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Original Article

MTSS1 is an independent prognostic biomarker for survival in intrahepatic cholangiocarcinoma patients

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Abstract: MTSS1 is a possible metastasis suppressor which has been proved to play a key role in metastasis of various tumors, yet its role in intrahepatic cholangiocarcinoma (ICC) remains unclear. In present study, we reported detection of MTSS1 expression in ICC and explored its clinical significances. Tissue microarrays containing 93 cases with ICC were constructed and immunohistochemistry was performed to detect MTSS1 expression on these arrays. PcDNA3.1-MTSS1 was transfected into QBC939 cell lines and cell function was measured by transwell assay. Data showed that MTSS1 expression was barely detectable in 56 cases (60.0%) of the 93 primary tumors and that lacking MTSS1 expression was significantly associated with tumor size, nodal metastases and advanced disease stage. In addition, survival analysis demonstrated that lacking MTSS1 expression also correlated significantly with tumor recurrence and poor outcome of patients with ICC. Meanwhile, enhanced expression of MTSS1 leaded to inhibition of the migration of QBC939 cell lines in vitro. These findings together support that MTSS1 may serve as a useful biomarker in predicting tumor recurrence and prognosis of ICC.

Keywords: MTSS1, intrahepatic cholangiocarcinoma, metastasis

Introduction

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary malignancy of the liver. Unlike hepatocellular carcinoma (HCC), ICC originates from the intrahepatic bile ductal epithelium and has extremely poor prognosis [1, 2]. The overall survival of patients with advanced, unresectable and untreated ICC is no more than 4 months, which is even worse than hilar cholangiocarcinoma [3]. Curative resection is considered to be the most favorable therapy to prolong the survival time of ICC patients to some extent [4]. Metastasis is regarded as the most major factor that attributes to the dismal outcome of ICC patients.

Tumor metastasis is a multistep and complex cellular biological process, which is now termed as invasion-metastasis cascade and is orchestrated by numerous molecules and signaling pathways in a genetic or epigenetic manner [5, 6]. During the whole process of metastasis, both activation of metastasis-promoting genes and inhibition of metastasis suppressors are critical to this sequential biological event. Metastasis suppressor refers to those genes that can affect the metastatic process at any step. Since metastasis suppressor genes were firstly discovered a few decades ago, more and more members have joined this suppressor family including nm23 [7, 8], FOXO4 [9], KAI1 [10, 11], KiSS1 [12, 13], etc.

MTSS1 (metastasis suppressor 1), initially termed as MIM (missing in metastasis), was firstly detected in the bladder cancer [14]. Till now, MTSS1 expression has been observed in many human malignancies such as glioblastoma [15], leukemia [16], lung cancer [17], kidney cancer [18], breast cancer [19] and liver cancer [20]. Researches revealed that MTSS1, which is an actin-binding protein, could regulate cell growth, invasion and migration through Sonic Hedgehog (SHH) or EGFR signaling pathway [18, 21, 22]. Although most studies demonstrate an anti-metastatic role of MTSS1 in human malignancies, some other researches revealed that MTSS1 may also promote growth or metastasis in melanomas [6], which suggests the complexity of MTSS1's function in human malignancies.

Table 1. Correlation between MTSS1 and clinicopathological parameters of ICC

Variables	N	MTSS1 Positive (%)	P
Age			
≤60	69	40 (58.0)	0.453
>60	24	16 (66.7)	
Gender			
Male	39	28 (71.8)	0.053
Female	54	28 (51.9)	
Size			
≤3 cm	34	26 (76.5)	0.017
>3 cm	59	30 (50.8)	
Positive Margin			
No	58	37 (63.8)	0.364
Yes	35	19 (54.3)	
T stage			
T1/T2	31	21 (67.7)	0.294
T3/T4	62	35 (56.5)	
Lymph node metastasis			
No	42	31 (73.8)	0.019
Yes	51	25 (49.0)	
Differentiation			
Well/moderate	77	45 (58.4)	0.443
Poorly	16	11 (68.8)	
TNM			
I-III	42	31 (73.8)	0.019
IV	51	25 (49.0)	

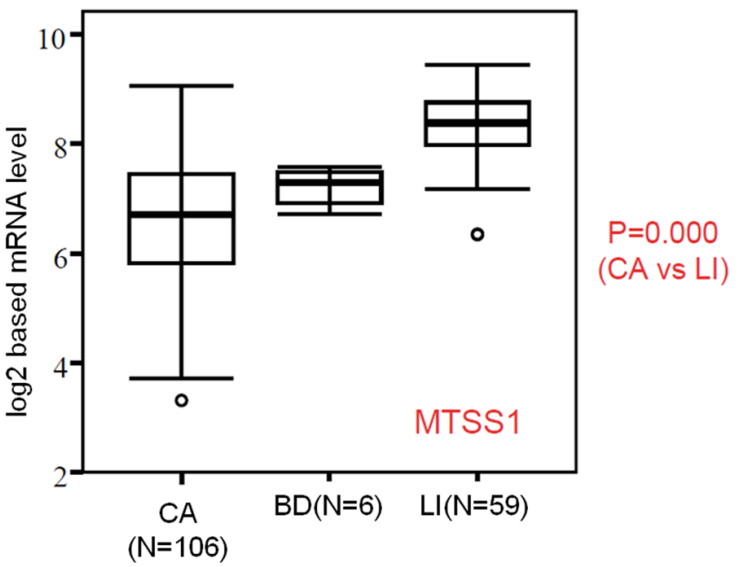

Figure 1. Expression of MTSS1 in cholangiocarcinoma. mRNA levels of MTSS1 transcript in the normal hepatocytes (LI), bile duct tissues (BD) and intrahepatic cholangiocarcinoma tissues (CA). *P<0.01.

In the present study we investigate the expression profile of MTSS1 gene in a cohort of human ICC tissues in order to elucidate its potential prognostic impact in ICC as well its correlation with clinic-pathological feature and overall survival. We also examined the biological effect of MTSS1 gene on migration in cholangiocarcinoma cells in vitro. Our results showed that MTSS1 expression is an independent prognostic factor in ICC and may serve as a valuable biomarker in predicting the outcomes of ICC patients.

Material and methods

Patients

This retrospective study consisted of 93 patients who were diagnosed as intrahepatic cholangiocarcinoma and were undergone surgery at Huashan hospital from 2007 to 2010. Histological diagnosis of each case was re-evaluated by two pathologists. Clinical data of patients were obtained from their medical records including sex, age, tumor size and TNM stage. 74.2% of patients (69/93) were less 60 years old and male-to-female ratio was 1:1.38 (39:54). Clinical follow-up data was available for the patients. The mean survival duration was 26 months, ranging from 1 to 59 months. The study was approved by the Ethic Committee of Huashan hospital. The detailed clinical information of the patients was listed in **Table 1**.

Tissue microarray and immunohistochemistry

Tissue microarray was constructed using a manual arrayer (Beecher Instruments) according to manufacturer's instructions.

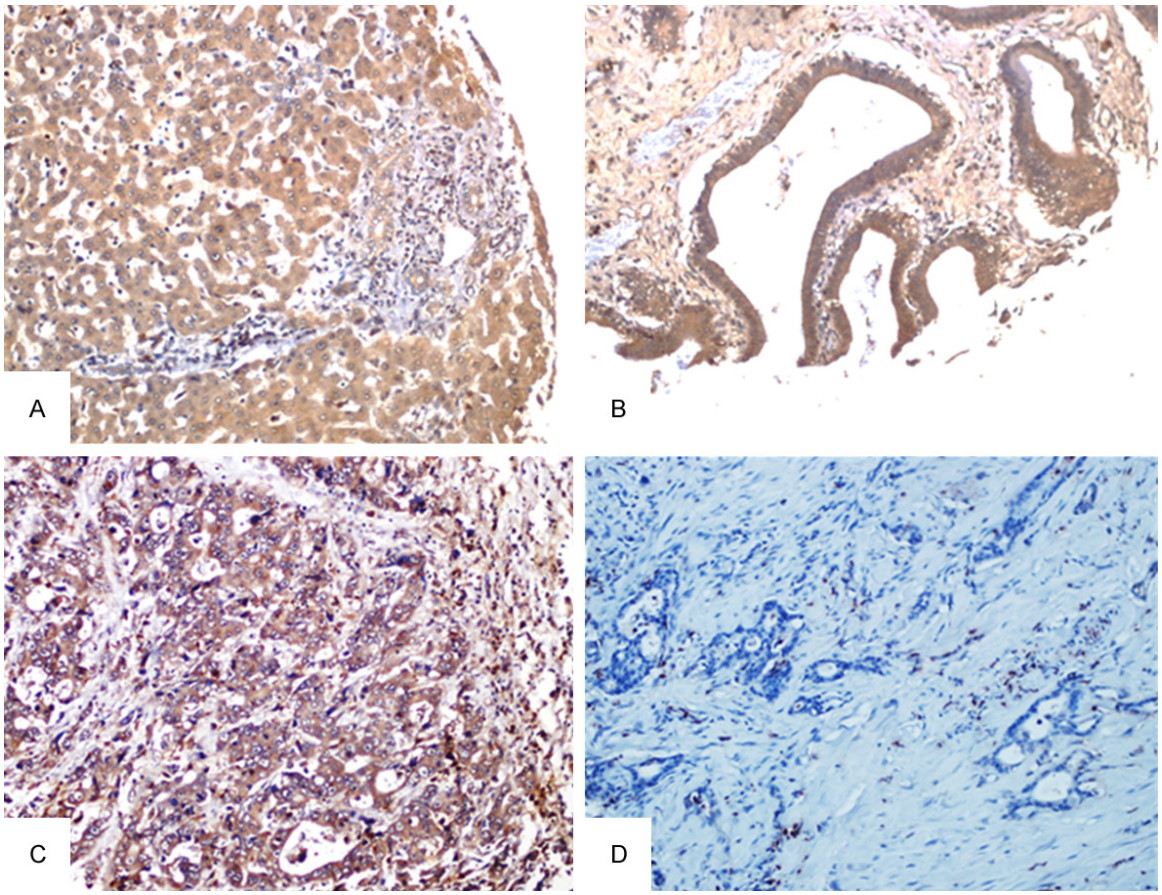

Figure 2. Analysis of MTSS1 expression in human ICC and adjacent normal specimens. A: Normal liver tissues; B: Bile duct tissues, C: Positive expression of MTSS1 in cancer cells; D: Negative staining of MTSS1 in tumors.

Three tissue cores (1.5 mm in diameter) were obtained from each case of intrahepatic cholangiocarcinoma, which included two replicas of cancer tissue and one matched non-neoplastic tissue. Four μm sections were prepared from paraffin-embedded tissue microarray blocks and then processed for immunohistochemical analysis of MTSS1 protein. The antibody against MTSS1 was purchased from Abcam Company (ab56780). Two-step Polymer method (Envision™, DAKO) was used to visualize the antibody. The positive signals were located in the cytoplasm. The staining results were evaluated by two pathologists and a semi-quantitative scoring method was applied as previously described [23].

Cell line and gene transfection

Human cholangiocarcinoma cell line, QBC393, was generously donated by Professor Yu, Changzheng hospital [24]. Cells were grown in the Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2. A full-length cDNA fragment of MTSS1 gene was cloned into the pcDNA3.0 vector to reconstruct the eukaryotic expression plasmid of MTSS1. The MTSS1 eukaryotic expression plasmid was stably transfected into QBC939 cells by lipofectamine™ 2000 transfection kit (Invitrogen). The positive transfectants containing MTSS1 gene or empty vectors were selected by selective medium with G418 for 2-3 weeks. MTSS1 overexpression was confirmed by Western blot analysis.

Western blotting

Cultured cells were lysed in the lysis buffer containing protease inhibitors cocktail (Sigma). Concentration of protein samples was determined by BioRad Protein Assay Kit II (BioRad Laboratories, Hercules, CA) according to the manufacturer' s protocol. 50 μg of protein from

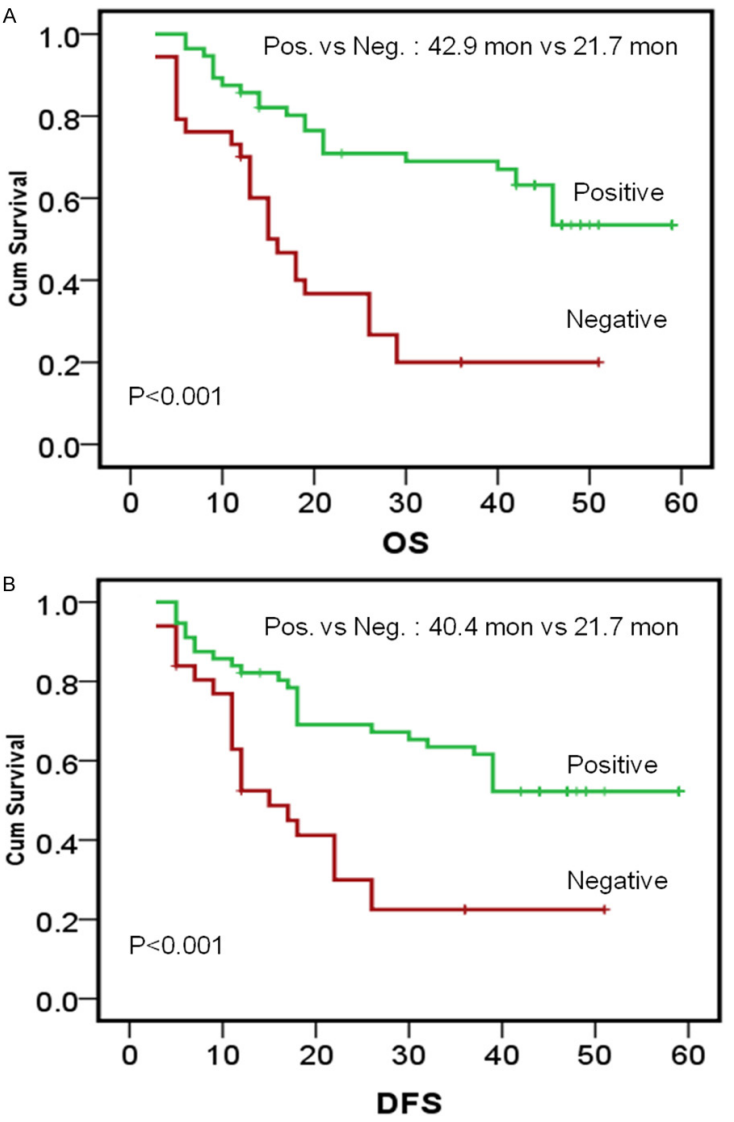

Figure 3. Prognostic significance of MTSS1 in ICC. A. Patients with positive MTSS1 expression had longer survival durations than those with negative MTSS1 expression in ICC (P<0.001). B. Cancers with positive MTSS1 expression had longer time to recurrence than those with negative MTSS1 expression (P<0.001).

cells was separated by 10-14% polyacrylamide SDS-PAGE gels and then electroblotted onto to a PVDF membrane (Bio-rad). The membrane was subsequently incubated with a mouse monoclonal primary antibody against MTSS1 at 4°C overnight. Anti-mouse secondary antibody conjugated to HRP plus an enhance chemiluminescent system was further used to visualize the target protein bands on X-ray film.

Cell migration in vitro

In vitro cell invasion was performed by Transwell® migration assay (Corning, USA). After 24 hours of incubation, cells had invaded through the pretreated extracellular matrix from the upper chamber to the low surface of the membrane. Extracellular matrix and cells on the upper surface of the membrane were carefully removed with cotton swabs. Then the membrane was fixed with 4% paraformaldehyde and cells on the lower surface of the membrane were stained with Giemsa. Cells were counted in 5 randomly selected fields under microscope and images were captured.

Statistical analysis

Statistical analysis was conducted using the SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL). Chi-square test was used to analyze the categorical data. The Kaplan-Meier method was performed to estimate the survival rate and Cox proportional hazards regression model for multivariate survival analysis was used to evaluate the predictable value of markers to recurrence and survival duration. All statistical tests were two-sided and p<0.05 was defined as statistically significant.

Results

Differential expression of MTSS1 transcript in the human liver and intrahepatic cholangiocarcinoma

The mRNA level of MTSS1 expression was assessed in silico using published cholangiocarcinoma microarray data from one patient cohort. Based on the mRNA amount levels, MTSS1 transcript was highly expressed in the normal hepatocytes (**Figure 1**). Bile duct tissues displayed the moderate level of MTSS1 expression. In contrast intrahepatic cholangiocarcinoma tissues showed significantly less amount of MTSS1 mRNA than that of normal hepatocytes (p<0.001). Although the mean amount of MTSS1 transcript was less than that of normal bile ducts, there was no statistical

Table 2. Univariate and multivariate analysis of variables associated with recurrence in patients with cholangiocarcinoma#

Variable	No.	Mean Survival (months)	*P* (univariate)	*P* (multivariate)	Hazard Ratio	95% CI
Age						
≤60 y	66	38.2	0.011			
>60 y	24	25.6				
Gender						
Male	36	25.1	0.005			
Female	54	39.8				
Tumor size						
≤3 cm	31	44.2	0.003			
>3 cm	59	26.8				
Positive margin						
No	58	42.0	<0.001			
Yes	32	19.2				
T stage						
T1/T2	31	52.5	<0.001	0.001	0.190	0.069 to 0.522
T3/T4	59	23.2				
Regional lymph nodes positive						
No	42	44.8	<0.001			
Yes	48	22.7				
TNM stage						
I-III	42	44.8	<0.001			
IV	48	22.7				
MTSS1						
Negative	34	21.7	<0.001	<0.001	0.277	0.136 to 0.564
Positive	56	40.4				
Differentiation						
Well/moderate	74	38.7	<0.001	0.006	0.313	0.137 to 0.711
Poor/undifferentiated	16	14.7				

#Three patients were unavailable for the exact recurrence time.

significance, possibly due to the limited cases of bile ducts involved.

MTSS1 expression profile in human cholangiocarcinoma

Since the results of mRNA amount revealed a slight decrease of MTSS1 in the intrahepatic cholangiocarcinoma, we analyzed the protein expression profile of MTSS1 in a cohort of 93 intrahepatic cholangiocarcinomas using immunohistochemistry to confirm the findings. We also investigated the correlation between MTSS1 overexpression and clinic-pathological parameters in intrahepatic cholangiocarcinoma patients. Histological analysis showed that MTSS1 protein overexpression was detected in both normal hepatocytes and intrahepatic bile ducts (**Figure 2A**, **2B**) while about two thirds of cholangiocarcinoma tissues (56/93, 60%) were MTSS1 positive (**Figure 2C**, **2D**). Furthermore, loss of MTSS1 expression was found to be significantly associated with larger tumor size (over 3 cm, p=0.017), lymph nodal metastasis (p=0.019) and advanced TMN stage IV (p=0.019). However, no significant association was detected between MTSS1 expression and other clinic-pathological features. Detailed information was listed in **Table 1**.

Prognostic significance of MTSS1 overexpression in the human intrahepatic cholangiocarcinoma

The mean cumulative survival duration of 90 patients with intrahepatic cholangiocarcinoma

Table 3. Univariate and multivariate analysis of variables associated with overall survival in patients with cholangiocarcinoma

Variable	No.	Mean Survival (months)	*P* (univariate)	*P* (multivariate)	Hazard Ratio	95% CI
Gender						
Male	39	25.8	<0.001			
Female	54	42.0				
Tumor size						
≤3 cm	34	42.5	0.008			
>3 cm	59	29.0				
Positive margin						
No	58	43.2	<0.001			
Yes	35	21.8				
T stage						
T1/T2	31	53.2	<0.001	0.002	0.205	0.073 to 0.570
T3/T4	62	24.9				
Regional lymph nodes positive						
No	42	47.2	<0.001	0.047	0.471	0.224 to 0.988
Yes	51	24.3				
TNM stage						
I-III	42	47.2	<0.001	0.047	0.471	0.224 to 0.988
IV	51	24.3				
MTSS1						
Negative	37	21.7	<0.001	<0.001	3.618	1.924 to 6.804
Positive	56	42.9				
Differentiation						
Well/moderate	77	39.0	<0.001	0.040	0.469	0.228 to 0.965
Poor/undifferentiated	16	17.7				

was 26 months (from 1 to 59 months). Three patients were lost. Patients with MTSS1 overexpression showed a much longer overall survival duration (OS) than those without MTSS1 expression (42.9 months *versus* 21.7 months, p<0.001) (**Figure 3A**). Moreover, MTSS1 expression status was significantly associated with tumor recurrence. The average month of disease-free survival (DFS) in patients without MTSS1 expression was much shorter than those with MTSS1 overexpression (21.7 months *versus* 40.4 months, p<0.001) (**Figure 3B**). The univariate survival analysis showed MTSS1 expression as well as almost other clinical factors significantly influenced tumor recurrence (**Table 2**) and patient survival (**Table 3**) in intrahepatic cholangiocarcinoma. In multivariate analysis, MTSS1 overexpression (p<0.001), T staging (p=0.001) and histological differentiation (p=0.006) were independent predictive factors (**Table 2**). Meanwhile, MTSS1 overexpression (p<0.001), T staging (p=0.002) and histological differentiation (p=0.04), regional lymph node metastasis (p=0.047) and TNM stage (p=0.047) were independent prognostic factors in the multivariate analyzing using Cox proportional hazards model (**Table 3**). Moreover, subgroup analyses of MTSS1 expression according to TNM staging revealed that the overall survival of patients with MTSS1 overexpression were better in every stage (**Figure 4A** and **4B**). However patients with MTSS1 overexpression showed a longer DFS duration in stages I to III, not in stage IV, than those with negative MTSS1 (**Figure4 C** and **4D**). These results suggest that MTSS1 might be more sensitive in clinical stages without lymph nodal or organic metastasis in predicting patient survival.

MTSS1 overexpression reduced cell migration in QBC393 cells in vitro

MTSS1 expression plasmid was stably transfected into QBC393 cells to investigate its bio-

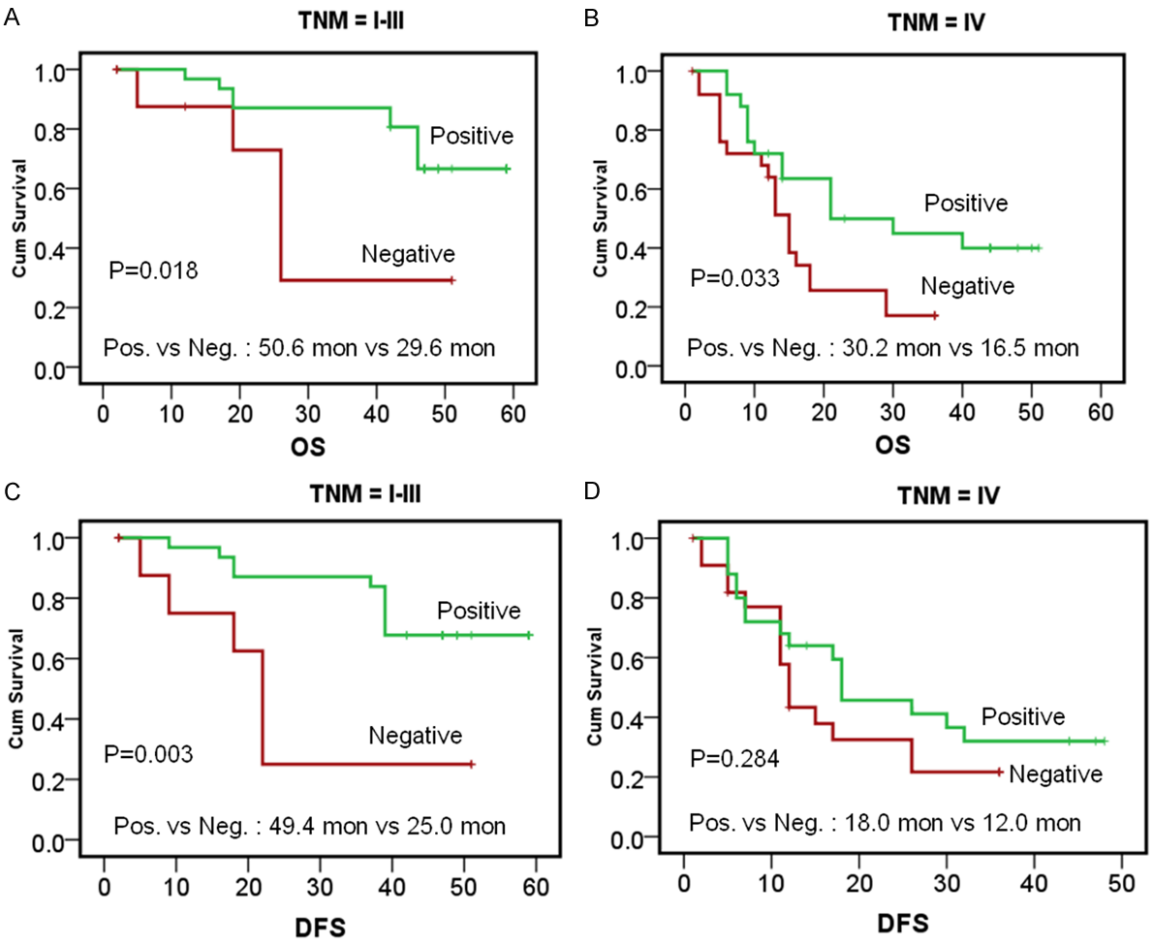

Figure 4. Subgroup analysis of MTSS1 according to TNM. A, B. Stage-specific survival curves showed patients with MTSS1 expression had a better survival than those without MTSS1 expression in each stage (P<0.05). C, D. Patients with MTSS1 expression had a longer time to recurrence than those without MTSS1 expression in stage I-III (P=0.003), not in stage IV (P=0.284).

logical effect on cultured cancer cells. MTSS1 overexpression was confirmed by Western blotting (**Figure 5A**). Enhanced expression of MTSS1 didn't affect the proliferation rate of QBC939 cell lines (**Figure 5B**). However, in vitro transwell migration assay showed that MTSS1 remarkably reduced cell migration capability of QBC393 compared to control cells (**Figure 5C**).

Discussion

Cholangiocarcinoma (CC) is a form of highly malignant and aggressive tumor that originates from the epithelium of biliary trees. Based on the anatomical location of bile ducts, it can be classified into intrahepatic, perihilar and extrahepatic cholangiocarcinoma. Although intrahepatic cholangiocarcinoma (ICC) accounts for only about 10% of total cases, ICC still carries the worst prognosis of cholangiocarcinoma patients who have undergone curative surgeries [25], nevertheless some studies show that the outcome of ICC appear to be slightly better than perihilar CC [3]. Despite the controversial views on the prognosis of ICC, it has been well recognized that metastasis is one of the leading cause of cancer-related death in ICC patient. Thus the main aim of this study is to determine the impact of a novel metastasis suppressor gene, MTSS1, in ICC, mainly focusing on its prognostic value and biological effect.

Since the first report suggested that MTSS1 might serve as a metastasis suppressor by Lee *et al* [14], it has been investigated in many types of human cancers including liver cancer and perihilar cholangiocarcinoma [15, 17, 26, 27]. But the role of MTSS1 gene in human

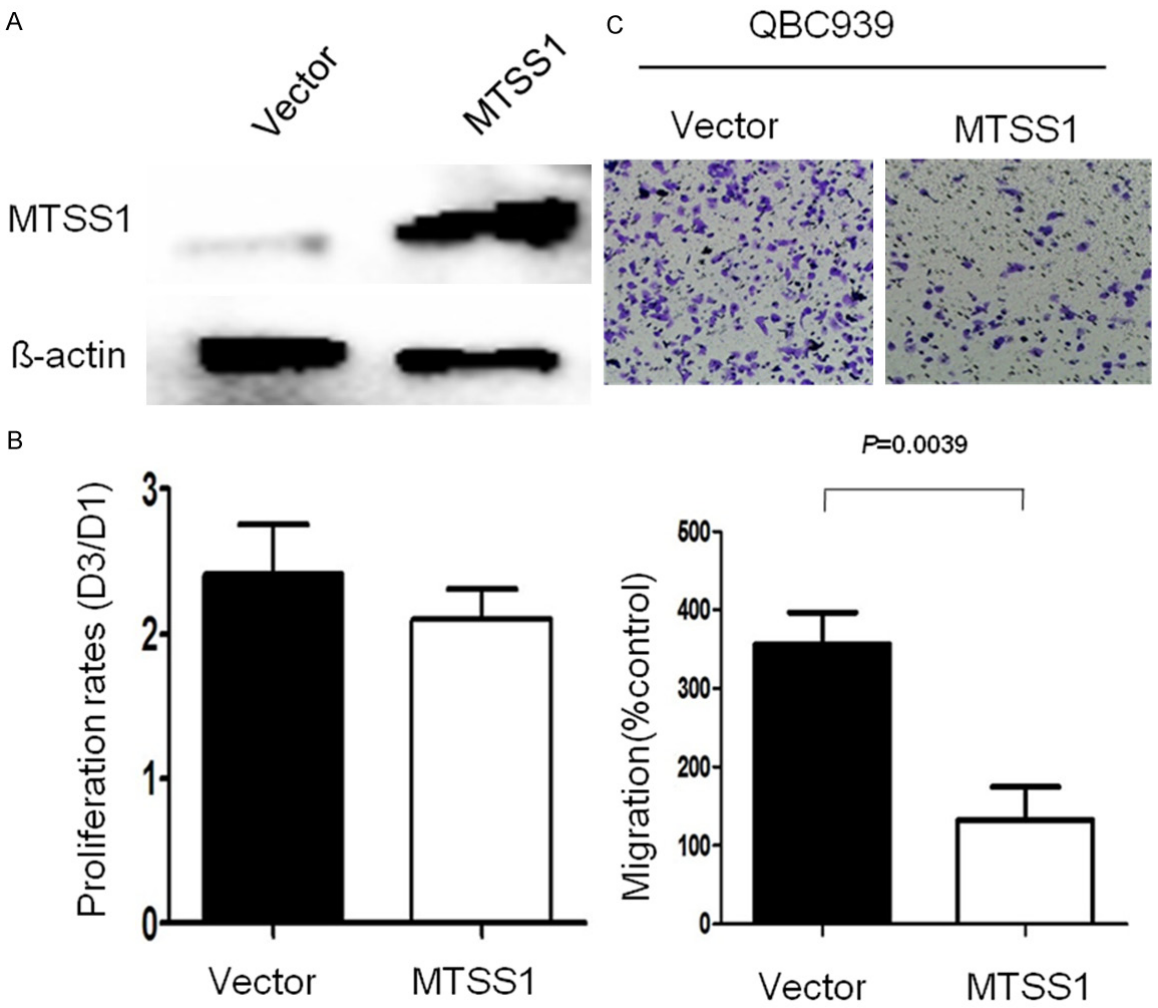

Figure 5. The effect of MTSS1 overexpression on the proliferation and migration ability of human cholangiocarcinoma cells in vitro. A. Western blotting analysis showed that the protein level of MTSS1 in QBC939 cells after transfection at 48 hours. B. The rate of proliferation of QBC939 cells after transfection of SPARCL1; C. Representative pictures of cell migration assay (upper) and the migration numbers of the QBC939 cell line transfected with MTSS1 and the control plasmid (lower).

malignancies still remains undefined. Several lines of evidence have indicated that MTSS1 downregulation was closely associated with clinical staging, lymph nodal metastasis and poor prognosis in cancer patients [17, 18, 26-30]. It has been demonstrated that DNA methylation, microRNA overexpression or ubiquitination-mediated destruction might be responsible to MTSS1 downregulation [19, 20, 31-33]. As a cytoskeletal scaffold protein, MTSS1 could regulate the interaction of actins, promotes cell-to-cell junctions and thus impairs cellular migration [21]. In the late stage of tumor progression, loss of MTSS1 expression could increase the ability of cancer cells to change shapes or induce EMT (epithelial-to-mesenchymal transition) via signaling pathways such as PDGF and RhoGTPase [34]. Besides its effect on migration, in vitro studies also reveal that MTSS1 could suppress cell growth through SHH pathway [15, 18].

In this context, expression of MTSS1 in the normal hepatocytes, bile ducts and ICC tissues was retrieved from published cancer microarray data. Compared with bile ductal epithelium, the mRNA amount of MTSS1 transcript was slightly decreased, yet no statistical significance was observed that might be due to limited cases of normal intrahepatic bile duct. Loss of MTSS1 is significantly correlated with some clinic-pathological parameters such as tumor

size, nodal metastasis and advanced TNM staging, all of which are predictors of poor prognosis. Both univariate and multivariate analyses indicate that MTSS1 overexpression is an independent prognostic factor in ICC. Similar results have been reported in hilar cholangiocarcinoma [26], ovarian cancer [30] and lung cancer [17] etc. Our data also show that the prognostic role of MTSS1 functions better in ICC patients without nodal or distant metastasis (TNM staging I-III) than those with metastatic lesions (TNM staging IV). These results suggest that MTSS1 protein might mainly play as a promoter in the relatively early phase of carcinogenesis and then be silence in the late stage due to loss of expression or degradation. Our in vitro experiment further supported the clinical observations that MTSS1 could impair cancer cells' capability of migration. However, it is also demonstrated that MTSS1 overexpression is associated with poor outcome in colorectal cancer [35] and interestingly even promote metastasis in early-stage melanoma [6].

All these above data indicate that MTSS1 is a multifunctional protein and its biological impact is still far beyond our understanding. Our present research is mainly expression-phenotype association study and hardly involved mechanistic considerations. Considering the complexity of MTSS1 in diverse cancers, further studies concerning molecular mechanisms of MTSS1 in ICC should be carried out in the future.

In conclusions, the present study revealed that loss of MTSS1 expression was associated with larger tumor size, lymph nodal metastasis and advanced TNM stage. Survival analysis and Cox proportional hazards regression model demonstrated that MTSS1 overexpression is a predictor for favorable prognosis of ICC patients. Our results indicate that MTSS1 might serve as a useful biomarker in early detection and survival surveillance in ICC.

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Disclosure of conflict of interest

None.

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