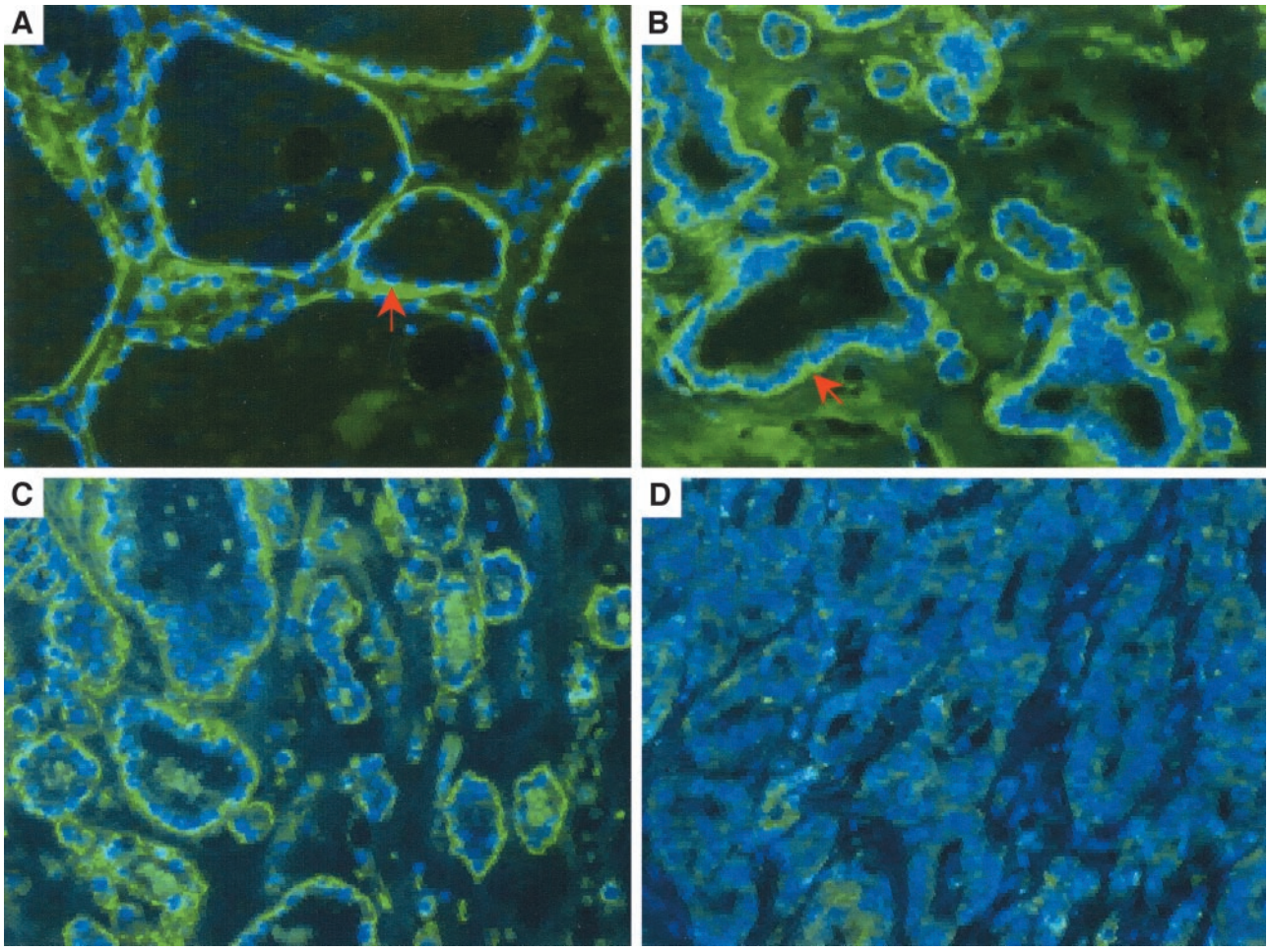


*Fig. 4* Immunodetection of HS in thyroid neoplasms. The sections of specimens derived from a thyroid follicular adenoma (*B*) and two papillary carcinomas (*C* and *D*) were dewaxed, rehydrated, and fixed with 1% paraformaldehyde. A section of the normal thyroid follicles adjacent to a thyroid papillary carcinoma was included as a control (*A*). The slides were incubated with anti-HS mAb (HepSS; working concentration, 5 μg/ml) at room temperature for 30 min. The slides were washed three times with PBS, followed by FITC-conjugated goat antimouse IgM. The slides were then washed and incubated with FITC-conjugated rabbit antigoat IgG. The slides were sealed and examined for HS under a fluorescence microscope.

amounts of HPR1 protein, whereas ARO81 and DRO90 cell lines expressed low levels of HPR1 mRNA but HPR1 protein was not detectable. The discrepancy of HPR1 mRNA and protein expression in some cell lines may be attributable to the difference in the stability of HPR1 mRNA and protein.

We then asked whether HPR1 protein expression correlated with HPR1 enzymatic activity in these cell lines, as measured by a novel ELISA method. Our results show that HPR1 activity in MRO87, NPA87, KAT10, and KAT-4 cell lines was about 5-fold higher than that in ARO81, DRO90, KAT-18, SW1736, and WRO82 cell lines (Fig. 6*B*). The KAK-1 cell line expressed an intermediate HPR1 activity. HPR1 activity in HPR1-transfected HT1080 cells, which was included as a positive control, was dramatically increased compared with HPR1 activity in pcDNA-transfected cells. These results show that the HPR1 enzymatic activities in these cell lines closely correlated with HPR1 protein levels as analyzed by Western blot.

*Table 2* HS composition in the BM inversely correlates with HPR1 expression

| | Number | HS positive (%) | *P* |
|---|---|---|---|
| Follicular adenoma | | | |
| HPR1 positive | 7 | 2 (29%) | |
| HPR1 negative | 8 | 6 (75%) | 0.2 |
| Total | 15 | 8 (53%) | |
| Papillary carcinoma | | | |
| HPR1 positive | 37 | 3 (19%) | <0.001 |
| HPR1 negative | 9 | 7 (78%) | |
| Total | 46 | 10 (22%) | |

We next analyzed whether HPR1 expression correlated with the metastatic and invasive potential of these cell lines. KAK-1 cells, which originated from a nonmetastatic benign follicular adenoma, expressed HPR1 protein and enzymatic activity at a moderate level but were unable to migrate through the uncoated Transwell inserts (Table 4). KAT-4 cells, an anaplastic cell line established from a patient with distant metastasis, expressed HPR1 protein and enzymatic activity at a high

*Table 3* HPR1 expression in thyroid neoplasms and its relationship with the clinicopathological parameters of PTCs

| | Number | Positive (%) | *P* |
|---|---|---|---|
| Tumor type | | | |
| Follicular adenoma | 19 | 5 (26%) | |
| Papillary carcinoma | 62 | 48 (77%) | |
| Follicular carcinoma | 3 | 3 (100%) | ≪0.001 |
| Medullary carcinoma | 4 | 3 (75%) | |
| Hürthle | 5 | 2 (40%) | |
| Total | 93 | 61 (66%) | |
| Metastasis (PTC) | | | |
| Yes | 30 | 27 (90%) | |
| No | 32 | 21 (66%) | 0.02 |
| Age (PTC) | | | |
| 18–39 | 25 | 21 (84%) | |
| >40 | 37 | 27 (73%) | 0.31 |
| Sex (PTC) | | | |
| Male | 18 | 12 (67%) | |
| Female | 44 | 36 (82%) | 0.32 |
| Tumor stage (PTC) | | | |
| T1 | 12 | 9 (75%) | |
| T2 | 34 | 27 (79%) | 0.85 |
| T3 | 16 | 12 (75%) | |

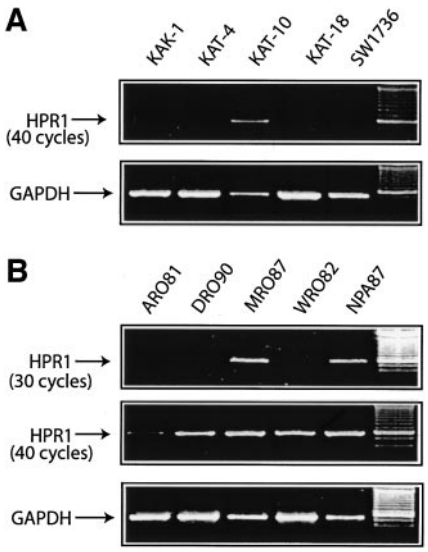


*Fig. 5* RT-PCR analysis of *HPR1* gene expression in thyroid tumor cell lines. Cells grown in 6-well plates were harvested on 60–80% confluence. Total cellular RNA was isolated using TRIzol (Life Technologies, Inc.) and quantitated by OD260 absorbance. RT-PCR reactions were conducted as described in "Material and Methods." Thirty PCR cycles were used to amplify GAPDH and HPR1 in cell lines shown in *B* (*top*). Forty cycles were used for the amplification of HPR1 cDNA in cell lines shown in *A* and *B* (*middle*). PCR product was separated on a 1.5% agarose gel and visualized by ethidium bromide staining under a UV transluminator. Results shown here are a representative of two independent experiments with similar results.

level but were also unable to migrate through the uncoated Transwell inserts and to penetrate the Matrigel-coated Transwell inserts (Table 4). In contrast, the other four metastatic anaplastic cell lines (KAT18, SW1736, ARO81, and WRO90) expressed little HPR1 and were able to migrate through the uncoated Transwell inserts and to invade the Matrigel-coated Transwell inserts (Table 4). MRO87 cells, a follicular carcinoma cell line from a patient with unknown metastasis status, expressed HPR1 at a high level but were unable to migrate through uncoated Transwell inserts and to penetrate the Matrigel-coated Transwell inserts (Table 4). WRO82, a follicular carcinoma cell line that was established from a patient with distant metastases (26), did not express HPR1 but was able to migrate and penetrate through uncoated and Matrigel-coated Transwell inserts (Table 4). The KAT-10 cell line, a metastatic PTC cell line, and NPA87, a poorly differentiated PTC cell line (27), expressed HPR1 at high levels but did not exhibit any motility or any invasive potential *in vitro* (Table 4). These results suggest that there is no correlation between HPR1 expression in thyroid tumor cell lines

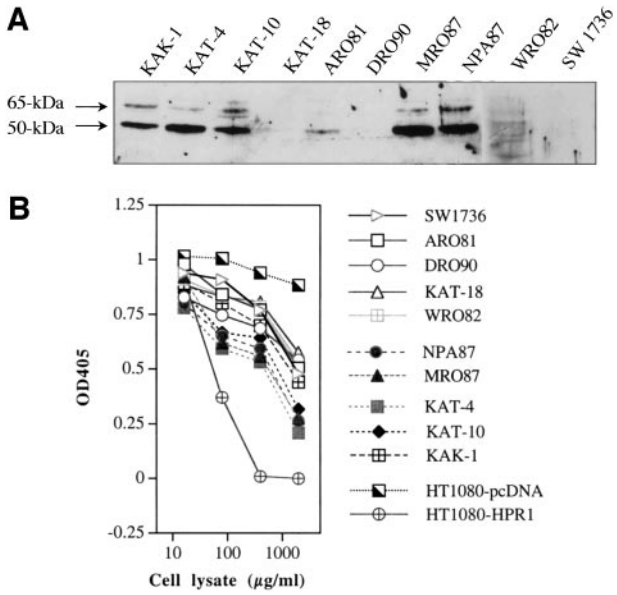


*Fig. 6* HPR1 protein expression and enzymatic activity in 10 thyroid tumor cell lines. *A*, Western blot analysis of HPR1 protein expression. Ten thyroid tumor cell lines were grown in 6-well plates. On 80% confluence, the cells were harvested and lysed in Triton X-100 lysis buffer. Cell lysates were prepared, and protein concentrations were quantitated by using a Bio-Rad protein assay kit. Equal amounts of protein (50 μg/lane) were loaded onto a 10% polyacrylamide-SDS gel. After electrophoresis, proteins were transferred onto a nitrocellulose membrane. HPR1 was detected by a rabbit anti-HPR1 antisera, followed by horseradish peroxidase-conjugated goat antirabbit IgG and enhanced chemiluminescence. *B*, detection of HPR1 activity by ELISA. The cells grown in 6-well plates were harvested and lysed in HPR1 assay buffer. The cell lysates were prepared, and protein concentrations were quantitated by using a Bio-Rad protein assay kit. Equal amounts of protein (50 μg/sample) were serially diluted 5-fold in HPR1 buffer and loaded to the Matrigel-coated ELISA plate and then incubated at 37°C for 16 h. Cell lysates prepared from pcDNA- and HPR1-transfected HT1080 cells were included as controls but were diluted with a starting concentration of 2 μg/sample because of high HPR1 enzymatic activity in HPR1-transfected HT1080 cells. The plate was then blocked with 5% BSA in PBS, followed by anti-HS-specific mAb and horseradish peroxidase-conjugated goat antimouse IgM antibody. Wells in triplicate omitting the secondary antibody were included as blanks. After development with the 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid substrate, the plate was read at OD405 absorbance in an ELISA plate reader. The OD450 values, after subtraction with the mean values of the three wells, are plotted against the dilution of the cell lysates. The data shown here are representative of two independent experiments with similar results.

*Table 4* HPR1 expression and the invasive and metastatic potential in a panel of thyroid tumor cell lines

| Cell line | Tumor type | Metastasis | HPR1 expression | | | Motility | Invasion |
|---|---|---|---|---|---|---|---|
| | | | mRNA | Protein | Activity | | |
| KAK-1 | FTA[a] | No | − | + | ++ | −[b] | −[b] |
| KAT-4 | ATC | Yes | − | +++ | +++ | − | − |
| KAT-18 | ATC | Yes | − | − | ± | ++ | + |
| SW1736 | ATC | Yes | − | − | + | ++ | + |
| ARO81 | ATC | Yes | + | ± | + | ++ | ± |
| DRO90 | ATC | Yes | + | − | + | ++ | + |
| MRO87 | FTC | Unknown | +++ | +++ | +++ | − | − |
| WRO82 | FTC | Yes | + | − | + | ++ | + |
| KAT-10 | PTC | Yes | + | ++ | +++ | − | − |
| NPA87[c] | PTC | Unknown | +++ | +++ | +++ | − | − |

[a] FTA, follicular thyroid adenoma; ATC, anaplastic thyroid carcinoma; FTC, follicular thyroid adenoma.
[b] −, <0.1% cells to be able to migrate through the uncoated or Matrigel-coated insert after incubation for 3 days.
[c] NPA87, a poorly differentiated PTC cell line.

grown *in vitro* and their intrinsic metastatic potential. Moreover, it seems that the cell lines expressing detectable HPR1 enzymatic activity tend to have low motility.

**Enforced HPR1 Expression Boosts the Invasive Potential of SW1736 and HT1080 Cells.** To further investigate the role of HPR1 expression in thyroid tumor metastasis, we tested whether overexpression of HPR1 in SW1736 cells, a HPR1-negative anaplastic tumor cell line, could increase their invasive potential. HT1080 cells, a human fibrosarcoma cell line commonly used in chemoinvasion experiments, were included as a positive control. HT1080 and SW1736 cells were transfected with the pcDNA3 empty vector or with the *HPR1* gene cloned in this vector. Western blot analysis revealed that HPR1 was expressed predominantly as the $M_r$ 50,000 active form (Fig. 7*A*) in both HPR1-transfected SW1736 and HT1080 cells but not in the pcDNA-transfected controls. HPR1 was detected in the supernatant as the $M_r$ 65,000 inactive form and on the membrane predominantly as the $M_r$ 50,000 form (data not shown). HPR1 expression in these two cell lines did not affect the growth curve because the doubling times of untransfected or HPR1-transfected HT1080 and SW1736 cells were not changed (data now shown). We next conducted ELISA to test whether HPR1 enzymatic activity was also correspondingly increased in these two transfected cell lines. As shown in Fig. 7*B*, the enzymatic activity of HPR1 in the cell lysates of both transfected cell lines was also dramatically increased compared with that in the pcDNA-transfected controls (Fig. 7*B*). Chemoinvasion assays revealed that very few pcDNA-transfected SW1736 and HT1080 cells were able to penetrate the Transwell inserts coated with 100 μg of Matrigel/insert within 24 h, whereas >50% of HPR1-transfected SW1736 and HT1080 cells were able to penetrate through the Matrigel-coated inserts (Fig. 7*C*). These results clearly show that increased HPR1 expression and activity in two tumor cell lines boosted their invasive potential, implying an important role for HPR1 in thyroid tumor metastases.

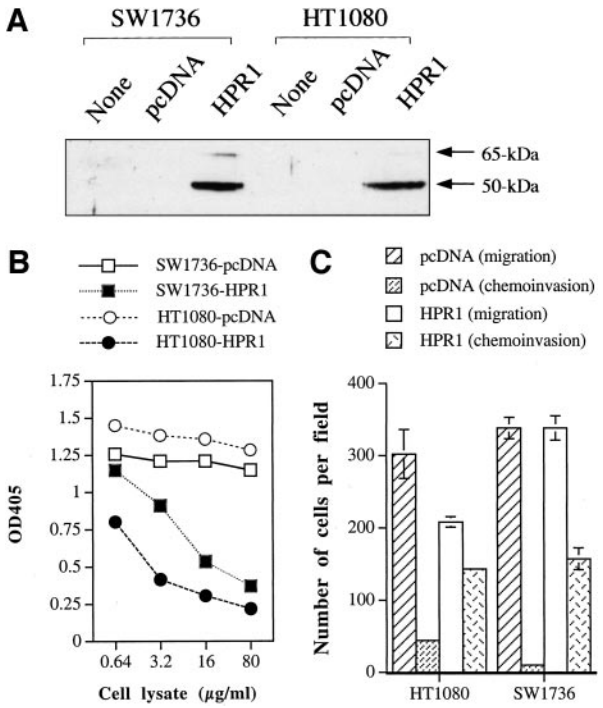


*Fig. 7* HPR1 overexpression in tumor cell lines boosted their invasive potential. HT1080 and SW1736 cells were transfected with the pcDNA3 empty vector or the same vector encoding the *HPR1* gene as described in "Materials and Methods." The cell lysates from pooled clones of each transfectant cell line were prepared similar to those in Fig. 6. Western blot and ELISA were conducted to examine HPR1 protein expression (*A*) and to quantitate HPR1 activity as described in Fig. 6, *A* and *B*, except the protein concentrations used were 2 μg/sample. *C*, cell motility and chemoinvasion assays. The Transwell inserts were uncoated or coated with Matrigel (100 μg/insert) for 2 h at room temperature. Single-cell suspensions were prepared and loaded into the Transwell inserts ($2 \times 10^4$ cells/insert) placed in the 24-well plates filled with the conditioned medium. After incubation for 24 h, the cells were harvested and stained. The stained polyethylene terephthalate membranes were sliced down and then mounted onto a hemacytometer. The cells in five fields were counted. The data are the mean of three independent experiments.

## DISCUSSION

Tumor metastasis is a complex process that requires multiple phenotypic and genetic alterations for tumor cells (28, 29). Previous studies have demonstrated that breakdown of the tissue

barrier by increased expression of matrix metalloproteinase 2 is critical for the metastasis of human PTCs (30–32). In the present study, we analyzed the expression of HPR1, an endoglycosidase capable of degrading HS, in thyroid neoplasms. Our results show that HPR1 was expressed at a significantly higher frequency in thyroid papillary carcinomas than follicular adenomas, indicating that HPR1 expression is associated with thyroid tumor malignancy. Additional studies show that HPR1 expression correlated with lower HS content in the BM and that HPR1-positive PTCs had a significantly higher metastasis rate (56%) than HPR1-negative ones (21%; $P < 0.05$). These observations suggest that HPR1 might break down tissue barriers and, thus, may significantly contribute to thyroid tumor metastases. Because 21 of the 48 HPR1-positive PTCs (44%) did not display a metastatic phenotype at the time of diagnosis and resection, and some follicular adenomas that are not malignant also expressed HPR1, HPR1 expression alone may not be sufficient to confer a metastatic capability to neoplastic thyroid cells. Rather, HPR1 may cooperate with other dysregulated molecules to establish the metastatic phenotype of thyroid neoplasms. This is consistent with two recent studies showing that HPR1 expression correlates with the metastatic potential of breast cancer (22) and with the invasive potential of hepatocellular carcinoma (18).

Alterations of the BM structure seem to play a key role in the morphogenesis of thyroid tumors. A previous study by Lu *et al.* (33) using a di-isopropanolnitrosamine-induced rat thyroid tumor model demonstrated that the follicular epithelial BM is interrupted or completely lost during the progression of thyroid lesions from benign nodular lesions to overt carcinomas. Consistent with these observations, our IF staining revealed a complete loss of HS in the vast majority of HPR1-positive thyroid papillary carcinomas. It seems that the loss of HS content in the BM is caused, in part, by the HPR1-mediated HS degradation. This is because HPR1 expression inversely correlated with the HS composition of PTCs and that the expression of some matrix proteins, such as fibronectin (33), and some HSGPs, such as syndecin-1 and glypican-1, are increased in a variety of malignancies such as thyroid (33) and pancreatic cancer (34, 35). Dysregulation or destruction of other matrix proteins may also contribute to the defective deposition of HS in the BM and ECM. Nevertheless, our present study using IF staining with a special HS-specific mAb to analyze HS content suggests that disassembling of the BM is an important step in the invasive growth of PTCs.

The degradation of HSPG on cell surfaces and in the ECM and BM not only triggers the breakdown of tissue barriers but also plays an important role in promoting angiogenesis and tumor cell proliferation by releasing many angiogenic molecules and growth factors stored in the ECM and BM. Emoto *et al.* (36) reported that intratissue ECM-free FGF-2 is significantly increased in PTCs compared with that in normal thyroid tissues. These authors further showed that two distinct fractions of HS in normal thyroid tissues contain FGF-2, but only one fraction of HS in thyroid papillary carcinomas contains FGF-2, which allows a subpopulation of FGF-2 to become bioavailable in the ECM (37). El-Assal *et al.* (18) showed that coexpression of HPR1 and basic FGF in hepatocellular carcinoma has a synergistic effect on tumor angiogenesis. Our present study shows that HPR1 expressed in thyroid papillary carcinomas was functional and that HS composition was absent in the BM of many HPR1-positive thyroid papillary carcinomas. These observations are consistent with two previous studies showing that relative amounts of HS are decreased in some PTCs (38, 39). On the basis of these observations, we propose that an increase of intratissue ECM-free FGF-2 and possibly other angiogenic molecules is attributable, in part, to increased HPR1 expression and HS degradation.

Anaplastic thyroid carcinoma is the most aggressive type of thyroid cancer and represents an advanced step of tumor progression. Although its occurrence rate is very low (about 2%) of all thyroid neoplasms in the United States, it is almost always fatal (4). Profiling of HPR1 expression, motility and invasive potential in a panel of thyroid tumor cell lines revealed that although the majority of anaplastic carcinoma cell lines were highly motile and invasive, most of them, except KAT-4, did not express detectable amounts HPR1 protein and enzymatic activity. It is possible that thyroid tumor cell lines grown in culture lost HPR1 expression. This may be because of different growth conditions such as oxygen tension. Consistent with this notion, Uno *et al.* (47) showed that HPR1 expression was undetectable in a human metastatic esophageal cancer cell line and a metastatic lung cancer cell line but was dramatically increased in human esophageal squamous cell carcinoma and lung adenocarcinoma. Ikuta *et al.* (40) found no correlation between HPR1 expression *in vitro* and the intrinsic metastatic potential in a panel of oral cancer cell lines. Nevertheless, our results suggest that invasion and metastasis of thyroid cancer can occur in the absence of *HPR1* gene expression and its enzymatic activity. Our transfection studies show that increased expression of HPR1 in the SW1736 cell line significantly enhanced its invasive potential *in vitro* in the artificial BM. This suggests that HPR1 is capable of promoting the metastatic potential of thyroid tumors.

A rapid and simple assay for quantitating HPR enzymatic activity should greatly facilitate screening for specific HPR inhibitors. However, most HPR assays involve time- and labor-intensive steps for isolation and purification of radiolabeled biosynthetic substrates (5, 10–12, 15, 41–45). The analyses themselves are also time-consuming and laborious. Freeman and Parish (46) recently developed a relatively easy HPR assay by quantitating the HPR-digested radiolabeled HS fragments (products) that are eluted through mini columns of HRG-coupled Sepharose beads (46). This method involves the radiolabeling of natural HS, purification of HRG, preparation of a HRG-Sepharose column, and quantitation of the HPR-digested, unbound heparan substrate. In the present study, we took advantage of a HS-specific mAb and developed a novel ELISA method to directly quantitate HPR1 activity in 96-well plates. HPR1 activity measured by this method correlated with the expression of the $M_r$ 50,000 mature enzyme in 10 thyroid tumor cell lines (Fig. 6*B*), whereas transfection of the *HPR1* gene in the HT1080 and SW1736 cell lines dramatically increased HPR1 activity (Fig. 7*B*). These results suggest that HPR1 enzymatic activity measured by this method is very specific and can be used to screen HPR1 inhibitors.

In summary, our present studies have demonstrated that HPR1 was expressed at a significantly higher frequency in

thyroid papillary carcinomas than in follicular adenomas/Hürthle cell adenomas. HPR1 expression in papillary carcinoma inversely correlated with the presence of HS, suggesting that HPR1 has a functional role. Additional studies have demonstrated that HPR1 was expressed in metastatic thyroid carcinomas at a higher frequency than that in nonmetastatic cancer and that HPR1 overexpression in thyroid neoplastic cell lines increased their invasive potential *in vitro*. These observations suggest collectively that HPR1 may facilitate the metastases of PTCs.

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