# βig-h3 Is Involved in the HAb18G/CD147-Mediated Metastasis Process in Human Hepatoma Cells

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**HAb18G/CD147, a new hepatoma-associated antigen cloned and screened from human hepatocellular carcinoma cDNA library, is closely correlated with metastasis process in human hepatoma cells. In the present study we aimed to identify the pivotal molecules of the HAb18G/CD147 signal transduction pathway. The investigation showed that βig-h3, a secretory extracellular matrix (ECM) protein, was upregulated in HAb18G/CD147-expressing human hepatoma T7721 cells and was downregulated by depressing HAb18G/CD147 expression. The expression of βig-h3, upregulated in human hepatoma cells, was positively relative to the expression of HAb18G/CD147 in different human hepatoma cell lines. By overexpressing βig-h3 in human SMMC-7721 hepatoma cells, we discovered that βig-h3 promoted cell adhesion, invasion, and matrix metalloproteinase (MMP) secretion potential. HAb18G/CD147-induced invasion and metastasis potential of human hepatoma cells can be attenuated by antibodies specific for βig-h3, and no significant differences on inhibitory effects were observed among T7721 cells incubated with antibodies for βig-h3 or HAb18G/CD147 or both types together. Taken together, our study suggests that βig-h3, regulated by the expression of HAb18G/CD147, is involved in the HAb18G/CD147 signal transduction pathway and mediates the HAb18G/CD147-induced invasion and metastasis process of human hepatoma cells.** Exp Biol Med 232:344–352, 2007

**Key words:** HAb18G; CD147; βig-h3; hepatoma cell; metastasis

## Introduction

CD147, a cell surface transmembrane glycoprotein widely expressed on many cell types, appears at especially high levels on human tumor cells (1–8). Elevated CD147 expression is correlated with tumor progression and invasion potential (9, 10). HAb18G, a new hepatoma-associated antigen, has an identical nucleic acid and amino acid sequence as CD147 (11). The transmembrane domain of HAb18G/CD147 contains a glutamic acid residue and a leucine zipper motif that is implicated in the protein association with the plasma membrane and the dimerization of DNA-binding proteins (12, 13). These structural characteristics may enable HAb18G/CD147 to interact with other membrane proteins, intracellular enzymes/factors as well as extracellular factors that are involved in the signaling pathways pertinent to the process of metastasis.

Our previous studies have demonstrated that HAb18G/CD147 promotes the invasion activity of human hepatoma cells by stimulating fibroblast cells to produce elevated levels of several matrix metalloproteinases (MMPs; Ref. 7). By transfecting HAb18G/CD147 cDNA into human SMMC-7721 hepatoma cells to obtain a cell line, T7721, that stably expressed HAb18G/CD147, we have confirmed that the overexpression of HAb18G/CD147 enhances the metastatic potential of human hepatoma cells by disrupting the regulation of $Ca^{2+}$ entry by nitric oxide (NO)/cGMP (11, 14).

The process of proliferation, invasion, and metastasis is a complex one, involving both the autonomy of the malignant cells and their interaction with the cellular and extracellular environments (14). The way for tumor cells to respond to cellular and extracellular stimuli is regulated through transduction of those signals and translation into cellular activities. Although many researchers are focused on the function of CD147 in tumor process, the mechanism is still not well understood. It is not clear whether CD147 is involved in signal transduction and cell adhesion or whether

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it directly serves as a signal-transmitting adhesion molecule or as a regulator of adhesion.

In this study we identified that the expression of βig-h3 is in positive correlation with HAb18G/CD147, and βig-h3 promotes hepatoma cell adhesion, invasion, and MMP secretion. Additionally, the potential effect of HAb18G/CD147 on the invasion and metastasis process in hepatoma cells was found to be mediated by its direct or indirect actions on expression of βig-h3.

## Materials and Methods

**Cell Culture.** The human SMMC-7721 hepatoma cells, FHCC-98 hepatoma cells, human QSG normal hepatic cells (all obtained from the Institute of Cell Biology, Academic Sinica, Shanghai, China), and T7721 cells stably overexpressing HAb18G/CD147 (14) were cultured with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 2% L-glutamine at 37°C in a humidified atmosphere of 5% $CO_2$. G418 was added into the medium for T7721 cells at a final concentration of 400 μg/ml.

**Differential Analysis of Microarray.** Dual-color competitive microarray hybridization analysis was performed from digestive gland mRNA extracts using the mytox chip v1.1 as described previously (15). In brief, total RNA was isolated from 7721 and T7721 cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cy3-labeled dCTP and Cy5-labeled dCTP (Amersham Biosciences, Freiburg, Germany) were directly incorporated into cDNA probes in a reverse transcription reaction, and were then analyzed using signal transduction–related microarray (Capital Biochip Corp., Beijing, China).

**Transient Small Interfering RNA to T7721 Cells.** The sense sequence for HAb18G/CD147 small interfering RNAs (siRNAs) was 5′-GATCCGTCGTCAGAACACATCAACTTCAAGAGAGTTGATGTGTTCTGACGACTTTTTTGGAAA-3′, and the antisense sequence was 5′-AGCTTTTCCAAAAAAGTCGTCAGAACACATCAACTCTCTTGAAGTTGATGTGTTCTGACGACG-3′. HAb18G/CD147 siRNA and the control siRNA (Ambion, Austin, TX) were cloned into the *Eco*RI and *Hin*dIII sites of pSilence r3h1 vector (Ambion). The T7721 cells, when at 70% to 80% confluence, were transfected with siRNA at a final concentration of 100 n*M* using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

**Western Blot Analysis.** The expression of βig-h3 and HAb18G/CD147 were analyzed by Western blotting as described previously (17). In brief, cells were suspended in serum-free medium at a density of $5 \times 10^5$/ml, and 2 ml of the cell suspension was seeded into a six-well plate. After culture for 48 hrs, the conditioned medium was collected. BCA protein assay kit (Pierce Biotechnology, Rockford, IL) was employed to determine the total protein density, and equal amounts of proteins were separated on 10% sodium dodecyl sulfide–polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to polyvinylidene fluoride (PVDF) microporous membrane (Millipore, Boston, MA). After blocking with 5% nonfat milk, the membrane was incubated for 2 hrs at room temperature with the designated antibody. Immunodetection was performed by using the Western-Light chemiluminescent detection system (Applied Biosystems, Foster City, CA).

**Quantitative Real-Time Polymerase Chain Reaction (PCR).** For quantitative analysis of βig-h3 mRNA in human hepatoma cells 7721, T7721, and FHCC-98, and human novel hepatic cell QSG, the standard curve for quantifying mRNA copy number was established by amplifying nine aliquots of templates with known copy numbers (serial dilution from $10^8$ to $10^{10}$ copies). The specific βig-h3 cDNA was synthesized as follows: total RNA extracted as previously described was reverse transcribed into the first-strand cDNA using first-strand cDNA synthesis kit ReverTra Ace (Toyobo, Osaka, Japan). Forward and reverse primer sequences for βig-h3 gene are 5′-CATTGAGAACAGCTGCATCG-3′ and 5′-AGTCTGCTCCGTTCTCTTGG-3′, respectively. Amplification of cDNA templates was performed in a 50-μl reaction volume consisting of 10 m*M* Tris-HCl, pH 8.3; 50 m*M* KCl; 1.5 m*M* $MgCl_2$; 200 μ*M* deoxynucleotide triphosphates; 0.2 μ*M* forward and reverse primers, 1 μl cDNA template from the reverse-transcribed product, and 1 unit Taq DNA polymerase (TaKaRa, Shiga, Japan) with the following cycling parameters: initial denaturation at 94°C for 3 mins, followed by 32 cycles at 94°C for 45 secs, 57°C for 45 secs, and 72°C for 45 secs, finally extended at 72°C for 10 mins.

TaqMan probes for βig-h3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-CGCTTTAGCATGCTGGTAGCTGCCA-3′ and 5′-CCTCAACTACATGGTTTAC-3′. GAPDH was used as internal reference housekeeping gene to assess the status of sample mRNA. Forward and reverse primers used for amplification of human βig-h3 were 5′-TGGATGTCCTGAAGGGAGACA-3′ and 5′-TCTCCGTCAGTCCTGCAGACT-3′, respectively. Forward and reverse primers used for amplification of GAPDH are 5′-CCATCAATGACCCCTTCATT-3′ and 5′-CATGGGTGGAATCATAATGGAAC-3′, respectively. The probes were labeled with the reporter fluorochrome 6-carboxyfluorescein (FAM) at the 5′ end and the quencher fluorochrome 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3′ end. Cycling and quantification was performed in DNA Engine Opticon (MJ Research, Hercules, CA) following the cycling conditions: initial denaturation at 94°C for 3 mins, followed by 30 cycles at 94°C for 45 secs, 60°C for 45 secs, and 72°C for 45 secs, finally extended at 72°C for 10 mins.

**Cell Adhesion Assay.** The wells of the 96-well culture plate were coated with Matrigel (BD Bioscience, Franklin Lakes, NJ) at a concentration of 5 μg/ml and incubated at 4°C overnight. The coated wells were blocked with PBS containing 2% bovine serum albumin (BSA) for

30 mins and then washed with PBS. Cells in serum-free medium containing 0.1% BSA were added to the wells ($2 \times 10^4$/well) and incubated at 37°C, 5% $CO_2$ for 30 to 60 mins with or without antibodies specific for βig-h3 and CD147. After the medium and nonattached cells were removed, 0.2% crystal violet was added for 10 min. The plate was gently washed with tap water and dried in air for 24 hrs. Then 0.1 ml of 5% SDS with 50% ethanol was added for 20 mins, and then the plate was read at 540 nm.

**Cell Invasion Assay.** The chemotactic cell invasion assay was performed using 24-well Transwell units (Millipore) with an 8-μm pore size polycarbonate filter according to the method described by Mensing *et al.* (15). Each lower compartment of the Transwell contained 600 ml of 0.5% FBS as a chemoattractant in RPMI 1640. The upper side of a polycarbonate filter was coated with Matrigel (5 mg/ml in cold medium) to form a continuous thin layer. Prior to addition of the cell suspension, the dried layer of Matrigel matrix was rehydrated with medium without FBS for 2 hrs at room temperature. Cells ($1 \times 10^5$) in 0.1 ml RPMI 1640 containing 0.1% BSA were added into the upper compartment of the Transwell unit and incubated for 24 hrs at 37°C with or without antibodies specific for βig-h3 and CD147 in a humidified atmosphere containing 5% $CO_2$. Cells remaining in the upper compartment were completely removed with gentle swabbing. The number of cells that had invaded through the filter into the lower compartment was determined by hematoxylin-eosin stain (HE stain).

**Zymography Experiments.** To check the expression and activation of MMPs, cells incubated with or without antibodies specific for βig-h3 and HAb18G/CD147 were cultured in serum-free medium and incubated at 37°C for 5 to 20 hrs. The conditioned media were collected by centrifugation, concentrated, and dialyzed. The dialyzed samples containing an equal amount of total proteins were mixed with sample buffer, incubated in a water bath (~55°C) for about 3 to 5 mins, and loaded onto a zymographic SDS gel containing gelatin (1 mg/ml) as previously described (16). The gels were washed in 2.5% Triton X-100 for 15 mins twice and incubated in incubation buffer (50 m*M* Tris-HCl [pH 8.8] containing 5 m*M* $CaCl_2$ and 0.02% [w/v] $NaN_3$) for 16 hrs. The gels were stained with Coomassie brilliant blue and destained. The zones of gelatinolytic activity were shown by negative staining.

**Clone βig-h3 Gene and Transient Transfected to 7721 Cells.** To clone βig-h3 cDNA, poly(A)+ RNA was isolated from FHCC-98 cells, which express high levels of βig-h3, and then reverse transcribed into the first-strand cDNA using superscript II reverse transcriptase and oligo(dT) primer (NEB, Ipswich, MA). Human βig-h3 cDNA PCR amplified using high-fidelity MasterAmpm DNA polymerase (New England Biotechnology, Beverly, MA) and synthetic primers (5′-TAACTCGAGGCTTGCC-CGTCGGTCGCTAGCT-3′, 5′-ATCCGCGGGCCTC-CAAGCCACGTGTAGATGT-3′) was then cloned into the *Xho*I and *Sac*I sites of pEGFP-C2 expression vector (Invitrogen), which express green fluorescent protein (GFP). The recombinant plasmid was transfected into 7721 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The expression of the enhanced green fluorescent protein (EGFP) was detected under a fluorescent microscope (Olympus, Nagoya, Japan). Schizolysis transfected 7721 cells 48 hrs after transfection. Western blot was adopted using lysate of transfected cells, as described above.

## Results

**βig-h3 Is Upregulated in T7721 Cells, Which Stably Overexpress HAb18G/CD147.** In our previous study, the overexpression of HAb18G/CD147 was shown to be able to enhance metastasis potential and elevate MMP secretion when it was tranfected into human 7721 hepatoma cells, hence renamed T7721 cells. To identify pivotal molecules in the HAb18G/CD147 signal transduction pathway in human hepatoma cells, we screened the differentially expressed genes by cDNA microarray between 7721 cells and T7721 cells. According to the final data from Capital Biochip Corp., the screening standard for a differentially expressed gene was that the fluorescence intensity ratio of T7721 to 7721 should be either $>2.0$ or $<0.5$. The final data showed that there were 13 differentially expressed genes, in which the expression of 7 genes was upregulated in T7721 cells compared with 7721 cells. We found that the expression of the transforming growth factor-β (TGF-β)–induced molecule βig-h3 in T7721 cells was upregulated significantly—4.4-fold over that in 7721 cells—whereas the expression of TGF-β1 was not obviously changed in both types of cells (Fig. 1), indicating that overexpression of HAb18G/CD147 may, at least in part, result in the upregulation of the βig-h3 gene, which encodes a secretory extracellular matrix (ECM) protein involved in cell growth, tumorigenesis, wound healing, apoptosis, and migration (16–21).

**Downregulation of HAb18G/CD147 Depresses the Expression of βig-h3.** To further investigate whether HAb18G/CD147 regulates the expression of βig-h3, HAb18G/CD147-specialized siRNA was used to suppress the expression of HAb18G/CD147. T7721 cells transfected with HAb18G/CD147-specialized siRNA reduced the expression of HAb18G/CD147 protein by 80% (Fig. 2). This reduction of HAb18G/CD147 was accompanied by concomitant decreases of βig-h3 expression in T7721 cells. These results suggest that HAb18G/CD147 may be involved in mediating the expression of βig-h3.

**Expression of βig-h3 Is in Positive Correlation with HAb18G/CD147 in Different Human Hepatoma Cells and Normal Hepatic Cells.** It has been reported that HAb18G/CD147 is highly expressed in hepatoma cells, especially in hepatoma cells with high metastasis potential, whereas it is lowly expressed in normal hepatic cells (11). Our results above suggest that βig-h3 expression may be in

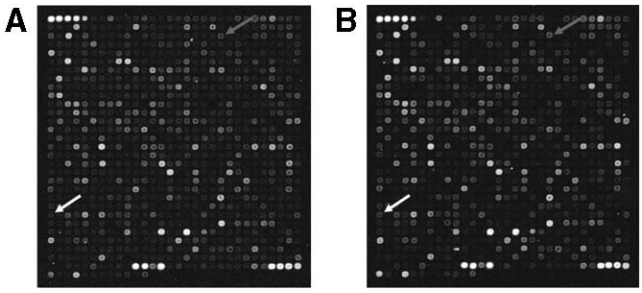


**Figure 1.** The overlay image of signal transduction-related microarray. (A) RNA of 7721 cells was directly labeled with Cy3 by reverse transcription, and RNA of T7721 cells was labeled with Cy5. The fluorescein labeled to RNA of 7721 and T7721 was opposite (B). The plots arrows point to present βig-h3 (row 24, column 1) and TGF-b1 (row 3, column 21).

positive correlation with HAb18G/CD147 expression in 7721 cells. To further confirm this conclusion, we tested the expression of HAb18G/CD147 in different human hepatoma cells and normal hepatic cells. Quantitative real-time PCR analysis results showed that the expression level of βig-h3 mRNA in hepatoma cells 7721, T7721, and FHCC-98 was higher than that in normal hepatic cells QSG. We also found that the βig-h3 mRNA level in FHCC-98 cells with high metastasis potential was much higher than that in 7721 cells with low metastasis potential (Fig. 3A). The results of Western blot showed that the expression level of βig-h3 protein was in accordance with the level of mRNA (Fig. 3B). These results further confirm that the expression of βig-h3 is in positive correlation with HAb18G/CD147 in hepatoma cells and suggest that βig-h3 may be related to invasion and metastasis process in human hepatoma cells.

**βig-h3 Enhances Hepatoma Cell Invasion and Metastasis Potential.** Given our finding that βig-h3 is highly expressed in human hepatoma cells, we overexpressed βig-h3 in 7721 cells by transiently transfecting recombinant plasmid pEGFP-C2/βig-h3. After 48 hrs, βig-h3 was highly expressed in transfected 7721 cells (Fig. 4).

As shown in Figure 5, the amount of attached cells observed in 7721 cells transfected with βig-h3, expressed as a percentage of total seeded cells, was 63.2% ± 2.8%, compared with 44.3% ± 1.6% in pEGFP-C2 vector–transfected 7721 cells ($P < 0.01$; Fig. 5A). Antibodies are useful tools for blocking or activation assay *via* binding with extracellular and transmembrane antigen or internalization. In the present study, the commercial polyclonal antibody for βig-h3 was introduced in the functional assay systems. The amount of attached cells was significantly decreased after treatment with antibodies specific for βig-h3 (29.1% ± 4.4%, $P < 0.01$). The ability of βig-h3–transfected 7721 cells to invade through Transwell chambers was greater than that of pEGFP-C2 vector–transfected 7721 cells: 42.9% ± 4.0% compared with 21.4% ± 3.1%, respectively ($P < 0.01$). Similarly, treatment with antibodies specific for βig-h3 decreased the invasion ability of βig-h3–transfected 7721 cells (15.2% ± 1.3%, $P < 0.01$; Fig. 5B).

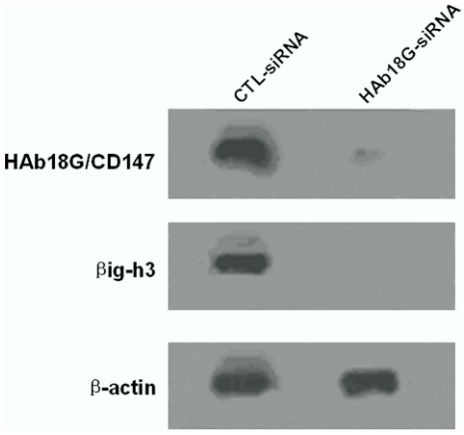


**Figure 2.** Downregulation of HAb18G/CD147 depresses the expression of βig-h3 protein in T7721 cells. T7721 cells were transfected with HAb18G/CD147-specific siRNA (HAb18G-siRNA) or control siRNA (CTL-siRNA) by Lipofectamine 2000 (Invitrogen). The βig-h3 protein expressions were determined by using Western blot analysis as described in Materials and Methods. Results are representative of triplicate experiments with similar results.

As shown in Figure 5C, MMP-2 and MMP-9 levels were enhanced by 62.3% ± 3.2% in βig-h3–transfected 7721 cells and were reduced by 40.8% ± 1.3% after treatment with antibodies specific for βig-h3 compared with 7721 cells transfected with pEGFP-C2 vector ($P < 0.01$). This finding suggests that βig-h3 may enhance invasion and metastasis potential of human hepatoma cells.

**βig-h3 Mediates HAb18G/CD147-Induced Metastasis of Human Hepatoma Cells.** To investigate the involvement of βig-h3 in the HAb18G/CD147-induced invasion and metastasis process in human hepatoma cells, we incubated T7721 cells with monoclonal antibodies specific for HAb18G/CD147 (developed in our lab), multiclonal antibodies specific for βig-h3 (Santa Cruz Biotechnology, Santa Cruz, CA), and both types of antibodies mixed to test the inhibitory effect on cell adhesion and invasion and MMP secretion.

Matrigel-coated plates and chambers were used to investigate the roles of βig-h3 and HAb18G/CD147 in metastasis-related processes in human hepatoma cells. The percentage of attached T7721 cells incubated with antibodies specific for βig-h3 and HAb18G/CD147 were significantly reduced (33.7% ± 8.7% and 28.3% ± 5.6%, respectively) compared with T7721 cells without either treatment (60.8% ± 11.3%; $P < 0.01$; Fig. 6A). Similar results also were observed in invasion tests. Compared with 29.7% ± 1.9% in T7721 cells without antibody treatment, the percentages of the invaded T7721 cells were 17.9% ± 2.4% in βig-h3 antibody group and 15.7% ± 1.4% in HAb18G/CD147 antibody group ($P < 0.01$; Fig. 6B).

Since MMPs are well known for promoting cancer metastasis by degradation of the extracellular matrix, the effect of βig-h3 and HAb18G/CD147 on metastatic potential could be mediated by MMPs. T7721 cells incubated with antibodies specific for βig-h3 and

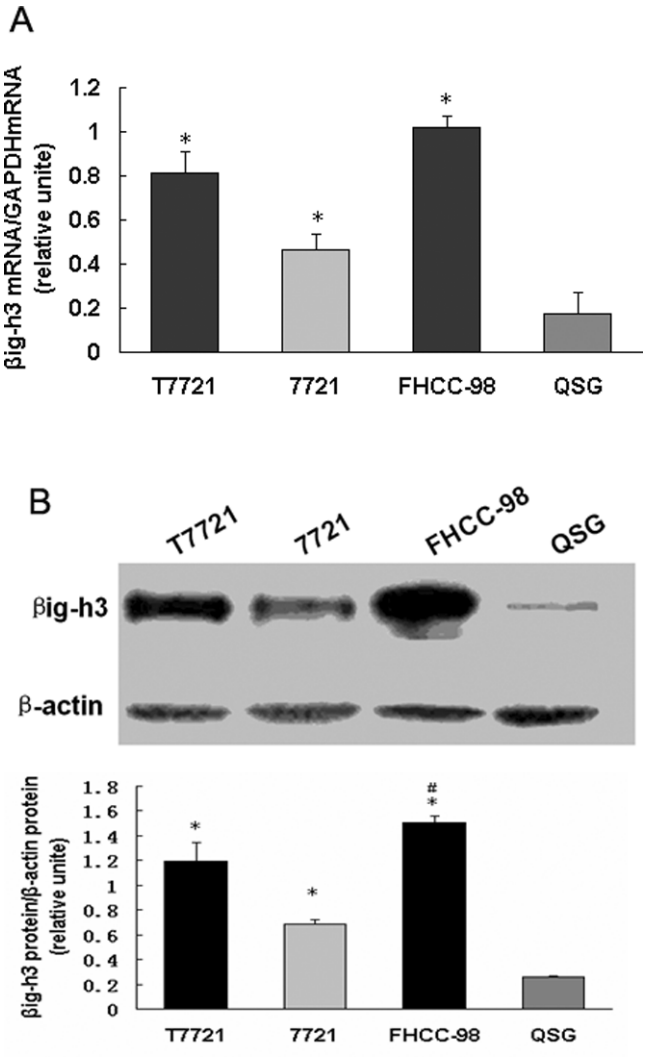


**Figure 3.** The mRNA (A) and protein (B) expressions of βig-h3 in different hepatoma cells and normal hepatic cells. The mRNA and protein expressions were tested using quantitative real-time PCR and Western blot analyses, respectively. The expression of βig-h3 mRNA was normalized by the expression of GAPDH mRNA, and βig-h3 protein was normalized by human β-actin expression. Data are shown as mean ± SE from three independent experiments. *$P <$ 0.01 vs. QSG cells; #$P <$ 0.01 vs. 7721 cells (Student's *t* test).

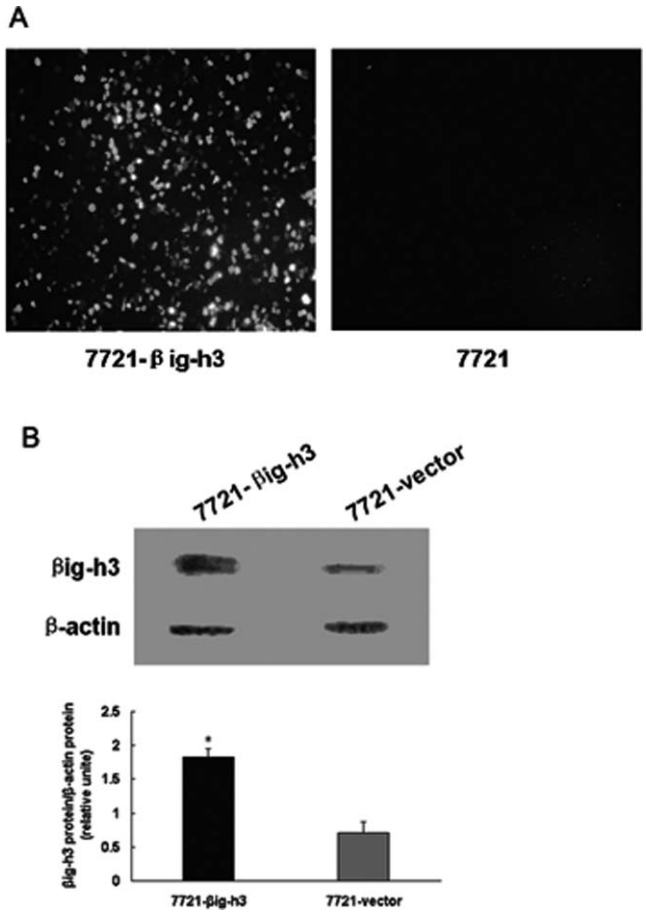


**Figure 4.** Intracellular expressions of the GFP (A) and protein expression of βig-h3 (B) in 7721 cells transfected with βig-h3. Cells were transfected with βig-h3 using a pEGFP-C2 expression vector (Invitrogen) as described in Materials and Methods. The expression of GFP was detected under a fluorescent microscope (Olympus). βig-h3 protein expressions were determined using Western blot analysis as described in Materials and Methods. βig-h3 protein was normalized by human β-actin expression and is shown as mean ± SE from three independent experiments. Cells transfected with the pEGFP-C2 vector alone served as control. *$P <$ 0.01 vs. pEGFP-C2 vector–transfected 7721 cells (Student's *t* test).

HAb18G/CD147 reduced MMPs levels by 40.8% ± 2.1% and 42.0% ± 5.5%, respectively, compared with T7721 cells without antibody treatment ($P < 0.01$; Fig. 6C).

The adhesion potential, invasion potential, and MMP levels of T7721 cells incubated with the two types of antibodies mixed were reduced compared with T7721 cells without antibody treatment, with the percentages of 27.0% ± 3.1% (adhesion potential), 14.6% ± 2.7% (invasion potential), and 51.5% ± 4.1% (MMP levels) for each ($P <$ 0.01). When comparing the groups treated with either antibody or both antibodies, we did not observe any significant difference in adhesion potential, invasion potential, or MMP seceration ($P > 0.05$). The results above indicate that βig-h3 might be involved in the progress of HAb18G/CD147-induced invasion and metastasis activity of human hepatoma cells.

## Discussion

Tumor growth and metastasis are multistep processes involving cell adhesion, proteolytic enzyme degradation of ECM, and motility factors that influence cell migration, in which the interaction between cells and ECM plays an essential role. The interaction of cells with ECM is mediated by contacts between cellular surface proteins and components of ECM, including laminin, fibronectin, and collagen (22). Invasion and metastasis are complicated and fatal cascades in human hepatoma, but the underlying molecular events remain largely unknown. Our previous studies implicated a hepatoma-associated antigen, HAb18G/CD147, in metastasis processes (11, 23, 24), and they

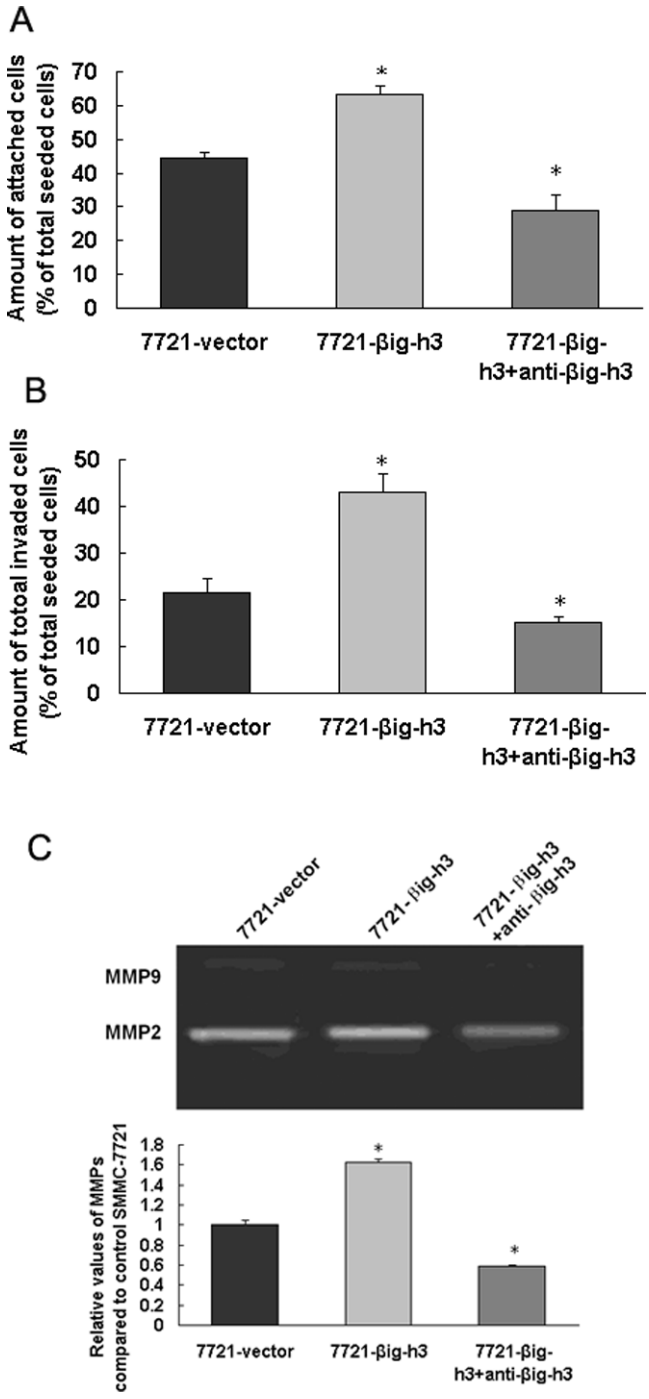


**Figure 5.** Adhesion potential (A), invasive potential (B), and MMP levels (C) of βig-h3–expressing 7721 cells incubated with or without antibodies specific for βig-h3. The invasion assay, invasive assay, and zymography experiment were adopted as described in Materials and Methods. Values are the means ± SE from six independent experiments. *$P < 0.01$ vs. pEGFP-C2 vector–transfected 7721 cells (Student's *t* test).

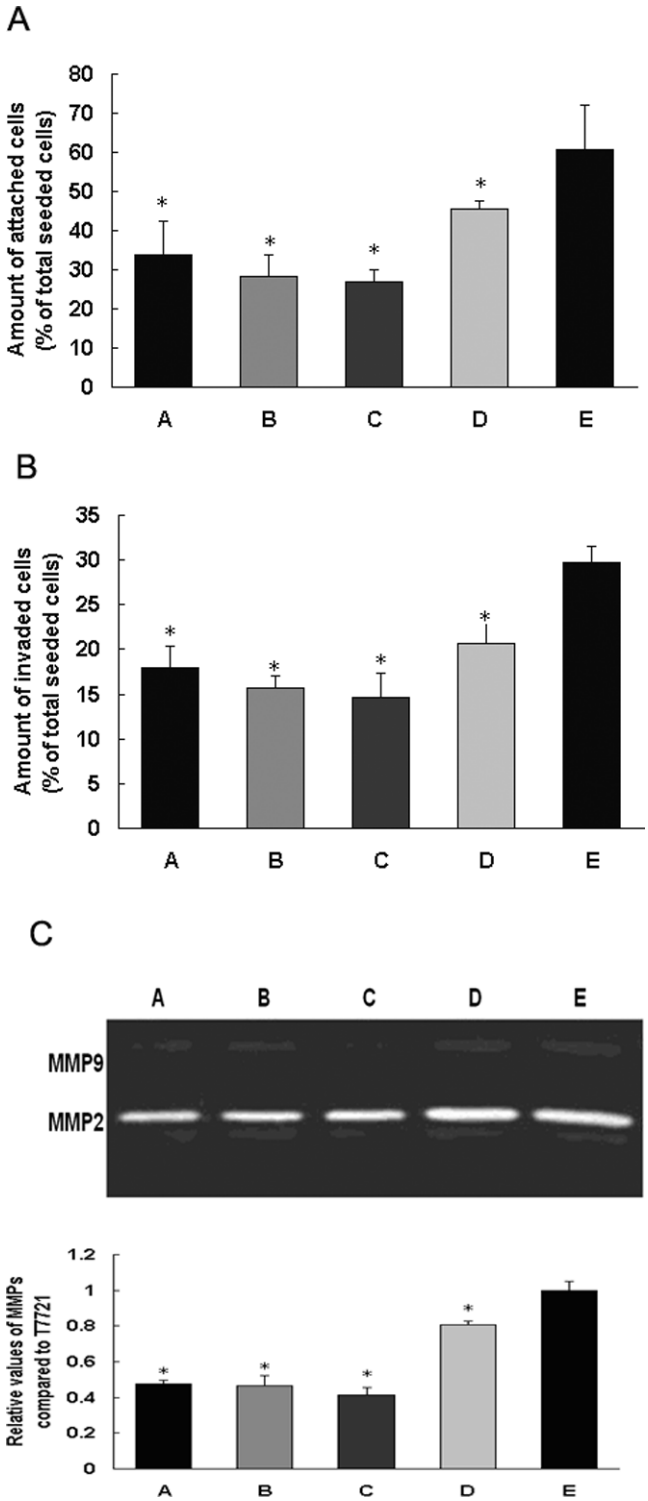


**Figure 6.** Adhesion potential (A), invasive potential (B), and MMP levels (C) of T7721 cells incubated with antibodies specific for βig-h3 (0.02 mg/ml), HAb18G/CD147 (0.1 mg/ml) and both types together. (A) T7721 + anti–βig-h3; (B) T7721 + anti-HAb18; (C) T7721 + anti–βig-h3 + anti-HAb18; (D) 7721; and (E) T7721. The invasion assay, invasive assay, and zymography experiment were adopted as described in Materials and Methods. Values are the means ± SE from six independent experiments. *$P < 0.01$ vs. T7721 cells (one-way ANOVA).

demonstrated the involvement of HAb18G/CD147 in the modulation of NO/cGMP-sensitive, store-operated $Ca^{2+}$ entry and the metastasis potential of human hepatoma cells (14, 25). However, ligands or receptors for HAb18G/CD147 remain obscure, and whether HAb18G/CD147 directly

interacts with extracellular molecules to execute its function is still unknown.

The present study demonstrated that expression levels of βig-h3, a secretory ECM protein, were positively relative to the expression of HAb18G/CD147 by cDNA microarray, quantitative real-time PCR, Western blot, and siRNA assay. The results also showed that the expression level of βig-h3 in hepatoma cells was much higher than that in normal hepatic cells, and in hepatoma cells with high metastasis potential it was much higher than that in hepatoma cells with low metastasis potential, which was coincident with the expression of HAb18G/CD147. This suggested that the expression of βig-h3 was mediated by HAb18G/CD147, and βig-h3 might be in involved in mediating the metastasis processes of hepatoma cells.

The regulatory mechanisms of the expression of βig-h3 are still obscure. βig-h3, first identified from the A549 lung adenocarcinoma cell line after long-term treatment by TGF-β1, could be similarly induced in H2981 cells (lung adenocarcinoma cell line), HEPM cells, human melanoma cells, mammary epithelial cells, keratinocytes, and fibroblasts. However, it has been reported that the same induced results were not observed in 293S cells (embryo kidney cell lines) and MCF-7, MDA453, and MDA468 cells (breast carcinoma cell lines; Refs. 16, 26). In the present study, the results of cDNA microarray indicated that βig-h3 was upregulated in T7721 cells compared with 7721 cells, but the expression of TGF-β1 had no obvious changes in the above two cell lines, suggesting that the upregulation of βig-h3 is not a universal result of TGF-β1 treatment.

It has been reported that CD147 stimulates the secretion of MMP-1 by activating a p38-dependent signaling pathway. This is the first available information regarding the mechanism by which tumor-associated molecules upregulate MMP synthesis in stromal fibroblasts. p38 is the only one of three major MAP kinases (ERK1/2, SAPK/JNK, and p38) that undergoes phosphorylation (an index of activation) in response to CD147 (27). So we supposed that HAb18G/CD147 regulates the expression of several proteins, such as MMP and βig-h3, possibly *via* a MAPK-dependent signaling pathway. Expression, localization, and the intracellular signal mechanism of βig-h3 were not yet well investigated. Studies to determine the signaling molecules in HAb18G/CD147 regulated βig-h3 expression pathway are in progress.

βig-h3, involved in cell growth, migration, apoptosis, wound healing, and tumorigenesis (16, 28–33), might function as either a promoter or an inhibitor of carcinogenesis, depending on cells and tumor types. The gain or loss of expression of βig-h3 might be involved in tumor formation and the acquisition of a metastatic phenotype in human cancer. Analysis of cultured human bronchial smooth muscle cells identified βig-h3 in both the ECM and the cytoplasm, and also in the nucleus, which suggests that it may have other as yet unknown functions in addition to its role as a structural ECM component. One possible explanation for nuclear localization is that the protein is secreted and subsequently taken up by cells, perhaps by receptor-mediated endocytosis. Alternatively, the protein may have multiple isoforms that potentially could be targeted to specific compartments; some isoforms are secreted and deposited in the ECM, whereas others lacking the signal peptide are translocated to the nucleus, transferring signals or regulating gene transcription (34). To detect its functions in human hepatoma carcinoma cells, we cloned βig-h3 without the signal sequence into a GFP vector. The present results obtained from assays for cell adhesion and invasion processes involved in metastasis indicated a greater metastatic potential of the βig-h3–expressing 7721 cells compared with 7721 cells transfected with vector as control. The fact that cell attachment and invasion could be greatly inhibited by antibody for βig-h3 further confirmed the results. MMPs have been implicated in several aspects of tumor progression, including invasion through basement membrane and interstitial matrices, angiogenesis, and tumor cell growth (35, 36). Our data obtained from a gelatin zymography assay showed that βig-h3 promoted MMP secretion, and the βig-h3 special antibody inhibited this stimulated effect. All of the results clearly demonstrated that βig-h3 as a promoting factor possibly enhances hepatoma cell invasion and metastasis potential.

Previous studies have demonstrated that HAb18G/CD147 promotes the invasive and metastatic ability of human hepatoma cells (7, 11), so what is the relationship between HAb18G/CD147 and βig-h3 in promoting invasion and metastasis potential? The phenomenon that the antibody specialized for βig-h3 inhibited HAb18G/CD147-induced invasion and metastasis potential can partly explain the question. The similar inhibitory effects on cell attachment and invasion and MMP secretion also were observed among T7721 cells incubated with antibodies for βig-h3, HAb18G/CD147, or both types together. These results indicated that βig-h3 may be involved in HAb18G/CD147-induced cell invasion and metastatic progress; that is, they cooperate in one signal transduction pathway promoting hepatoma cell metastasis.

Integrins are cell surface adhesive receptors composed of α- and β-chain heterocomplexes, which mediate the physical and functional interactions between a cell and its surrounding ECM, thus serving as bidirectional transducers of extracellular and intracellular signals in the processes of cell adhesion, proliferation, differentiation, apoptosis, and tumor progression (37). Berditchevski *et al.* documented that CD147 may form complexes with integrin $\alpha_3\beta_1$ (37). Conversely, caveolin-1 may associate with CD147 from CD147-$\alpha_3$ integrin complexes, thereby diminishing both CD147 clustering and CD147-dependent, MMP-1–inducing activities (24). Moreover, βig-h3, which contains an amino terminal signal peptide, four internal FASI repeat domains, and an RGD (Arg-Gly-Asp) motif, may interact with $\alpha_3\beta_1$, $\alpha_1\beta_1$, $\alpha_6\beta_4$, $\alpha_v\beta_3$, or $\alpha_v\beta_5$ in various tumor cells (30, 31, 33,

38, 39). These findings shed new light on the opportunity for integrin (especially $\alpha_3\beta_1$) to act as a bridge between HAb18G/CD147 and βig-h3 to form a trimer, and then regulate HAb18G/CD147-induced metastasis of human hepatoma cells. The investigation of the relationship of HAb18G/CD147, βig-h3, and integrin is underway in our laboratory.

In summary, all the results suggest that βig-h3, the expression of which is regulated by HAb18G/CD147, may be involved in the HAb18G/CD147 signal pathway, and it plays important roles in invasion and metastatic processes in human hepatoma cells, although the precise mechanism calls for further attention.

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1. Guo H, Majmudar G, Jensen TC, Biswas C, Toole BP, Gordon MK. Characterization of the gene for human EMMPRIN, a tumor cell surface inducer of matrix metalloproteinases. Gene 220:99–108, 1998.
2. Igakura T, Kadomatsu K, Kaname T, Muramatsu H, Fan QW, Miyauchi T, Toyama Y, Knuo N, Yuasa S, Takahashi M, Senda T, Taguchi O, Yamamura K, Arimura K, Muramatsu T. A null mutation in basigin, an immunoglobulin superfamily member, indicates its important roles in peri-implantation development and spermatogenesis. Dev Biol 194:152–165, 1998.
3. Guo H, Li R, Zucker R, Toole BP. EMMPRIN (CD147), an inducer of matrix mellatoproteinase synthesis, also binds interstitial collagenase to the tumor cell surface. Cancer Res 60:888–891, 2000.
4. Wang XH, Chen ZN, He SY, Wang XQ. Immunohistochemical study of the colocalization of CD147 and MMP-2 in human hepatocellular carcinoma cell line. Chin J Histochem Cytochem 10:270–272, 2001.
5. Kanekura T, Chen X, Kanzaki T. Basigin (CD147) is expressed on melanoma cells and induces tumor cell invasion by stimulating production of matrix metalloproteinases by fibroblasts. Int J Cancer 99:520–528, 2002.
6. Saxena DK, Oh-Oka T, Kadomatsu K, Muramatsu T, Toshimori K. Behaviour of a sperm surface transmembrane glycoprotein basigin during epididymal maturation and its role in fertilization in mice. Reproduction 123:435–444, 2002.
7. Ding NZ, He CQ, Yang ZM. Quantification of basigin mRNA in mouse oocytes and preimplantation embryos by competitive RT-PCR. Zygote 10:239–243, 2002.
8. Sun J, Hemler ME. Regulation of MMP-1 and MMP-2 production through CD147/extracellular matrix metalloproteinase inducer interations. Cancer Res 6:2275–2281, 2001.
9. Polette M, Gilles C, Marchand V, Lorenzato M, Toole BP, Tournier JM, Zucker S, Birembaut P. Tumor collagenase stimulatory factor (TCSF) expression and localization in human lung and breast cancers. J Histochem Cytochem 45:703–709, 1997.
10. Muraoka K, Nabeshima K, Murayama T, Biswas C, Koono M. Enhanced expression of a tumor-cell-derived collagenase-stimulatory factor in urothelial carcinoma: its usefulness as a tumor marker for bladder cancers. Int J Cancer 55:19–26, 1993.
11. Chen ZN, Yang Z, Mi L, Jiang JL, Guo XN. Structure and function analysis of hepatoma associated factor HAb18G. Chin J Cell Mol Immunol 15:34–39, 1999.
12. Seulberger H, Lottspeich, Risau W. The inducible blood-brain barrier specific molecule HT7 is a novel immunoglobulin-like cell surface glycoprotein. EMBO J 9:2151–2158, 1990.
13. Fossum S, Mallett S, Barclay AN. The MRC OX-47 antigen is a member of the immunoglobulin superfamily with an unusual transmembrane sequence. Eur J Immunol 21:671–679, 1991.
14. Jiang JL, Zhou Q, Yu MK, Ho LS, Chen ZN, Chan HC. The involvement of HAb18G/CD147 in regulation of store-operated calcium entry and metastasis of human hepatoma cells. J Biol Chem 276:46870–46877, 2001.
15. Mensing H, Albini A, Krieg T, Pontz BF, Muller PK. Enhanced chemotaxis of tumor-derived and virus transformed cells to fibronectin and fibroblast-conditioned medium. Int J Cancer 33:43–48, 1984.
16. Skonier J, Bennett K, Rothwell V, Kosowski S, Plowman G, Wallace P, Edelhoff S, Disteche C, Neubauer M, Marquardt H, Rodgers J, Purchio AF. βig-h3: a transforming growth factor-b-responsive gene encoding a secreted protein that inhibits cell attachment in vitro and suppresses the growth of CHO cells in nude mice. DNA Cell Biol 13: 571–584, 1994.
17. Escribano J, Hernando N, Ghosh S, Crabb J, Coca-Prados M. cDNA from human ocular ciliary epithelium homologous to βig-h3 is preferentially expressed as an extracellular protein in the corneal epithelium. J Cell Physiol 160:511–521, 1994.
18. Billings PC, Herrick DJ, Kucich U, Engelsberg BN, Abrams WR, Macarak EJ, Rosenbloom J, Howard PS. Extracellular matrix and nuclear localization of βig-h3 in human bladder smooth muscle and fibroblast cells. J Cell Biochem 79:261–273, 2000.
19. Bron AJ. Genetics of the corneal dystrophies: what we have learned in the past twenty-five years. Cornea 19:699–711, 2000.
20. Dighiero P, Ellies P, Valleix S, D'Hermies F, Savoldelli M, Pouliquen Y, Legeais JM, Renard G. Avellino dystrophy, current diagnostic criteria. J Fr Ophtalmol 23:735–737, 2000.
21. Dighiero P, Valleix S, D'Hermies F, Savoldelli M, Pouliquen Y, Legeais JM, Renard G. Clinical, histologic, and ultrastructural features of the corneal dystrophy caused by the R124L mutation of the BIGH3 gene. Ophthalmology 107:1353–1357, 2000.
22. Konishi M, Yamada M, Nakamura Y, Mashima Y. Immunohistology of kerato-ephithelin in corneal stromal dystrophies associated with R124 mutation of the BIGH3 gene. Curr Eye Res 21:891–896, 2000.
23. Lim M, Martinez T, Jablons D, Cameron R, Guo H, Toole B. Tumor-derived EMMPRIN (extracellular matrix metalloproteinaseinducer) stimulates collagenase transcription through MAPK p38. FEBS Lett 441:88–92, 1998.
24. Tang W, Hemler ME. Caveolin-1 regulates matrix metalloproteinases-1 induction and CD147/EMMPRIN cell surface clustring. J Bio Chem 279:11112–11118, 2004.
25. Jiang JL, Chan HC, Zhou Q, Yu MK, Yao XY, Lam SY, Zhu H, Ho LS, Leung KM, Chen ZN. HAb18G/CD147-mediated calcium mobilization and hepatoma metastasis require both C-terminal and N-terminal domains. Cell Mol Life Sci 61:2083–2091, 2004.
26. Skonier J, Neubauer M, Madisen L, Bennett K, Plowman GD, Purchio AF. cDNA cloning and sequence analysis of βig-h3, a novel gene induced in a human adenocarcinoma cell line after treatment with transforming growth factor-beta. DNA Cell Biol 11:511–522, 1992.
27. Lim M, Martinez T, Jablons D, Cameron R, Guo H, Toole B, Li JD, Basbaum C. Tumor-derived EMMPRIN (extracellular matrix metalloproteinase inducer) stimulates collagenase transcription through MAPK p38. FEBS Lett 441:88–92, 1998.
28. Rawe IM, Zhan Q, Burrows R, Bennett K, Cintron C. Beta-ig molecular cloning and in situ hybridization in corneal tissues. Invest Ophthalmol Vis Sci 38:893–900, 1997.
29. Kim JE, Kim EH, Han EH, Park RW, Park IH, Jun SH, Kim JC, Young MF, Kim IS. A TGF-beta-inducible cell adhesion molecule, βig-h3, is downregulated in melorheostosis and involved in osteogenesis. J Cell Biochem 77:169–178, 2000.
30. Thapa N, Kang KB, Kim IS. βig-h3 mediates osteoblast adhesion and inhibits differentiation. Bone 36:232–242, 2005.
31. Bae JS, Lee SH, Kim JE, Choi JY, Park RW, Park JY, Park HS, Sohn YS, Lee DS, Bae LE, Kim IS. βig-h3 supports keratinocyte adhesion,

migration, and proliferation through α3β1 integrin. Biochem Biophys Res Commun 294:940–948, 2002.

32. Kim JE, Kim SJ, Jeong HW, Lee BH, Choi JY, Park RW, Park JY, Kim IS. RDG peptides released from βig-h3, a TGF-beta-induced cell-adhesive molecule, mediate apoptosis. Oncogene 22:2045–2053, 2003.
33. Park SW, Bae JS, Kim KS, Park SH, Lee BH, Choi JY, Park JY, Ha SW, Kim YL, Kwon TH, Kim IS, Park RW. βig-h3 promotes renal proximal tubular epithelial cell adhesion, migration and proliferation through the interaction with alpha3beta1 integrin. Exp Mol Med 36: 211–219, 2004.
34. Billings PC, Herrick DJ, Howard PS, Kucich U, Engelsberg BN, Rosenbloom J. Expression of βig-h3 by human bronchial smooth muscle cells: localization to the extracellular matrix and nucleus. Am J Respir Cell Mol Biol 22:352–359, 2000.
35. Ogata R, Torimura T, Kin M, Ueno T, Tateishi Y, Kuromatsu R, Shimauchi Y, Sakamoto M, Tamaki S, Sata M, Tanikawa K. Increased expression of membrane type 1 matrix metalloproteinase and matrix metalloproteinase-2 with tumor dedifferentiation in hepatocellular carcinomas. Hum Pathol 30:443–450, 1999.
36. Theret N, Musso O, Turlin B, Lotrian D, Bioulac-Sage P, Campion JP, Boudjema K, Clement B. Increased extra-cellular matrix remodeling is associated with tumor progression in human hepatocellular carcinomas. Hepatology 34:82–88, 2001.
37. Berditchevski F, Chang S, Bodorova J, Hemle ME. Generation of Monoclonal Antibodies to Integrin-associated Proteins. Evidence that $\alpha_3\beta_1$ complexes with EMMPRIN/basigin/OX47/M6. J Biol Chem 272: 29174–29180, 1997.
38. Ohno S, Noshiro M, Makihira S, Kawamoto T, Shen M, Yan W, Kawashima-Ohya Y, Fujimoto K, Tanne K, Kato Y. RGD-CAP (βig-h3) enhances the spreading of chondrocytes and fibroblasts via integrin α1β1. Biochim Biophys Acta 1451:196–205, 1999.
39. Kim MO, Yun SJ, Kim IS, Sohn S, Eunjoo HL. Transforming growth factor-β-inducible gene-h3 (βig-h3) promotes cell adhesion of human astrocytoma cells in vitro: implication of $\alpha_6\beta_4$ integrin. Neurosci Lett 336:93–96, 2003.