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RESEARCH

Loss of MTSS1 Expression is an Independent Prognostic Factor for Hilar Cholangiocarcinoma

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Abstract Metastasis suppressor 1 (MTSS1) is a novel metastasis suppressor gene in a variety of cancers. This study aimed to detect MTSS1 expression in normal and cancerous tissue specimens from Chinese patients with hilarcholangiocarcinoma to determine the association with clinicopathological parameters and survival. Tissue microarrays containing normal and tumor specimens were constructed using paraffin blocks from 61 patients for immunohistochemical analysis of MTSS1 expression. A subgroup of these tissues was verified by Western blot analysis. MTSS1 protein was expressed in 24 of 61 cases (39.3 %) of tumor tissues, compared to that in 22 of 26 (84.6 %) of non-neoplastic bile duct epithelium and in 26 of 26 (100 %) of adjacent normal liver cells. Loss of MTSS1 expression was associated with lymph node metastases of cholangiocelluarcarcinoma and tumor cell de-differentiation. MTSS1 expression inversely associated with tumor recurrence and overall survival of the patients by univariate and multivariate analyses. MTSS1 expression was significantly decreased in human hilarcholangiocarcinoma and lost MTSS1 expression was associated with pooroverall survival and tumor recurrences in cholangiocarcinoma patients. Thus, MTSS1 expression represents an independent predictor for tumor recurrence and overall survival in patients with cholangiocarcinoma.

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Introduction

Hilarcholangiocarcinoma is an extremely lethal tumor with overall five-year survival rates of less than 20 %. Due to the location at the biliary tree, this deadly disease is usually diagnosed at later stages, which precludes the effectiveness of conventional therapies [1–3]. However, early stage hilarcholangiocarcinoma can be easily treated by R0 resection with negative histological margins, and this intervention improves the five-year survival rateto 30–50 % [1, 2, 4–6]. The etiology and pathogenesis of cholangiocarcinoma remain to be defined, and a number of pathologic conditions resulting in either acute or chronic biliary tract epithelial injury may predispose an individual to malignant transformation. Thus, more research on hilarcholangiocarcinoma is urgently needed for improvement of early diagnosis, initiation and development of more effective treatment strategies, and better prediction of the patient outcome.

The metastasis suppressor 1 (MTSS1) gene, mapped to human chromosome 8q24.1, is expressed in non-metastatic tumor cell lines, but remarkably absent in metastatic or invasive cancer cells [7–10]. Previous studies indicated that MTSS1 protein might play a critical role in regulating cytoskeletal dynamics, cell proliferation, and carcinogenesis in various organ sites (7–13). For example, MTSS1 expression has been clinically detected as dramatically decreased in cancers of the prostate [7], bladder [7, 9], stomach [11]and mammary glands [10, 12]. Moreover, decreased MTSS1 was found to be associated with tumor progression and poor survival (9–11), which suggests a potential role in suppressing cancer development. Loss of expression of MTSS1 protein was shown to be induced by DNA methylation of its gene promoter (8). Nevertheless, other studies have shown that MTSS1 was highly expressed in hepatocellular carcinoma [13]. The MTSS1 protein has been characterized as a sonic

hedgehog-responsive gene that potentiates Gli-dependent transcription; MTSS1 appears to regulate target gene expression through its association with the Gli complex, which is responsible for epidermal growth [14].

To date, there are no data available about MTSS1 expression in cholangiocarcinoma. The aims of this study were to detect MTSS1 expression in a large number of hilarcholangio carcinomatissue specimens and to investigate the potential association of MTSS1 expression with clinicopathological features and survival of hilarcholangiocarcinoma patients. Our data showed the loss of MTSS1 expression might serve as a useful biomarker in predicting the outcome of patients with cholangiocarcinoma.

Material and Methods

Tissue Samples and Tissue Microarray Construction

Sixty-one patients with primary hilarcholangiocarcinoma were identified for study participation from Eastern Hepatobiliary Hospital and Changzheng Hospital, Shanghai, China between 2005 and 2007. Haematoxylin-eosin (HE)-stained sections of resected tumor samples were prepared and reviewed by two pathologists to ensure the quality of paraffin-embedded tissue blocks. Medical records of these patients were reviewed for extraction of clinicopathological data, including age at diagnosis, sex, tumor size, depth of invasion, presence of nodal metastases, and the American Joint Committee on Cancer stage. The mean age of patients at tumor resection was 55 years old (range: 31–79 years old). There were 45 (73.8 %) males and 16 (26.2 %) females. None of these patients received any pre-surgery treatment. The median follow-up period of time for these patients was 16 months (range: 1–59 months). Three patients were lost during follow-up and four patients died of other diseases without recurrence [15]. The Institutional Review Board of both Eastern Hepatobiliary Hospital and Changzheng Hospital approved the use of the tissues and clinical information and informed consent was obtained from each patient or their guardians.

Tissue blocks (tumor and patient-matched normal tissue specimens) were retrieved from the respective Pathology Departments and used to construct the two paraffin-embedded tissue microarray (TMA) blocks using a manual arrayer (Beecher Instruments, Sun Prairie, WI, USA) as previously described [15].

Protein Extraction and Western Blot Analysis

Whole-cell lysates were prepared from both normal and cancerous tissue specimens using a sodium dodecyl sulfate (SDS) lysis buffer. After quantification of the isolated protein, each patient's sample was separated in SDS-polyacrylamide gel electrophoresis gels and then detected by standard Western blotting technique. The primary antibody was rabbit polyclonal antibody against human MTSS1 (1:500 dilution; Abnova, Buckingham, UK) and the secondary antibody was horseradish peroxidase-linked donkey anti-rabbit IgG antibody (1:5000; Amersham, Arlington Heights, IL, USA). The anti-β-actin antibody was used as an internal control to normalize sample loading. The targeted protein bands were detected by an enhanced chemiluminescence kit from Amersham, according to the manufacturer's instructions.

Immunohistochemistry

Four-micron paraffin-embedded sections from the tissue microarrays were prepared as described previously [15], and immunohistochemical staining of MTSS1 protein was conducted using a rabbit polyclonal anti- MTSS1 antibody (1:50; Abnova, Caltag-Medsystems Ltd., Buckingham, UK). A Streptavidin-Biotin kit (#KIT-9720; Maixin, Fuzhou, China) was used to visualize antibody binding to the tissues. Counterstaining was performed with haematoxylin. Control sections were incubated with buffer only, instead of the primary antibody.

Review and Scoring of Immunohistochemically-Stained Sections

The stained TMA sections were independently evaluated and scored under a microscope by two pathologists without any knowledge of clinical outcome. Any discrepancy was dissolved by re-evaluation of the sections by these two pathologists. A semi-quantitative scoring system was used to assess MTSS1 expression, as described previously [16]. Briefly, MTSS1 staining was graded on a binary scale of 0 or 1 (0, negative staining [<10 % of tumor cells stained cytoplasmically]; 1, positive staining [≥10 % of tumor cells stained cytoplasmically])as described previously [17] (Figure S1).

Statistical Analyses

The data were summarized as high and low immunohistochemical expression of MTSS1 protein and then statistically analyzed using Chi-square test. Within-group correlations of continuous and ordinal variables were assessed using Pearson's correlation coefficient or Spearman's rank correlation coefficient. The Kaplan-Meier method was used to estimate survival rates, and the log-rank and the Wilcoxon rank sum tests were performed to assess survival differences between groups. The Cox proportional hazards model for multivariate survival analysis was used to assess predictors related to survival. A two-sided *P*-value of less than 0.05 was considered statistically significant. Analyses were

performed using the SPSS statistical software program for Microsoft Windows (SPSS Inc., Chicago, IL, USA).

Results

Differential Expression of MTSS1 Protein in Cholangiocelluar Carcinoma and Adjacent Normal Tissues

Data from both immunohistochemistry and Western blotting showed that all cholangiocytes of normal bile ducts and the normal hepatocytes had moderate or strong staining of MTSS1 (Fig. 1a and b). In contrast, MTSS1 expression was significantly decreased in tumor cells of cholangiocelluar carcinoma, ascompared to that in adjacent normal tissues(Fig. 1c–f). Particularly, MTSS1 expression was observed in 22 of 26 (84.6 %) non-neoplastic bile duct epithelium and 26 of 26 (100 %) adjacent normal liver cells. MTSS1 expression was only found in 24 of 61 (39.3 %) cases of primary hilarcholangiocelluar carcinoma, significantly lower than that inpara-neoplastic normal bile ducts ($P<0.001$).

Loss of MTSS1 Expression Associated with Tumor Lymph Node Metastasis and Pathology Grade

To delineate the clinical significance of the loss of MTSS1 expression, we associated it with clinicopathological parameters from hilarcholangiocarcinoma patients. In particular, MTSS1 expression was found to be more frequently lost in tumors with regional lymph nodemetastasis (25.0 %) than in N0-stage tumors (55.2 %; $P=0.003$). Moreover, MTSS1 expression was significantly higher in well-to moderately-differentiated tumors than in low-to un-differentiated tumors (55.9 % *vs*. 18.5 %; $P<0.008$). However, there was no significant association between MTSS1 expression and other clinicopathological factors, such as tumor size, nerve invasion, tumor invasion, and clinical stage Table 1.

Association of MTSS1 Expression with Prognosis and Recurrence of Hilarcholangiocarcinoma Patients

The median cumulative survival duration in these patients was 16 months, ranging from 1 to 59 months. MTSS1 expression

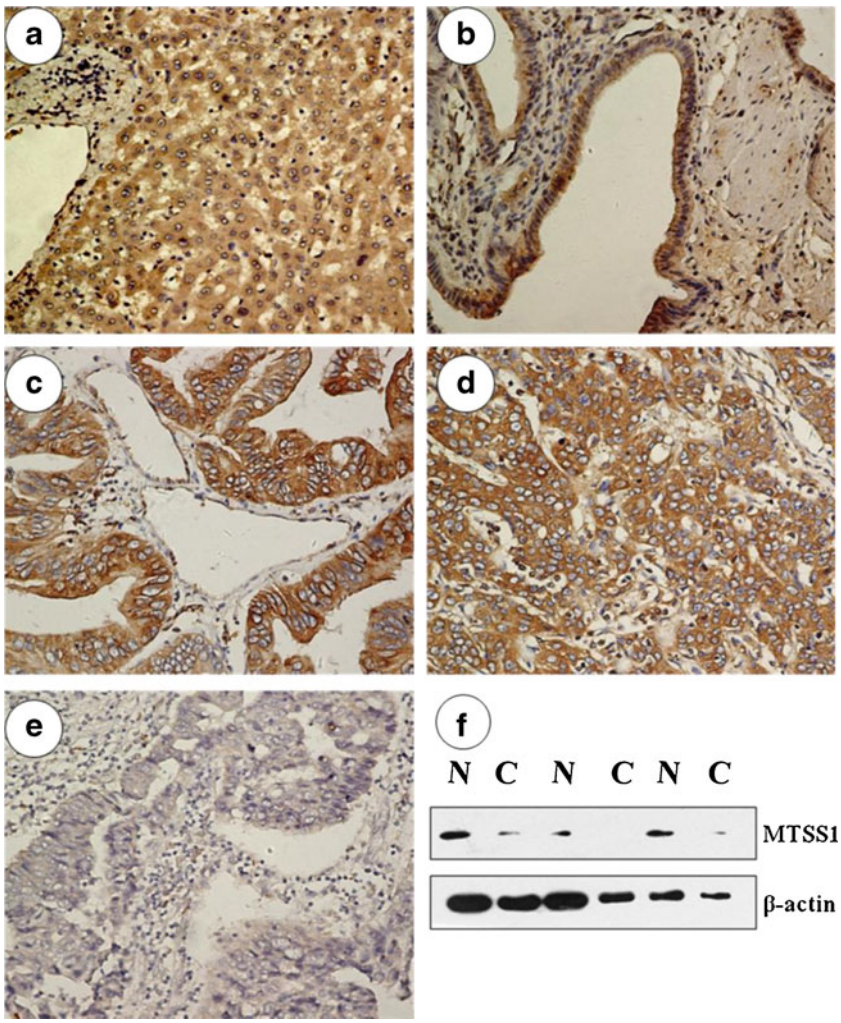

Fig. 1 Expression of MTSS1 in hilarcholangiocarcinoma and non-neoplastic bile ducts by immunohistochemistry (**a**–**e**) and Western blotting (**f**). **a** Adjacent normal liver tissues; **b** Normal (non-neoplastic) bile ducts; **c**, **d** Positive expression; **e** Negative expression. Original magnification: 200×. MTSS1 expression was decreased in cancer (C) but not in normal tissues (N)

Table 1 Association of MTSS1 expression with clinicopathological parameters of hilarcholangiocarcinoma patients

	No. of patients (%)	MTSS1 expression (%)	*P*-value
Age, years			
≤55	32(52.5)	14(43.8)	0.459
>55	29(47.5)	10(34.5)	
Gender			
Male	45 (73.8)	18(40.0)	0.860
Female	16 (26.2)	6(37.5)	
Clinical stage			
I–III	13(21.3)	7(53.8)	0.338
IV	48(78.7)	17(35.4)	
Tumor stage,pT			
T1/T2	13(21.3)	7(53.8)	0.338
T3/T4	48(78.7)	17(35.4)	
Tumor differentiation			
Well/Moderate	34(55.7)	19(55.9)	0.003
Poor	27(44.3)	5(18.5)	
Lymph nodemetastasis,pN			
No	29(47.5)	16(55.2)	0.016
Yes	32(52.3)	8(25.0)	
Nerve invasion			
No	34(55.7)	13(38.6)	0.842
Yes	27(44.3)	11(40.7)	
Tumor size, cm			
<3	22(36.1)	6(27.3)	0.147
≥3	39(63.9)	18(46.2)	
Total cases	61	24(39.3)	

in the tumor tissues was associated with longer disease-free survival of the patients. Specifically, patients with MTSS1-negative tumors had a median survival of 14 months, whereas patients with MTSS1-positive tumors had a median survival of 42 months (P=0.005) (Fig. 2a). The other factors in univariate analysis were tumor invasion (P=0.0085), lymph node metastasis (P=0.0012), residual tumor margin (R1 or R2) (P=0.0295), tumor differentiation (P=0.0027), and TNM stage (P=0.0085). Multivariate analysis by Cox regression revealed that complete resection (P=0.045), lymph node metastasis (P=0.015) and MTSS1 (P=0.021) were independent prognostic factors. Furthermore, patients with MTSS1-positive tumors were associated with a future recurrence at an average of 37 months, whereas patients with MTSS1-negative tumors had recurrence at an average of 12 months (P=0.0025) (Fig. 2b). Other factors that associated with future recurrence inunivariate analysis were tumor invasion (P=0.0039), residual tumor margin (R1 or R2) (P=0.022), tumor cell differentiation (P=0.0008), lymph node metastasis (P=0.0005), and TNM stage (P=0.0039). Multivariate analysis using the Cox proportional hazards model for all significant variables in univariate analysis showed that tumor invasion (P=0.009), lymph node metastasis (P=0.029), and MTSS1 (P=0.021) were all independent predictors for tumor recurrence.

Discussion

In the present study, we enrolled 61 patients with cholangiocelluar carcinoma, collected their clinicopathological data, and constructed tissue microarrays using the paraffin blocks obtained from these patients. We then performed immunohistochemical staining of MTSS1 protein in both normal and hilarcholangiocarcinoma tissue specimens. Our data showed that MTSS1 expression was significantly reduced in hilarcholangiocarcinoma specimens, as compared to the patient-matched normal tissue specimens. Further statistical analyses showed that the loss of MTSS1 expression was associated with lymph node metastases of cholangiocelluar carcinoma and tumor cell de-differentiation.MTSS1 expression was inversely associated with overall survival of the patients and tumor recurrence. The univariate and multivariate analyses revealed that detection of MTSS1 expression is an independent predictor for both overall survival of the

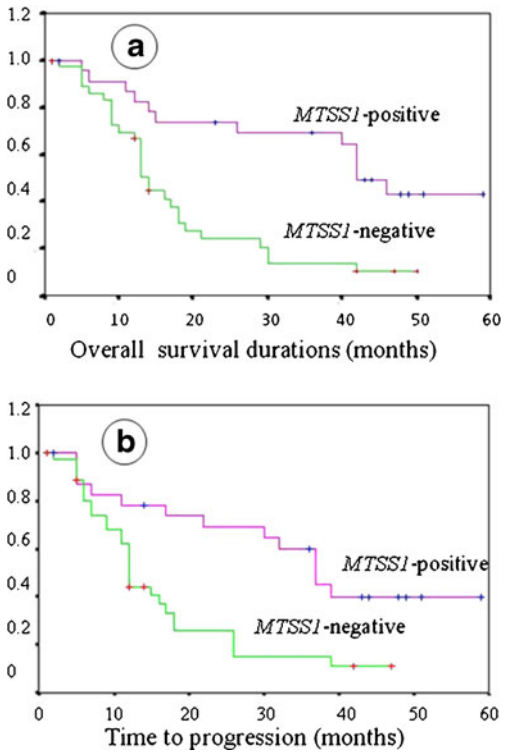

Fig. 2 Kaplan-Meier analysis of overall survival of and recurrence in hilarcholangiocarcinoma patients according to MTSS1 expression. **a** Overall survival was significantly worse in patients with MTSS1-negative tumors (medial survival, 14 months) than in those with MTSS1-positive tumors (median survival, 42 months; P<0.001). **b** Disease-free survival was significantly worse in patients with MTSS1-negative tumors (medial survival, 12 months) than in those with MTSS1-positive tumors (median survival, 37 months; P<0.001)

patients and future recurrence. Future studies with larger patient populations will be carried out to confirm this finding.

Cholangiocarcinoma is a relatively rare disease, accounting for only about 2 % of all cancers diagnosed worldwide. The long-term survival of the patients remains dismal, although great advances have been made in therapeutic strategies available to treat this cancer at early stages. Numerous clinicopathological factors that affect clinical outcome of hilarcholangiocarcinoma have been investigated, including TNM stage, curative resection, and lymph node metastasis of the tumor [1, 2, 4–6, 18, 19]. Of these, complete resection (R0 resection) has been the most frequently used factor to predict long-term survival [19, 20]. Data from our previous studies have also shown that those patients with extensive tumor invasion and lymph node metastasis survive for a much shorter period and experience higher rates of recurrence than those with localized tumors and less invasion [21, 22]. The data from this study indicated that lymph node metastasis and curative resection were independent factors for the long-term survival of patients with hilarcholangiocarcinoma. Moreover, R0 resection was able to achieve long-term survival, as compared to resections with macroscopic- and microscopic-positive margins. In addition, TNM stage did not seem to be an independent predictor for overall survival or tumor recurrence in these patients.

Molecular events underlying the development of hilarcholangiocarcinoma are gradually being elucidated, and a number of studies have provided information on gene expression patterns that may prove useful as biomarkers to predict the overall survival in patients after resection of hilarcholangiocarcinoma. For example, previous studies have shown that differential gene expressions of MMP [23], p27 [24], MVD [25], or Anxa1 [15] can distinguish tumors from normal tissues and these genes' expressions can be used for early tumor detection and survival prediction. In contrast, other studies have failed to find a correlation between some of these markers and prognosis of the patients [26]. In our current study, we detected, for the first time, differential MTSS1 expression in hilarcholangiocarcinoma and the corresponding normal tissues, suggesting this gene's potential as a novel tumor biomarker. Indeed, previous studies of other types of cancers have demonstrated the usefulness of MTSS1 protein as a biomarker [7, 9, 11, 12]. Our current data showed that MTSS1 expression was significantly reduced in hilarcholangiocarcinoma tissues, as compared to the normal tissues. The loss of expression of MTSS1 protein was associated with lymph node metastasis and tumor de-differentiation, but was not associated with tumor stage and size, nerve invasion, or tumor invasion. This result was in agreement with previous studies in other cancers, including those of the stomach and breast. Moreover, the loss of MTSS1 expression was associated with shorter overall survival of the patients and with more frequent recurrence. Multivariate analysis confirmed this finding and identified MTSS1 as an independent factor for predicting future recurrence and overall survival of patients with cholangiocarcinoma. However, future study will be needed to independently verify these findings.

Our current data indicate that MTSS1 protein may play an important role in suppression of hilarcholangiocarcinoma development and progression, although the functions of MTSS1 protein in hilarcholangiocarcinoma are still not fully elucidated. Molecularly, MTSS1 contains a C-terminal WH2 (WASP homology domain-2) domain, which is a small actin monomer-binding motif that interacts with Rac, an actin and actin-associated protein, to modulate lamellipodia formation during cell migration [27–30]. Enhanced expression of MTSS1 has been demonstrated to suppress the invasive, migration, growth and adherence properties of breast cancer cell lines, and knockdown of MTSS1 has been shown to dramatically enhance these properties [10]. Further studies are needed to explore the functions and the underlying mechanism of MTSS1 in hilarcholangiocarcinoma.

It is important to note that our current study has some limitations. First, most of the patients used in this study were diagnosed with stage IV disease, which may have introduced some bias in the analysis and results. Second, the study is associative and was carried out without any mechanistic considerations. Therefore, future study with a larger sample size and more diversified tumor stages will be needed to verify our current findings before clinical use of MTSS1 as tumor marker.

In conclusion, the present study demonstrated that loss of MTSS1 expression was a common event in hilarcholangiocarcinoma and associated with lymph node metastasis and poor differentiation of hilarcholangiocarcinoma. After independent confirmation of these findings is achieved, detection of MTSS1 expression could be useful as a tumor biomarker in early detection and survival prediction for patients with hilarcholangiocarcinoma.

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