

Enhanced expression of trophinin promotes invasive and metastatic potential of human gallbladder cancer cells

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Abstract

Purpose To study effects of trophinin on the metastatic potential of human gallbladder cancer cells and its potential mechanism.

Materials and methods Expression of trophinin in the highly metastatic GBC-SDH_i cells was investigated by real time RT-PCR and western blot. Recombinant expression plasmid vector of the human trophinin gene was constructed and transfected into GBC-SD cells. Effects of trophinin on the invasion of GBC-SD cells were investigated by adhesion assay and invasion assay in vitro. The siRNA was used to down-regulate the expression of trophinin. Some genes related to the invasion and metastasis of cancer were determined by real time RT-PCR and western blot. The pulmonary metastasis regulated by trophinin was determined in the nude mice.

Results Overexpression of trophinin in GBC-SDH_i cells was confirmed compared with its parental counterparts. Up-regulation of trophinin enhanced the in vitro invasion in the GBC-SD/TRO cells. The enhancement was associated with

increasing integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1 expression. The results were further demonstrated by RNA interference experiment in vitro. In in vivo study, we also demonstrated that trophinin-transfected gallbladder cancer cells had more pulmonary metastases than the vector-transfected one or its parental counterparts.

Conclusion Overexpression of trophinin leads to a more invasive phenotype and metastatic potential in human gallbladder cancer, at least in part, through regulating integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1 expression.

Keywords Gallbladder cancer · Invasion · Metastasis · Trophinin

Introduction

Gallbladder cancer is one of the commonly diagnosed malignancies in bile duct system. Although steady progress has been achieved in clinical treatment, the prognosis of the gallbladder cancer is very poor. Because of the absence of characteristic early symptoms, the majority of cases are diagnosed at a late stage when most patients already have occult or overt metastasis.

Although a number of molecules have been implicated in the complex process of cancer metastasis, the precise mechanisms promoting gallbladder cancer metastasis still remain unclear to date. The poor understanding of the molecular mechanisms is due, in part, to the lack of ideal gallbladder cancer cell line and animal model for study. Cancer cell lines provide an important resource for cancer gene discovery as well as functional studies. Several established human gallbladder cancer cell lines are available. However, most of these were derived from various histological sources with different genetic background, and are

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polyclonal and composed of cell populations that are heterogeneous in metastatic phenotype, making them difficult to use as models in identifying critical metastasis-related genes. The GBC-SD cell line is a novel human gallbladder cancer cell line. The animal models of the cell line also have been developed and characterized by our institute. In the previous study, we have isolated GBC-SDH_i cell clone with high invasive phenotype in vitro was fished out (Chang et al. 2007c). The invasive phenotype and metastatic potential of GBC-SDH_i were confirmed in a surgical orthotopic implantation model of gallbladder cancer in nude mice. The differences in metastatic potential of the genetically related cell lines characterized in this model make it a valuable system for understanding the biology of gallbladder cancer metastasis.

Trophinin (TRO) is a membrane protein that potentially mediates the initial adhesion between human embryo and uterine epithelial cells through a unique apical cell adhesion (Fukuda and Nozawa 1999; Fukuda et al. 1995; Suzuki et al. 1998). The processes of human embryo implantation, which include rapid proliferation and invasion of trophoblasts, are similar to the aggressive behaviors of malignant cancer cells. Hatakeyama et al. (2004) observed that trophinin could enhance invasiveness of the cells and promotes metastasis of testicular germ cell tumor. Chen et al. (2007) also identified trophinin as an enhancer for cell invasion and a novel prognostic factor for early stage lung cancer. However, the role of trophinin in gallbladder cancer metastasis is unclear.

Based on these findings of the trophinin protein, we hypothesized that increased trophinin expression may be involved in gallbladder cancer development and metastasis. To test this hypothesis, expression of trophinin in the highly metastatic GBC-SDH_i cells was investigated and a trophinin high-expression cells by reintroduced human trophinin cDNA into GBC-SD cells was generated. Results showed that trophinin gene enhances the invasive and metastatic potential of GBC-SD cells in vitro and in vivo, accompanied by up-regulation of integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1 expression. These results were further supported by the data obtained from RNAi experiments in vitro. These data provide functional evidence that trophinin may be a novel mediator of metastasis promotion in human gallbladder cancer.

Materials and methods

Cell line and animal

The human gallbladder cancer cell line GBC-SD (obtained from Professor Wang in Department of General Surgery, Qilu Hospital of Shandong University in P. R. China) was

maintained in a RPMI 1640 containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. The medium was changes every 2–3 days, and cells were harvested by treatment with 0.25% trypsin/0.53 mM EDTA solution in a laminar flow hood during their logarithmic phase of growth. All culture medium components were obtained from Gibco BRL (Grand Island, NY). BALB/c-*nu/nu* nude mice, 4 weeks old, were obtained from Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China), and housed in laminar flow cabinets under specific pathogen-free conditions with food and water ad libitum. All experiments on mice were conducted in accordance with the guidelines of NIH for the Care and Use of Laboratory Animals. The study protocol was also approved by Guangdong Medical Experimental Animal Care Committee.

Trophinin expression

Real time RT-PCR analysis was performed to detect the mRNA expression of trophinin in GBC-SDH_i and GBC-SD cells. Total RNA was extracted from cultured GBC-SDH_i and GBC-SD cells using Trizol reagent (Invitrogen, San Diego, CA) according to the manufacturer's instructions and quantified spectrophotometrically at 260 and 280 nm. After reverse transcription, the products and the primers were used for real-time quantitative PCR of trophinin. (Upstream primer: 5'-AGGGAAGAGTTAGGCGATGAT-3', downstream primer: 5'-TTGGGCTCTGGCCTCAATT-3', 69 bp) The reaction was performed using the DNA Engine Opticon™ 2 real time RT-PCR detection system (MJ Research, USA) with SYBR Green I. HPRT1 was chosen as internal control. Each experiment was performed in 25 μ l of reaction volume including 1.25 μ l of 20 \times SYBR Green, 1 μ l of first-strand cDNA (50 ng RNA), 2 μ l of each 5 nmol primer, 0.15 μ l Hotstar Taq DNA polymerase, 2.5 μ l of 10 \times amplification buffer, 2.5 μ l of 25 mmol/l MgCl₂, 0.5 μ l of 10 mmol/l solution of dNTP mixture, and 15.1 μ l of dH₂O. In the last tube, 1 μ l of ddH₂O was added as a nontemplate control. The conditions of amplification cycles were as follows: 40 cycles consisting of denaturation at 94°C for 20 s, annealing at 58°C for 20 s, and extension at 72°C for 20 s. The Opticon 2 apparatus was used to measure the fluorescence of each sample in every cycle at the end of the extension, and the comparative threshold cycle ($2^{-\Delta\Delta C_T}$) method was used to allow the quantification of the mRNA of the gene. For each sample, results were normalized against HPRT1. All samples were run in triplicate. After PCR, a melting curve was obtained by increasing the temperature from 65 to 95°C with a temperature transition rate of 0.1°C/s. The melting curves of all final PCR products were analyzed. The differences in melting temperature

of PCR products were allowed to distinguish genuine products from nonspecific products and primer dimers. To ensure that the correct product was amplified in the reaction, all samples were also separated on 1.2% agarose gel electrophoresis. All PCR conditions and primers were optimized to produce a single product of the correct size.

Western blot analysis was performed to detect the protein expression of trophinin. Protein was extracted from the cultured cells using ice-cold modified RIPA lysis buffer containing 50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mM Na_3VO_4 , and 1 mM NaF (all protease and phosphatase inhibitors from Sigma-Aldrich Co., St. Louis, MO), and then quantitated with the bicinchoninic acid (BCA) assay kit with BSA as a standard (Pierce, Rockford, IL). Equal amounts of protein (50 μg) from different cells were separated by 10% SDS-PAGE and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). After treating with 5% nonfat dry milk in $1 \times$ TBST (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) for 1 h at room temperature, the membranes were then incubated for 2 h at room temperature with mouse anti-human monoclonal antibodies against trophinin (from Dr. Fukuda) in TBST containing 1% nonfat dry milk followed by horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 h at room temperature. Target proteins were detected by enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech) and exposure to Biomax ML film (Eastman Kodak, Rochester, NY). Images were captured by Alpha Image 950 documentation system and analyzed by NIH Image version 1.62. Relative protein in different cell lines was normalized to the signal intensity of β -actin as an internal control.

Stable transfected clone selection

Human trophinin expression vector was constructed using pcDNATM3.1 Directional TOPO[®] Expression Kit (Invitrogen, San Diego, CA) according to the published method with some modifications (Chang et al. 2007b). To construct the human trophinin expression vector, the entire open reading frame (ORF) of human trophinin gene was amplified from recombinant plasmid vector in which the full-length trophinin gene had been inserted (from Dr. Fukuda) by PCR using high-fidelity Platinum[®] Taq DNA Polymerase. The resulting PCR fragment for subcloning was purified from agarose gels using 3S Spin DNA Agarose Gel Purification Kit (Shanghai Shenneng Bocai Biological Science & Technology Company Ltd., Shanghai, China) according to the manufacturer's instructions. The TOPO[®] cloning reaction in a total volume of 6 μl including 3 μl

PCR product, 1 μl Salt Solution, 1 μl Sterile water and 1 μl TOPO[®] vector was mixed gently and incubated for 10 min at room temperature. Then the cloning reaction was placed on ice and proceeded to be transformed into competent *E. coli*. The resulting construct was named as pcDNA3.1D/TRO.

The recombinant vector was identified by automated sequencing analysis. To determine the effects of trophinin on the invasive and metastatic potential of GBC-SD cells, cells were transfected with either resultant constructs or empty vector by using Lipofectamine[™] 2000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions, and selected in the presence of 800 $\mu\text{g}/\text{ml}$ Geneticin (G418 sulfate; Invitrogen) for 4 weeks. The trophinin-positive colonies were identified by RT-PCR and Western blot analysis. In this study, the clone in which trophinin gene was successfully transfected was named as GBC-SD/TRO clones (two positive clones were selected, named TRO-1 and TRO-2, respectively). The one only transfected with pcDNA3.1D vector was named as GBC-SD/vector. For all functional and biological assays, cells between 70 and 90% confluence were used with viability >95%.

Expression analysis of trophinin transfectants

For real time RT-PCR analysis, total RNA isolated from the control and trophinin transfected cells were reverse-transcribed, and the human trophinin products were amplified by real time RT-PCR according to the methods as above described. HPRT1 was amplified as an internal control.

For western blot analysis, proteins extracted from the control and trophinin transfected cells were separated by 10% SDS-PAGE and then transferred onto PVDF membranes. After incubated with monoclonal antibodies against trophinin, target proteins were detected according to the method as above described. Relative proteins in different cells were normalized to β -actin as an internal control.

Adhesion assay

A total of 2 μg matrigel was coated in each well of 96-well culture plate and 20 μl medium containing 3% bovine serum albumin was added to the well. Then the well were incubated at 37°C for 1 h and washed twice with warm PBS. A total of 100 μl cell suspension that contained 5×10^4 cells of different clones were added to each well. After incubated for 1 h, the wells were washed twice with PBS. We used the wells that were seeded corresponding cells 6 h ago as the control. The Cell Counting Kit-8 (CCK8) was used to detect the optical density (OD) value of each well. The adhesive rate was calculated according to the following formula: Rate = (the trial OD/the control OD) \times 100%.

In vitro invasion assays

In vitro invasion assays were performed to analyze the invasive potential of parental, vector, and trophinin transfected cells with Matrigel invasion chamber (Becton Dickinson Labware, Bedford, MA) as described previously with some modifications (Chang et al. 2007a). Each well insert was coated with 100 μ l of a 1:3 dilution of Matrigel in serum-free culture medium. Then, a mixture of 200 μ l medium with 10% FBS, 200 μ l supernatant of corresponding cell culture, and 200 μ l supernatant of NIH/3T3 cell culture was added to the lower chambers as a chemoattractant, and 1×10^5 cells in 250 μ l of serum-free medium were added to the top of this Matrigel layer. The cells were incubated at 37°C for 24 h. The cell suspension was aspirated, and excess Matrigel was removed from the filter using a cotton swab. Then, the filters were fixed in 10% formalin and stained with H&E. Cells which had invaded through the Matrigel and reached the lower surface of the filter were counted under a light microscope at a magnification of $\times 200$. Five fields selected randomly should be counted for each sample.

Real time RT-PCR and western blot analysis

The mRNA and protein expression of matrix metalloproteinase-1 (MMP-1), MMP-2, MMP-7, MMP-9, transcriptional factor Ets-1, tissue inhibitor of MMP-1 (TIMP-1), TIMP-2, urokinase-type plasminogen activator (uPA), uPA receptor (uPAR), cathepsin D, maspin, cystatin C, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), RhoC, P21, IGF-1, IGF-1R, IGF-2, Cyclin A, Cyclin D1, Cyclin E, E-cadherin, C-jun, C-fos, Tropomyosin 4 (TPM4), and TGF α in the control cells and trophinin transfected cells were determined by real time RT-PCR and western blot analysis, respectively, as described previously. All primary antibodies and HRP-conjugated secondary antibodies for western blot analysis were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Amer-

sham Pharmacia Biotech (Arlington Heights, IL), respectively. All experiments were performed in triplicate.

RNAi experiments

To further demonstrate the role for the trophinin gene in the progression of human gallbladder cancer, we used the RNAi technique to down-regulate the trophinin gene expression. Three chemical synthesized siRNAs were purchased from Shanghai GeneChem Co., Ltd (Shanghai, China). The siRNA sequences for trophinin are: T1 (5'-CGAGACUAGCAAGAUGAAAtt-3'), T2 (5'-CCACAGAA GAGGACAGUGUtt-3'), and T3 (5'-GCCCAAAUUAAC UUGGCAGtt-3') according to the published paper. (Chen et al., 2007) The siRNAs were transfected into subconfluent GBC-SDH_i cells using Lipofectamine™ 2000 reagent following the manufacturer's recommended protocol. The knockdown level of trophinin gene was analyzed by real time RT-PCR and western blot methods as described above. After 48 h of post-transfection, the effects of reduced trophinin expression on the invasion capacity and expression of integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1 in GBC-SDH_i cells were determined by in vitro invasion assay, real time RT-PCR and western blot analysis, respectively, as described previously. The primers were designed using Primer 3 software (Table 1). All experiments were performed in duplicate.

Metastasis assays in nude mice

Cells were harvested, washed with PBS, and resuspended in sterile PBS at a density of 1×10^6 viable cells/200 μ l. Different GBC-SD cells were inoculated via the lateral tail vein of 4-week-old nude mice. Animals were divided into four groups, including GBC-SD, GBC-SD/vector, GBC-SD/TRO-1, and GBC-SD/TRO-2 groups, and each group had eight animals. Lungs were harvested 4 weeks after tumor cell inoculation, fixed, and stained with Bouin's solution. The number and area of metastatic lung nodules

Table 1 Primers for PCR and their annealing temperatures

Gene		Primer sequence	Product size (bp)	Annealing (°C)
Integrin $\alpha 3$	Up	5'-GGAAGGAACAAAGACAGGCAAAC-3'	150	58
	Down	5'-GGTAGTGGTGAGTGAGAAGTGGC-3'		
MMP-7	Up	5'-GTTTAG,AAGCCAAACTCAAGG-3'	232	55
	Down	5'-CTTTGACACTAATCGATCCAC-3		
MMP9	Up	5'-TGGGCTACGTGACCTATGACAT-3'	150	60
	Down	5'-GCCCAGCCCACCTCCACTCCTC-3'		
Ets-1	Up	5'-GGGTGACGACTTCTGTGTTG-3'	274	57
	Down	5'-GTTAATGGAGTCAACCCAGC-3'		
HPRT1	Up	5'-TGACACTGGCAAAACAATGCA-3'	93	60
	Down	5'-GGTCCTTTTACCAGCAAGCT-3'		

were determined by examination under a dissecting microscope equipped with a millimeter-squared eye grid.

Statistical analysis

Data are expressed as the mean \pm standard deviation (SD) of at least three independent experiments performed in triplicate. Calculation of means and SD was performed using Excel software (Microsoft Office for Windows 2000). Statistical analysis was performed using the software of Statistical Package for the Social Sciences (SPSS) version 11.5 for Windows and $P < 0.05$ was considered as statistically significant.

Results

Expression of trophinin in GBC-SDH_i and GBC-SD cells

To confirm the roles of trophinin in GBC-SD cells, real time RT-PCR and western blot analysis of trophinin were performed. As shown in Fig. 1, results of replicate samples from GBC-SDH_i and GBC-SD cells confirmed up-regulation of trophinin in GBC-SDH_i, which indicated the functional relevance of trophinin in gallbladder cancer development and metastasis ($P < 0.05$).

Construct of human trophinin expression vector and stable transfections

The entire ORF of human trophinin gene was amplified and then subcloned into the pcDNA3.1D expression vector. The resultant construction was confirmed automated DNA sequencing. To investigate the effects of trophinin overexpression on the metastatic phenotype of gallbladder cancer cells, stable trophinin transfectants of GBC-SD were established. As shown in Fig. 2, real time RT-PCR and western blot analysis revealed higher levels of trophinin expressions in two trophinin transfectants (GBC-SD/TRO-1 and GBC-SD/TRO-2) when compared with the parental or vector control ($P < 0.05$). To confirm their stable nature, 3 months later (about passage 20), western blot and real time RT-PCR were performed to confirm the up-regulation of trophinin (data not shown). The transfectants were used before passage 20 in all cases to minimize the impacts of clonal diversification and phenotypic instability.

In vitro effect of trophinin overexpression on gallbladder cancer cells

Adhesion assay

The cell adhesive ability was one of key determinants for tumor metastasis. We examined the adhesive potential to

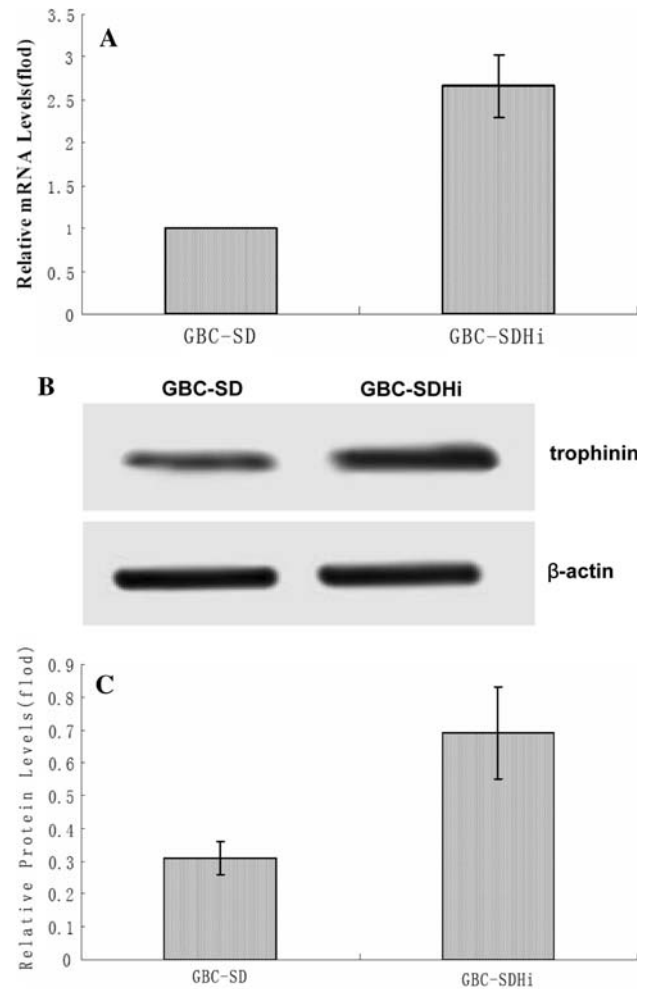


Fig. 1 Real time RT-PCR, and western blot analysis of trophinin differentially expressed in GBC-SDH_i and GBC-SD cells. **a** Quantitative real time RT-PCR values for trophinin mRNAs obtained from both two cell lines. Targets were normalized to reactions performed by using HPRT1 and the fold change was determined with the LightCycler analysis software. Means from three independent experiments are shown. **b** Representative immunodetection of trophinin was shown. Total proteins were separated by 10% SDS-PAGE gels and transferred to PDVF membrane. Immunoblotting was performed by monoclonal antibodies against trophinin, HRP-conjugated secondary antibody, and detected by ECL detection kit. Equal protein loading was evidenced by detection of β -actin level using a monoclonal β -actin antibody. **c** Relative protein expression of trophinin in different cell lines was normalized to the signal intensity of β -actin as an internal control. Images were captured by Alpha Image 950 documentation system and analyzed by NIH Image version 1.62

Matrigel of different cells using CCK8 assay. As shown in Fig. 3a, the values of transfected cells were higher than those of the control ($P < 0.05$).

In vitro invasion assays

In vitro invasion assays were performed to determine the effect of trophinin on cell invasion based on the Boyden

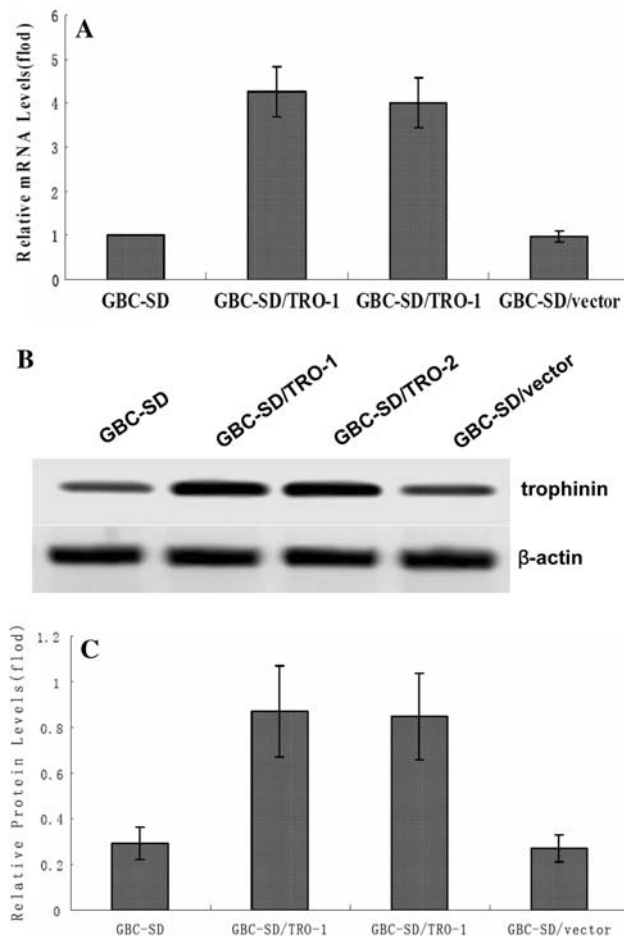


Fig. 2 Expression of trophinin in the trophinin transfectants and control cells. **a** Quantitative real time RT-PCR values for trophinin expression in different GBC-SD cells. Targets were normalized to HPRT1. **c** Western blot analysis of trophinin expression in different GBC-SD cells. β -actin was shown as an internal control. **d** The relative expression of trophinin protein in different cell lines was normalized to β -actin as an internal control

chamber assay. The Matrigel matrix served as a reconstituted basement membrane in vitro. The number of cells migrating through the Matrigel matrix was counted, and the result was presented in Fig. 3b. The trophinin transfectants showed higher invasive capacity than either parental or empty vector control cells ($P < 0.05$).

Trophinin regulate expression of integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1

To further investigate the molecular mechanisms underlying trophinin mediated invasion phenotype in vitro, we focused on the several recognized invasion- and metastasis-associated genes. Real time RT-PCR and western blot analysis showed that trophinin transfectants constitutively enhanced the mRNA and protein expression of integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1 as compared with vector

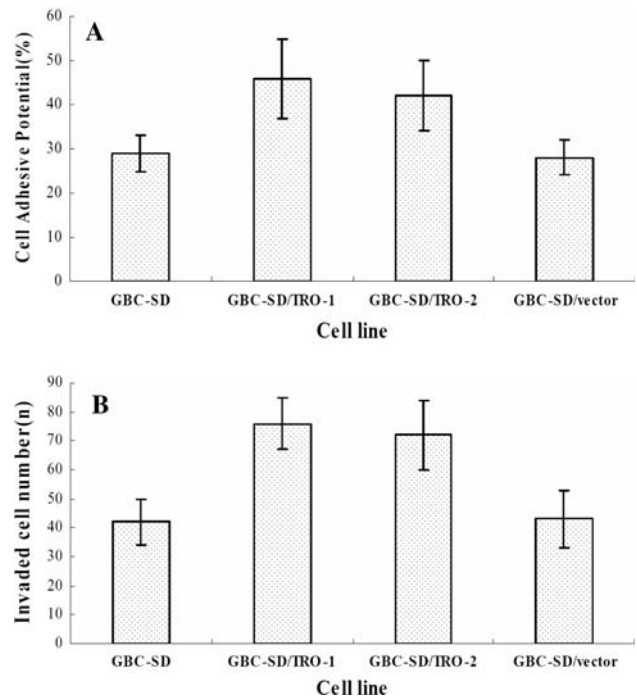


Fig. 3 Stimulation of the invasive potential of GBC-SD cells by trophinin in in vitro studies. **a** The cell adhesive potential of different cells was compared. The potential of cells with trophinin transfected was higher than that of the control cells. **b** In vitro matrigel invasion assay, the trophinin transfected cells were more invasive than its parental counterparts ($P < 0.05$). Results were expressed as the mean \pm SD of three independent experiments in the bar graphs

control transfected or parental cells (Fig. 4). However, we did not find any significant differences in MMP-1, MMP-2, TIMP-1, TIMP-2, uPA, uPAR, cathepsin D, maspin, cystatin C, VEGF, bFGF, RhoC, P21, IGF-1, IGF-1R, IGF-2, Cyclin A, Cyclin D1, Cyclin E, E-cadherin, C-jun, C-fos, TPM4, and TGF α expression between trophinin transfectants and control (data not shown).

Reduced trophinin expression decreases tumor invasion in vitro and down-regulates integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1 expression

We used the RNA interference technique to silence the trophinin gene in GBC-SDH₁ cells to determine whether trophinin expression was critical to the invasive phenotype of gallbladder cancer cells in vitro and expression of selected genes involved in cell invasion and metastasis. As shown in Fig. 5, a significant reduction in trophinin expression was detected by real time RT-PCR and western blot ($P < 0.05$). In addition, reduced trophinin expression in gallbladder cancer cells resulted in a significant decrease in the invasive potential in vitro and concomitant down-regulation of integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1 expression as compared with parental cells ($P < 0.05$).

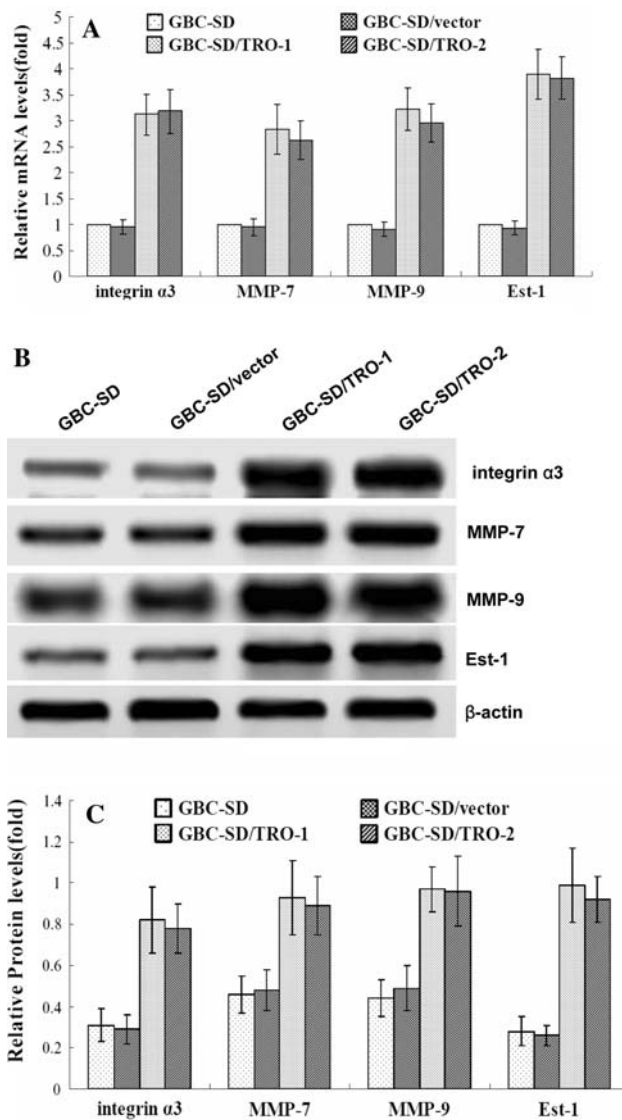


Fig. 4 Expression of trophinin in gallbladder cancer cells regulated integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1 expression in vitro. **a** Quantitative real time RT-PCR values for integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1 expression in different cells. Targets were normalized to reactions performed by using HPRT1. **b** Western blot analysis of integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1. β -actin was shown as an internal control. **c** The relative expression of integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1 protein in different cell lines was normalized to the signal intensity of β -actin

Promotion of pulmonary metastasis in nude mice by trophinin transfection

The effect of trophinin expression on pulmonary metastasis was further assayed in the nude mice. At the experimental endpoint, lungs were examined physically at autopsy and the overt surface metastases were observed. Results revealed that both the number and total area of overt surface metastases in the lungs of trophinin transfected group were bigger than the control group ($P < 0.05$) (Fig. 6). The

data suggested that up-regulation of trophinin could promote GBC-SD metastasis in vivo.

Discussion

Until recently, the specific mechanisms that actually promote gallbladder cancer metastasis still remain unclear. In the previous study, GBC-SDH_i, a highly metastatic cell clone of parental GBC-SD cells, was isolated and maintained. The differences in metastatic potential of the genetically related cell lines make them valuable systems for understanding the molecular mechanisms underlying gallbladder cancer metastasis. In this study, we demonstrated that trophinin, which was considered as an important adhesion molecule during embryo implantation, showed significantly increased expression levels in the highly metastatic GBC-SDH_i cells compared with its parental counterparts. It suggested that trophinin might play a role in the metastatic potential of GBC-SDH_i cells.

It is a complicated procedure with many steps for malignant carcinoma cells to form invasion and metastasis in vivo. The steps include the tumor cell detaches from the primary tumor, invades the extracellular matrix (ECM) and basement membrane (BM), adheres to some macromolecular protein, secretes some proteolytic enzymes to degrade ECM, migrates and invades through the basement membranes of blood and lymph vessels, embolizes, arrests and binds to vascular endothelium at secondary organs, extravasates, and invades the secondary organ. This process is reminiscent of the process of embryonic implantation, which is the release of the unfertilized egg from the ovary, transportation of the embryo through the oviduct and uterus, and then invasion of the blastocyst to the endometrium (Cross et al. 1994; Strickland and Richards 1992). Among the proteins involved in embryonic implantation, trophinin is a membrane protein that potentially mediates the initial adhesion between human embryo and uterine epithelial cells (Suzuki et al. 1999). Considering its role in embryonic implantation while blastocyst invades the endometrium, trophinin may also mediate the invasion process of cancer cells. Chen et al. (2007) demonstrated that ectopic expression of trophinin could enhance lung cancer cell invasion. Knock down of endogenous trophinin by siRNA in lung cancer cell lines led to a decrease in invasion ability. These findings were in agreement with a study of Hatakeyama et al. (2004) that trophinin enhanced invasiveness of the cells and promotes metastasis of testicular germ cell tumour. However, until now, there was little report about the role of trophinin in the metastasis of other cancers including gallbladder cancer.

To better understand the possible role of the trophinin gene in the invasion and metastasis of gallbladder cancer,

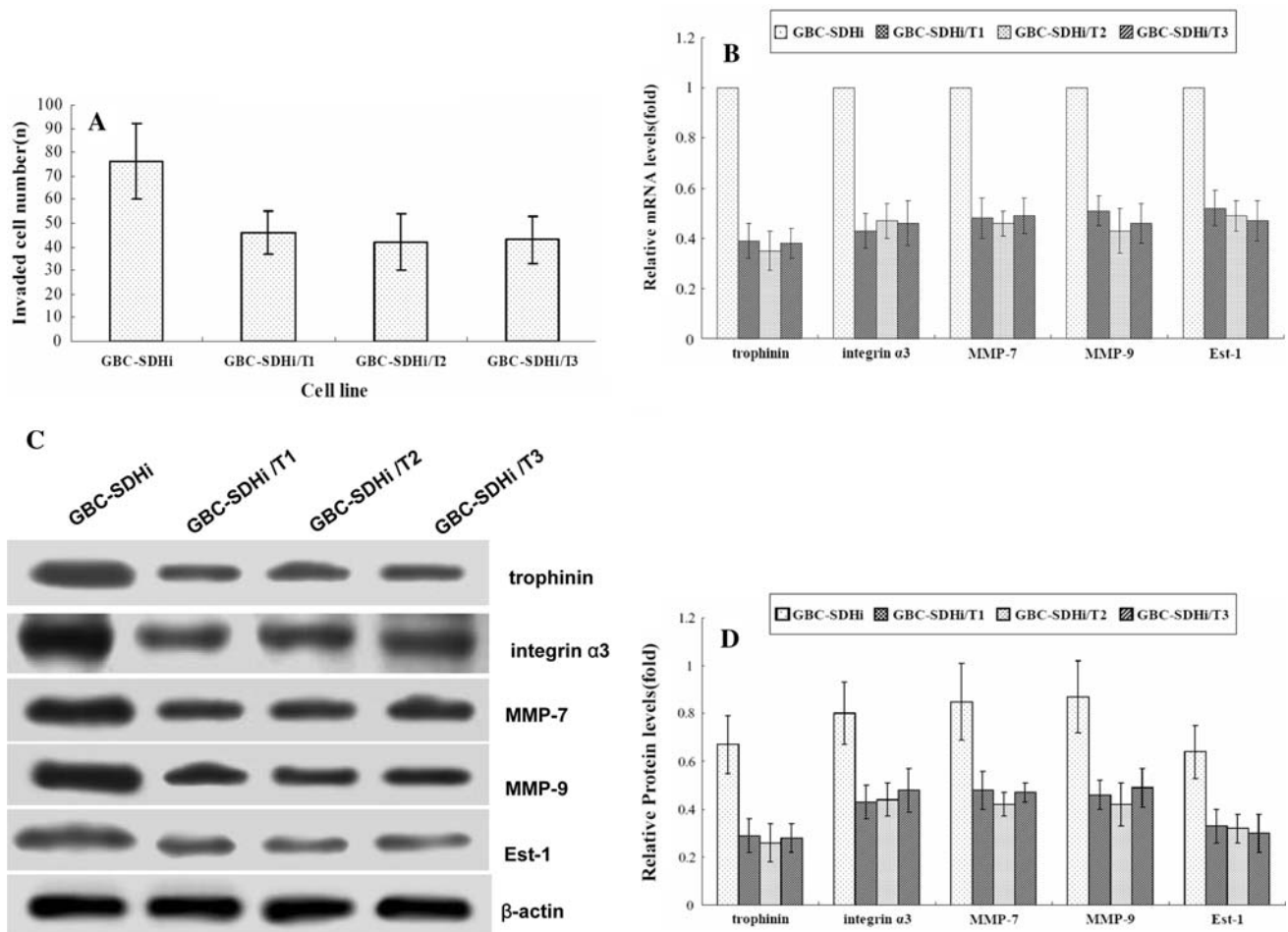


Fig. 5 Reduced trophinin expression in GBC-SDH₁ cells decreased the invasion capacity in vitro and downregulated integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1 expression. **a** In vitro invasion assays of the invasive potential of GBC-SDH₁ and siRNA-targeted GBC-SDH₁ cells using Matrigel invasion chamber. **b** Quantitative real time RT-PCR values for trophinin, integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1

expression in different cells. **c** Representative western blot analysis of trophinin, integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1 expression in different cells. **d** Relative protein expression of trophinin, integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1 in different cell lines was normalized to the signal intensity of β -actin as an internal control

we constructed recombinant plasmid vectors and transfected it into GBC-SD cells. After generating stable GBC-SD/TRO transfectants, we tested them for any alterations in the invasive and metastatic phenotype. Our results showed that trophinin significantly enhances the invasive and metastatic potential of GBC-SD cells in vitro and in vivo. All these may be related to up-regulation of integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1 levels.

Integrin $\alpha 3$ belongs to the integrin family members and the integrin family serves as adhesion receptors for extracellular matrix proteins and cellular counterligands (Kumar 1998). These adhesion receptors are heterodimers of transmembrane glycoproteins (α and β subunits); various combinations of α and β subunits produce polymorphisms of ligand specificity. A number of reports have demonstrated that alterations in integrin expression profiles in cancer cells are frequently associated with their malignant phenotypes, including invasive and metastatic potentials (Sun

et al. 2007; Yao et al. 2007). The expression of integrin $\alpha 3\beta 1$ was demonstrated to be positively correlated with the increased invasiveness of the tumors (Morini et al. 2000; Nishimura et al. 1996; Pochee et al. 2003). These studies suggest that integrin $\alpha 3$ is involved in the invasive and metastatic potentials of cancer cells. The mechanisms that integrins modulate cancer cell migration and invasion are not entirely known, but some reports have shown that the integrin was implicated in the production and/or activation of MMP-7 and MMP-9 (Chen et al. 2005; Fouchier et al. 2007; Impola et al. 2004; Mitra et al. 2003; Rolli et al. 2003). MMPs are a family of more than 20 members of zinc-dependent neutral endopeptidases that plays a key role in degrading the extracellular matrix and basement membrane in various cancers and therefore promotes metastasis and angiogenesis (Deryugina and Quigley 2006). The Ets transcription factors were also reported to be involved in tumor metastasis through the promotion of angiogenesis

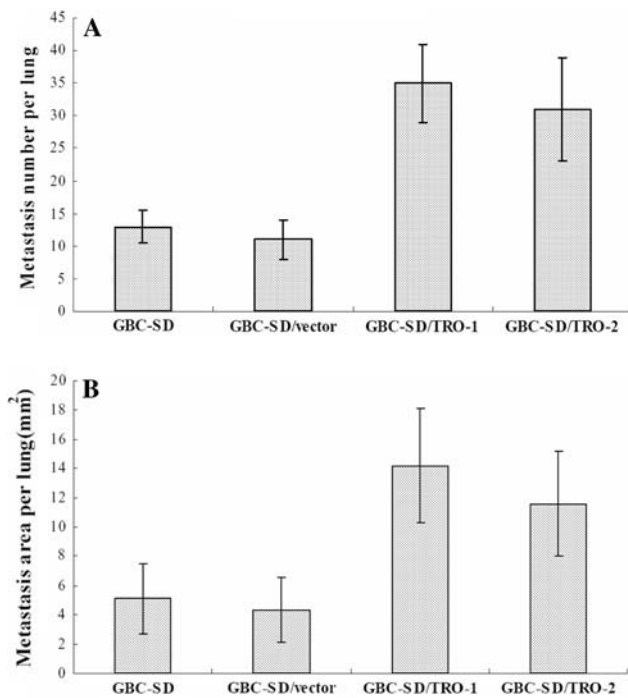


Fig. 6 Stimulation of the pulmonary metastasis of GBC-SD cells by trophinin in in vivo studies. Representative metastasis numbers (a) and total area (b) per lung in nude mice bearing GBC-SD, GBC-SD/vector, GBC-SD/TRO-1, and GBC-SD/TRO-2 tumors by the 4 weeks of post-inoculation

and the expression of MMPs (Hou et al. 2004; Ozaki et al. 2000; Sasaki et al. 2001; Yamamoto et al. 2004). Kato et al. (2002) found that the Ets family of transcription factors regulated the expression of integrin $\alpha 3$. These observations suggest that integrin $\alpha 3$, Ets and MMPs cooperatively promote adhesion and invasion of cancer cells during the metastatic processes.

In the present study, we demonstrated that overexpression of trophinin could up-regulate the expression of integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1 accompanied with the increased in vitro invasive potential in GBC-SD cells. The results were confirmed in trophinin knockdown cells. It was identified that trophinin forms a complex through interacting with two other cytoplasmic proteins, tustin and bystin, during the process of human embryo implantation. In the present study, we also investigated the expression of tustin and bystin in different GBC-SD cell. However, we could not find the obvious difference among them. Further investigation is warranted to clarify it.

In this study, RNAi technique was utilized to reduced trophinin expression in GBC-SDH_i cells with relatively higher pulmonary metastatic rate and trophinin expression level. The results showed a significant decrease in the invasive potential of GBC-SDH_i cells in vitro as well as the expression of integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1. The

results supported the data obtained from gene transfection experiments in GBC-SD cells.

To evaluate the roles of trophinin in gallbladder cancer metastasis in vivo, we inoculated the trophinin transfected and their control cells into nude mice via the lateral tail vein. The results also showed that trophinin could enhance the pulmonary metastasis in vivo. To address the relevance of trophinin in human gallbladder cancer, we also examined trophinin expression in some human gallbladder cancer samples and found positive expression in several cases. Due to the small patient number, in this study we could not draw a conclusion (data not shown). Of course, additional work in larger cohorts of patients is needed to investigate its role.

In conclusion, our data indicated that trophinin was differentially expressed between highly metastatic potential GBC-SDH_i and lower metastatic potential GBC-SD cells. Furthermore, we demonstrated that overexpression of trophinin promoted the invasive and metastatic potential of gallbladder cancer in vitro and in vivo. Conversely, experimental inhibition of trophinin expression decreased their progression, partially via regulation of integrin $\alpha 3$, MMP-7, MMP-9, and Ets-1 expression. These results confirmed our hypothesis that trophinin expression contributed to gallbladder cancer invasion and metastasis.

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