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Inhibitory Role of Plasminogen Activator Inhibitor-1 in Invasion and Proliferation of HLE Hepatocellular Carcinoma Cells

Yasushi Morita,[1] Yoshitake Hayashi,[2,3] Taichi Kanamaru,[1] Takashi Itoh,[1] Satoshi Suzuki,[1] Masahiro Yamamoto,[1] Yoshikazu Kuroda[1] and Hiroshi Itoh[2]

[1]First Department of Surgery and [2]First Department of Pathology, Faculty of Medicine, Kobe University, 7-5-2 Kusunoki-cho, Chuo-ku, Kobe 650-0017

Plasminogen activator inhibitor (PAI)-1, a serine protease inhibitor, inactivates urokinase-type plasminogen activator (uPA) and regulates degradation of the extracellular matrix; whether it functions for or against tumor progression, however, has been the subject of controversy. To assess the role of PAI-1 in invasion and proliferation of hepatocellular carcinoma (HCC) cells, HLE cells were transfected with a vector capable of expressing an antisense PAI-1 transcript. Analysis of seven stably transfected clones (PAI-1$^-$) showed reductions of 81% in PAI-1 mRNA by northern blot analysis and 63% in the cellular PAI-1 antigen level by enzyme-linked immunosorbent assay (ELISA). There was no change in the levels of secreted PAI-1 or PAI-2. The activity of cellular uPA increased by 54%, without change in the protein level or the secreted uPA activity evaluated by ELISA. Morphologically, PAI-1 antisense induced a spindle shape with narrower cytoplasmic processes in HLE cells. The forced inhibition of PAI-1 increased the invasion and the growth of PAI-1$^-$ cells by 75% and 82%, respectively. These results suggest that PAI-1 plays a role in inhibiting invasion and proliferation, and the balance between uPA and PAI-1 expression is important to assess the invasiveness of HCC cells.

Key words: Hepatocellular carcinoma — Plasminogen activator inhibitor-1 — Antisense — Invasion — Proliferation

The plasminogen activation system is closely associated with the proteolytic processes in the invasion steps of cancer cells.[1–3] In these processes, the expression of urokinase-type plasminogen activator (uPA) and uPA receptor (uPAR) in cancer cells converts plasminogen to plasmin which in turn degrades the extracellular matrix (ECM) in conjunction with collagenases,[4] thus facilitating the migration of cancer cells. This uPA-mediated proteolytic system has also been shown to play a role in cellular growth. The activation of hepatocyte growth factor (HGF) by uPA is closely associated with the growth of hepatocytes[5] and with the invasion and tumorigenicity of human leiomyosarcoma cells.[6] The activity of uPA in normal cells is regulated by its inhibitors, plasminogen activator inhibitor (PAI)-1 and PAI-2, which block uPA by internalizing uPA protein[7,8]; thus the balance between uPA and PAIs expression is important in determining the activity of uPA. It has been recognized that this balance is disturbed in some cancers, resulting in the degradation of ECM, invasion and metastasis with enhanced plasmin generation. Some reports have shown that PAI-2 suppresses the degradation of ECM, invasion and metastasis with enhanced plasmin generation in several human cancer cells.[9,10] The precise role of PAI-1 in tumor progression is, however, still unclear. Several groups have shown that PAI-1 expression is positively associated with poor prognosis in some cancers,[11–13] and PAI-1 weakens the attachment of cancer cells to the ECM by disrupting the binding of vitronectin to uPAR on cancer cells, thus facilitating cancer cell migration.[14,15] On the other hand, PAI-1 suppresses metastasis in murine models of prostate carcinoma and melanoma.[16,17]

We have reported that the plasminogen activation system is positively related to the invasion and postoperative recurrence of hepatocellular carcinoma (HCC),[18] suggesting that the inhibition of uPA-mediated proteolysis may contribute to the inhibition of HCC progression.

In this study, we assessed the role of PAI-1 in growth and invasion of HLE HCC cells by transfecting the cells with a vector capable of expressing an antisense PAI-1 transcript and analyzing the effects on cellular invasion and proliferation.

MATERIALS AND METHODS

Preparation of PAI-1 antisense constructs A quantity of 5 μg of total RNA isolated from HLE cells was reverse-transcribed with random primers using a commercial kit (First Strand Synthesis Kit; Pharmacia, Inc., Piscatamay, NJ). To obtain a 1479 bp (1 to 1465) partial PAI-1 cDNA fragment containing the linker sequences, the cDNA was

[3] To whom correspondence should be addressed.
E-mail: hayashiy@mailgate.kobe-u.ac.jp

amplified by polymerase chain reaction (PCR) using synthetic primers (5′-GCTCGGATGCAGATGTCTCCAGCC-3′, 3′-GGCTAGCTTGGAGACCTTAAGGGAGT-5′; nonspecific *Nhe*I and *Xho*I restriction enzyme recognition sequences are in bold; Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) that included *Nhe*I and *Xho*I recognition sites. The amplified cDNA fragment was subcloned into the *Nhe*I and *Xho*I sites of pMAMneo (CLONTECH Laboratories Inc., Palo Alto, CA).

Transfection and selection The PAI-1 antisense construct (6 μg) was transfected into HLE cells with "SuperFect" (Quiagen Inc., Valencia, CA). The cells were grown in medium containing 200 μg/ml of G418 (Calbiochem-Novabiochem Co., San Diego, CA) and 10^{-6} *M* dexamethasone (Sigma Chemical Co., St. Louis, MO) to stimulate MMTV-LTR promoter in pMAMneo.[19] Hereinafter G418-resistant cells are referred to as PAI-1$^-$ and control cells transfected with pMAMneo without the insert are referred to as HLE-M. These transfectants were maintained in RPMI 1640 (Nissui, Tokyo) containing 10% fetal bovine serum (FBS) and 10^{-6} *M* dexamethasone.

Quantitation of uPA, PAI-1 and PAI-2 antigen The amounts of uPA, PAI-1 and PAI-2 protein were measured with commercial antigen assay kits, "TintElize uPA," "TintElize PAI-1" and "TintElize PAI-2" (Biopool A.B., Umeå, Sweden). For detection of uPA, PAI-1 and PAI-2, the medium and the cell lysates were 10-fold and 5-fold concentrated, respectively, prior to assay.

Determination of uPA activity The uPA activity was measured with a commercial kit, Chromolize™ uPA (Biopool A.B.). To determine secreted and cellular uPA activity, the medium and the cell lysates were treated as stated above.

Northern blot analysis Total RNA (20 μg) was electrophoresed on a 1% agarose gel containing 7.5% formaldehyde, transferred to nylon membranes and hybridized with digoxygenin (DIG)-labeled PAI-1 cDNA probe for 16 h at 50°C. The PAI-1 cDNA probe was prepared by amplifying 851 bp PAI-1 cDNA (1265 to 2115) with a PCR DIG Probe Synthesis Kit (Boehringer Mannheim GmbH Biochemica). Quantitation of the bands was carried out with the software NIH Image 1.55 (National Institutes of Health, Bethesda, MA).

***In vitro* invasion assays** A suspension (0.2 ml) of HLE, HLE-M and PAI-1$^-$ cells at a density of 80,000 cells per ml in RPMI 1640 containing 1% bovine serum albumin (CALZYME Laboratories, Inc., San Luis Obispo, CA) (RPMI-BSA) was added to a Matrigel invasion chamber (Becton Dickinson, Bedford, MA), which was placed into a well of a 24-well culture plate containing 0.5 ml of RPMI-BSA. After 48 h, the cells remaining on the upper side of the filter ("noninvasive cells") were removed with a cotton swab and the cells attached to the underside of the filter ("invasive cells") were counted with an MTT-kit (Chemicon International Inc., Temecula, CA). The invasion index (%) was determined as the number of invasive cells/the number of invasive and noninvasive cells×100.

Assay for cell growth A suspension of HLE, HLE-M and PAI-1$^-$ cells (0.1 ml) at a density of 10^5 cells per ml in RPMI 1640 containing 10% FBS was added to the wells of a 96-well culture plate. The next day (day 1), the medium was changed to RPMI 1640 containing 1% FBS, and the cells were counted with an MTT-kit on days 1, 3 and 6.

RESULTS

To cause an imbalance between PAI-1 and uPA expression, HLE cells were transfected with an anti PAI-1 expression vector and selected based on G418 resistance. Seven clones of PAI-1$^-$ were isolated and compared to cells transfected with the empty vector and with untransfected parental cells.

Construction integration was confirmed in all clones by PCR amplification using their genomic DNA as a template with the primers stated in "Materials and Methods." Northern blot analysis showed that the level of PAI-1 mRNA in PAI-1$^-$ clones was 20 to 83% of that of untransfected HLE and 19 to 77% of that of HLE-M (Fig. 1, A and B). PAI-1$^-$-3, PAI-1$^-$-4 and PAI-1$^-$-5, whose PAI-1 mRNA expression levels were 19, 40 and 42% of that of the control cells, respectively, were characterized. Enzyme-linked immunosorbent assay (ELISA) showed that the levels of the cellular PAI-1 protein in PAI-1$^-$-3, PAI-1$^-$-4 and PAI-1$^-$-5 were 45, 38 and 60% of that of HLE, and 44, 37 and 58% of that of HLE-M, respectively ($P<0.01$). There were no significant differences in secreted PAI-1 among PAI-1$^-$, HLE-M and HLE (Fig. 2). The levels of the cellular and secreted uPA protein as analyzed by ELISA were also similar among PAI-1$^-$, HLE-M and HLE (Table I). Antisense transfection caused little change in the PAI-2 level (data not shown). The activity levels of cellular uPA in PAI-1$^-$-3, PAI-1$^-$-4 and PAI-1$^-$-5 were 35, 54 and 32% higher than that of HLE-M, and 29, 47 and 26% higher than that of HLE, respectively ($P<0.05$). There were no significant differences in the level of secreted uPA activity among PAI-1$^-$, HLE-M and HLE (Fig. 3). The levels of cellular and secreted uPA proteins as analyzed by ELISA were also similar among PAI-1$^-$, HLE-M and HLE (Table I).

The analysis of cell morphology demonstrated that PAI-1$^-$-4 cells exhibited a spindle shape with narrower cytoplasmic processes than control cells. In addition, they were characterized by the formation of a gland-like space, with or without papillary structure, or the presence of mucus in tumor cells, or both (Fig. 4).

To assess whether the forced reduction of PAI-1 results in increased invasion of HCC cells, an *in vitro* invasion

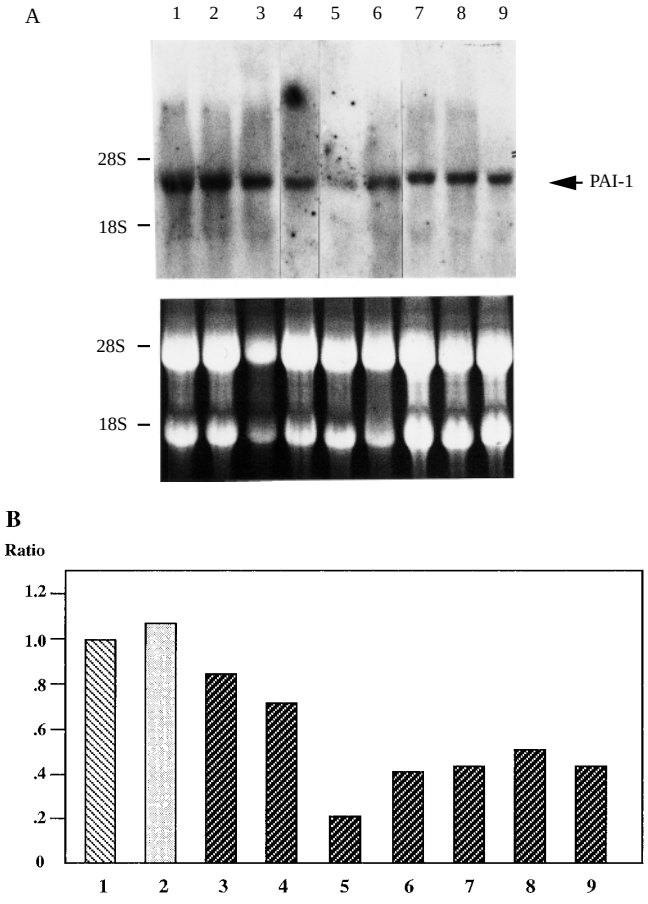

Fig. 1. (A) Expression of PAI-1 mRNA in each cell line by northern blot analysis. The bottom panel of (A) shows ethidium bromide staining of 28S and 18S ribosomal RNA. (B) Quantitation of PAI-1 mRNA expression by NIH-Image. Lane 1, HLE; lane 2, HLE-M; and lanes 3–9, PAI-1⁻ cells are indicated.

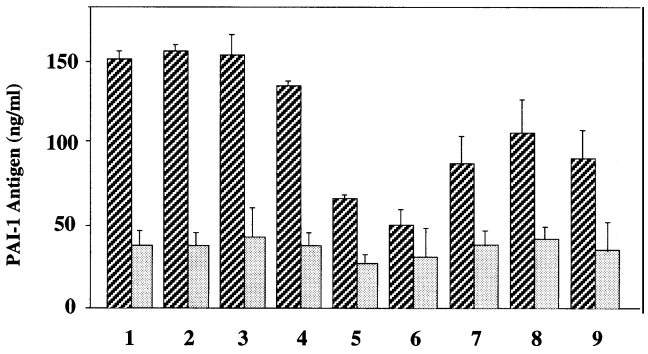

Fig. 2. Concentration of PAI-1 antigen in cell lysates (cellular PAI-1) (indicated by the dark columns) and in medium (secreted PAI-1) (indicated by the light columns). Lane 1, HLE; lane 2, HLE-M; and lanes 3–9, PAI-1⁻ cells are indicated.

Table I. Concentration of uPA in Serum-free Supernatant and in Cell Lysates Determined by ELISA

	Secreted uPA (ng/ml)	Cellular uPA (ng/ml)	
HLE	5.96±1.53	5.12±2.07	NS
HLE-M	5.11±1.96	5.31±2.32	
PAI-1⁻-3	6.09±2.01	6.14±2.22	
PAI-1⁻-4	6.00±1.62	6.79±2.06	

NS: not significant.

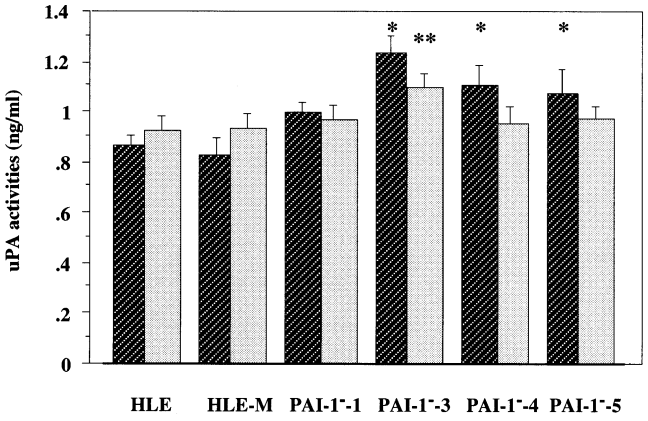

Fig. 3. uPA activities in cell lysates (cellular uPA activity) (indicated by the dark columns) and in medium (secreted uPA activity) (indicated by the light columns). *,** $P<0.01$ vs. HLE and HLE-M.

assay was done to assess the ability of cells to migrate through a Matrigel-coated filter. The assay yielded the following invasion indices: HLE (29.4±2.0%), HLE-M (28.2±2.8%), PAI-1⁻-1 (30.7±3.6%), PAI-1⁻-3 (48.9±2.9%), PAI-1⁻-4 (42.0±3.1%) and PAI-1⁻-5 (41.5±2.5%) ($P<0.01$) (Fig. 5). On Matrigel, HLE and HLE-M cells were largely clustered, whereas PAI-1⁻ cells were single or in small, sparse groups. Analysis of cell growth showed that PAI-1⁻ cells exhibited a higher proliferation rate than HLE or HLE-M; the numbers of PAI-1⁻-3, PAI-1⁻-4, PAI-1⁻-5, HLE-M and HLE cells on the 6th day of culture were $5.8\pm0.4\times10^4$, $6.9\pm0.7\times10^4$, $5.2\pm0.4\times10^4$, $3.7\pm0.2\times10^4$ and $3.4\pm0.9\times10^4$ per well, respectively ($P<0.01$) (Fig. 6).

DISCUSSION

The aim of this study was to analyze the change of phenotype of HLE HCC cells following forced inhibition of PAI-1 to cause an imbalance between PAI-1 and uPA expression. The balance of uPA and PAI-1 expression is probably disturbed in some invasive cancer cells,[17, 20] just

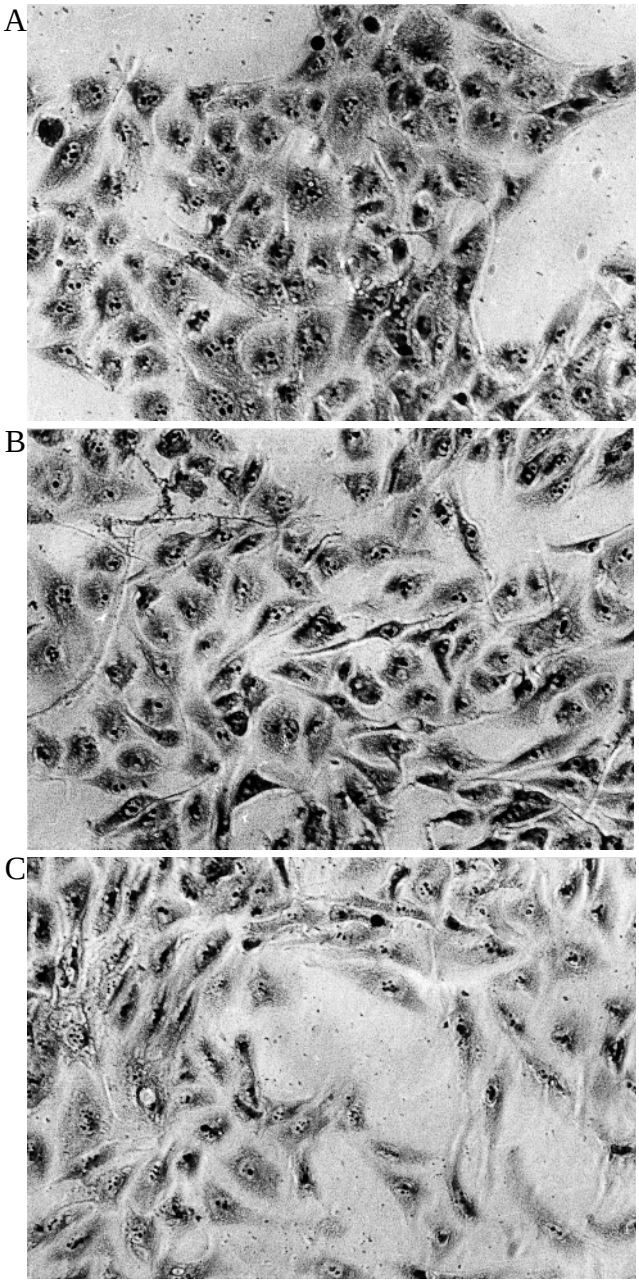

Fig. 4. Cell morphology. All the cells were fixed with ethanol and stained with 0.02% Giemsa stain. (A) HLE, (B) HLE-M and (C) PAI-1$^-$-3 are indicated.

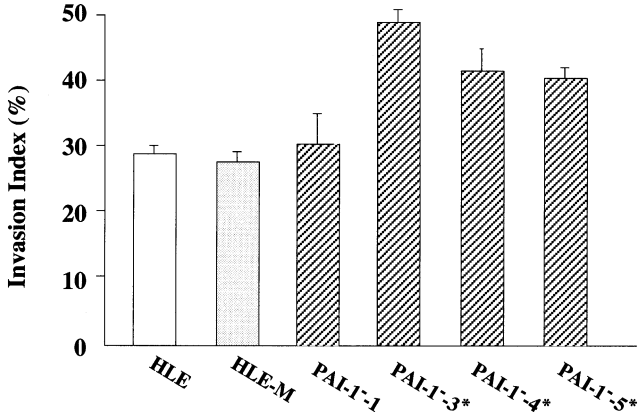

Fig. 5. *In vitro* invasion assay: invasion index (%) of each cell line. ∗ P<0.01 vs. HLE and HLE-M.

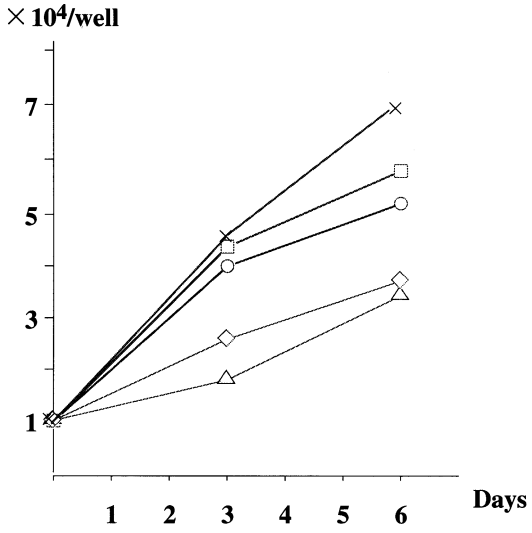

Fig. 6. Growth curves of HLE (△), HLE-M (◇), PAI-1$^-$-3 (×), PAI-1$^-$-4 (○) and PAI-1$^-$-5 (□).

as the invasive ability of cancer cells is affected by the imbalance of metalloproteinases and their inhibitors.[21]

Initially, our results showed that HLE cells produce PAI-1, although it has been reported that PAI-1 is not produced by the epithelial cancer cells but by the stromal cells in the tumor.[22] This suggested that the balance of expression of uPA and PAI-1 may depend on the autocrine system of cancer cells as well as the paracrine system and network of cancer cells and stromal cells, and also showed that the imbalance of expression of these molecules within cancer cells may facilitate invasion.

Forced inhibition of PAI-1 expression decreased cellular PAI-1 antigen and increased uPA activity, whereas secreted PAI-1 and uPA activity did not change, and the level of secreted uPA in PAI-1$^-$ cells is similar to that in control cells. These results may suggest that PAI-1 produced in cancer cells leaves the cells and inactivates secreted uPA protein first, and then free PAI-1 returns to

the cancer cell surface and inactivates cell-bound uPA, supporting the theory proposed by Mimuro *et al.*[23] that PAI-1 has a high affinity for some constituents of the ECM and once bound to the ECM, has a longer half life. Moreover, our results may also suggest that the induction of high levels of PAI-1 in cancer cells is necessary to inhibit cell-bound uPA and consequent cellular uPA activity and degradation of the ECM around cancer cells.

Morphologically, the cells exhibited a spindle shape with narrower cytoplasmic processes after PAI-1 antisense transfection, as though the cells were proceeding to a more undifferentiated cell type. These changes may indicate that PAI-1 influences a large number of genes and that the transfection of the anti-PAI-1 vector therefore affects these genes.

The role of PAI-1 in tumor progression, especially in invasion and metastasis, is still a subject of controversy, and it has been concluded that PAI-1 can be effective in controlling metastasis, depending on the type and location of the primary tumor. Although PAI-1 suppresses uPA activities and ECM degradation in colon cancer, prostate cancer and melanoma,[16, 17, 23] the role of PAI-1 in invasion and proliferation of HCC cells has not been reported. Our data showed that forced inhibition of PAI-1 increased the invasion index of HCC cells and suggested that the imbalance between uPA and PAI-1 expression and the consequent uPA activity in cancer cells may be important for the invasion of HCC. If such is the case, the independent analysis of uPA or PAIs may not be of use in predicting cancer prognosis.

uPA activity is closely associated with tumor cell growth[24, 25] and PAI-1 transfection has been shown to reduce the growth of human prostate carcinoma,[16] suggesting that PAI-1 has a role in suppressing tumor cell growth. Our data also suggest that uPA activity regulated by PAI-1 is closely associated with the growth of HCC cells. Jeffers *et al.* have reported that hepatocyte growth factor (HGF) activated by uPA increases cellular growth in leiomyosarcoma.[6] Since long-acting PAI-1 also inhibits the growth of prostatic cancer cells not expressing uPA,[25] we are not able to explain the mechanism of cellular growth in terms of the uPA-HGF network alone. Thus, PAI-1 may have another mechanism which directly inhibits cell growth or regulates other growth factors.[26]

In this study, HCC cells with an imbalance between uPA and PAI-1 leading to a uPA dominant state showed an increase of uPA activity, invasion and cell growth. We intend to examine the effect of a PAI-1 dominant state in HCC cells, in forthcoming studies. Since we did not examine the role of PAI-1 in the migration of HCC cells in the present study, we can not comment on the theory that PAI-1 is positively associated with the spreading of tumor cells.[14] Further studies may be necessary to reveal the relationship between PAI-1 expression and the progression or prognosis of HCC.

In conclusion, PAI-1 inhibits invasion and proliferation, and the balance between uPA and PAI-1 expression is important in determining the invasiveness of HCC cells, suggesting that forced expression of PAI-1 may prevent the progression of HCC.

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