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Research Article

Decreased Expression of Metastasis Suppressor 1 Correlates with Poor Prognosis of Patients with Rectal Adenocarcinoma

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ABSTRACT

Background: Venous thromboembolic events (VTE) are common causes of morbidity and mortality in glioblastoma patients. Mutation in the isocitrate dehydrogenase 1 enzyme (IDH1) is frequent in secondary glioblastoma and results in altered metabolomics.

Objectives: This study evaluates whether IDH-1 status correlates with incidence of VTE in glioblastoma patients.

Methods: Observational study of 398 cases of patients with glioblastoma, who all underwent surgery in a regional Neurosurgical centre between April 2012 and December 2014. IDH -1 status and Tissue factor (F3) protein expression were assessed by immunohistochemistry. Deep venous thrombosis (DVT) and pulmonary embolism (PE) were diagnosed by Doppler ultrasound and pulmonary CT angiogram respectively.

Results: 336 cases were wild type (WT) IDH-1 (94.1%) and 21 cases were IDH-1 mutated (R132H) (5.9%). 51 patients had a thromboembolic event (15.3%), with all cases of VTE in WT IDH-1 tumors, a rate of 21.8% within this group. IDH-1 status had a significant correlation with VTE (p=0.033 Fisher exact test). As expected, mutant IDH was associated with prolonged patient survival (p=0.024 Log rank). The mean expression in IDH-1 wild type GBM was 7.14 and in R132h mutant GBM was 4.87 (log2 scale). This was highly statistically significant with a corrected P value of less than 0.0001.

Conclusion: A significant association exists between IDH1 status in glioblastoma patients and the risk of VTE. Patients with wild type IDH-1 appear at high risk of VTE and appropriate precautions should be considered.

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Highlights

Results from qPCR, Western blot and IHC were showed rectal adenocarcinoma cells and tissues with low *MTSS1* expressions. Multivariate and univariate analyses indicated *MTSS1* was an independent prognostic factor.

Introduction

Rectal cancer is the third most common form of cancer in men (16.5%) and the second in women (10.0%) [1]. The long-term outcomes of rectal cancer patients remain poor despite advances in new treatments over the past decades [2-4]. According to the International Agency for Research on Cancer (IARC), rectal adenocarcinoma is the third most common cancer and the fourth deadliness cancer worldwide [5]. Rectal adenocarcinoma occurs in a manner similar to that of other cancers and is caused by the accumulation of altered genetic materials [6, 7]. Also, past studies have shown that elevated interleukin-6 (IL-6)/JAK/STAT3 signalling is one of the key pathways involved in the initiation, development and formation of rectal adenocarcinoma [7]. Given its high mortality, early diagnosis of rectal adenocarcinoma is critical to improve the survival rate. Hence, identification of novel biomarkers of rectal adenocarcinoma will be of great importance in promoting the survival rate of patients with rectal adenocarcinoma.

Metastasis suppressor 1 (*MTSS1*, also known as *MIM* for missing in metastasis) was identified to play a role in invasion and metastasis of bladder cancer [8]. Previous studies have shown that *MTSS1* was a significant cancer-related gene and possessed important functions in many human cancers, including pancreatic cancer, breast cancer, ovarian cancer, and cervical carcinoma [9-12]. Correspondingly, down-regulation of *MTSS1* was shown to be correlated with the poor prognosis of patients with oesophageal squamous cell carcinoma [13]. Mechanistically, *MTSS1* can interact with the cytoskeleton and assemble in actins to maintain cell shape and cell-cell junction stability, and *MTSS1* may serve as an adaptor molecule to regulate the intracellular signalling pathway and actin remodelling in acute myeloid leukemia [8, 14]. However, the expression status of *MTSS1* and its clinical significance in rectal adenocarcinoma remain unclear.

Many known prognostic factors including the stage, metastasis and size of residual tumor can predict survival and recurrence. Nevertheless, patients bearing the same above-mentioned factors may have different prognosis, and other factors, including biomarkers may also exist and affect patient's outcomes after treatments. Therefore, it is important to identify new prognostic factors that can better predict the prognosis of patients and assist in the selection of treatment direction. In the present study, we employed molecular and histo-biochemical approaches to analyse the expression levels of *MTSS1* in rectal adenocarcinoma cell lines and tissues obtained from patients and examined the correlation between its expression and the prognosis of patients with rectal adenocarcinoma.

Materials and Methods

I Cell Culture

LoVo, SW480, and HRC-99 were obtained from American Type Culture Collection (ATCC, USA), and cultured in DMEM with high glucose medium (Life Technology, USA) supplemented with 10% fetal bovine serum (Gibco BRL Co.Ltd., USA), 1% non-essential amino acid (Life Technology, USA), and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a 37°C incubator with 5% CO_2-humidified atmosphere.

II Tissue Samples and Patient Information

All patients were recruited at the First Affiliated Hospital of Hainan Medical University between 1/1/2005 and 12/31/2010. Medical records were reviewed to obtain demographic and clinical data including age, pathologic differentiation stages such as T, N, M, Dukes, and UICC, surgical methods, recurrence, and follow-up results. All patients were diagnosed with rectal adenocarcinoma by different pathologists, and those patients who had previously received radiotherapy or chemotherapy were excluded from this study. All patients were treated at the First Affiliated Hospital of Hainan Medical University. The specimens used for Western blot and quantitative PCR (qPCR) analysis were obtained from four pairs of matched fresh tumor tissues and adjacent normal tissues. The tumor purity in these tissue sections for RNA and protein analysis was tested by using a histopathological protocol. In addition, a total of 180 cancer tissue samples and 50 benign rectal tumor tissue samples were collected for IHC.

III qPCR

cDNA was synthesized with iScript™ cDNA Synthesis Kit (Promega, USA) on RNA that was isolated from cultured cells and primary tumor tissues. qPCR was performed with a pair of primers specifically designed for targeting human *MTSS1*: forward, 5'-GAAGGCATCCTGGGATAGA-3', and reverse, 5'-TATCAGACGCCACCCTCTTC-3'. qPCR conditions were as follows: 1 cycle of initial denaturation at 95°C for 10 min, 45 cycles of 95°C for 15s, 60°C for 60s and 72°C 15s, and 1 cycle of final extension at 72°C 7 min. *GAPDH* was used as an internal reference gene and the sequences of *GAPDH* primers are: forward, 5'-CGAGATCCCTCCAAAATCAA-3', and reverse, 5'-TGTGGTCATGAGTCCTTCCA-3'. All reactions were run in three replicates. Relative expression levels of *MTSS1* were calculated by $2^{-\triangle\triangle Ct}$ formula.

IV Western Blot

Whole protein lysates were purified from cultured cells or tissues with radioimmunoprecipitation assay buffer (RIPA buffer) (Cell Signaling Technology, Danvers) that contained a complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). A total of 20 μg protein of each sample was separated in 10% SDS-PAGE and transferred to PVDF membranes (Invitrogen, CA, USA). The PVDF membrane was blocked in 5% skimmed milk (with 0.1% Tween 20 and Tris-buffered saline) for 1 hour at room temperature (RT). Thereafter, the membrane was incubated with anti-*MTSS1* antibodies (1:1000, Abcam, UK) at 4°C overnight, followed by another incubation with a horseradish peroxidase-conjugated goat anti-rabbit antibody for 1 hour at RT. GAPDH was used as an internal control. Chromogenic reaction was performed to detect the protein expression level.

V Immunohistochemistry

Tissue sections (4 μm thickness) were dewaxed, hydrated, and boiled for 10-15 min in the EDTA-antigen-repair buffer for antigenic retrieval. After then, slides were treated with methanol containing 3% H_2O_2, blocked in1% BSA, and incubated with the primary *MTSS1* antibody (1:200, Abcam, UK) at 4°C overnight. The slides incubated with goat serum were used as a negative control. Thereafter, the slides were incubated with biotinylated anti-rabbit secondary antibodies (Santa Cruz Biotechnology, USA), followed by incubation with the streptavidin-horseradish peroxidase complex (Abcam, UK). After then, 3'-diaminobenzidine and 10% Mayer's hematoxylin were added for chromogenic reaction.

The expression levels of *MTSS1* were evaluated by the product of the positive-cell-proportion and the staining-intensity score. The staining intensity was scored by two pathologists independently. The proportion of tumor cells was graded according to the following rules: the proportion of positive tumor cells corresponding to grade 0-4 was less than 5%, 6-25%, 26~50%,51%~75% and more than 75%, respectively. In addition, the grade 0-4 level of intensity of staining corresponded to colourless (no staining), light yellow (weak), yellow brown (moderate) and brown (strong), respectively. The cut-off values for the *MTSS1* expression were the mid-values of all products. An optimal cut-off value was identified as follows: less than or equal to a score of 4 was defined as low expression of *MTSS1* in the tumor tissue, and greater than or equal to 6 was defined as high expression.

VI Statistical Analyses

All statistical analyses were performed by using SPSS software (version:16.0). The correlation between the *MTSS1* expression level and clinical features of tumors was determined by Pearson's chi-square and Fisher exact test. The correlation between the *MTSS1* expression level and progression-free survival (PFS) and overall survival (OS) was analysed by Kaplan-Meier curve. Survival curves were compared by logarithmic rank test. PFS was defined as the total time from the initiation of the treatment to the onset of recurrence or progression, which was determined by imaging data and clinical evaluation. OS was defined as the total time from the beginning of surgery to the death of patients or the last follow-up time conducted in this study. Multivariate Cox regression analysis was performed for univariate and multivariate analysis of all clinicopathological variables. That was found to be significant by univariate analysis. A p value less than 0.05 was considered statistically significant.

Results

I *MTSS1* Expression was Downregulated in Rectal Adenocarcinoma Cells

We first performed qPCR and Western blot to examine the expression of *MTSS1* at both transcription and protein levels in three rectal adenocarcinoma cell lines, LoVo, SW480, HRC-99 and normal rectal tissues. As shown in (Figure 1A), the transcription of *MTSS1* was at least 4-fold lower in the rectal adenocarcinoma cell lines compared with that in the normal rectal cells. Consistent with this observation, Western blot analysis also showed that the *MTSS1* was highly expressed in the normal rectal tissues but weakly expressed in the rectal adenocarcinoma cell lines (Figure 1B). Thus, the *MTSS1* expression is downregulated in rectal adenocarcinoma cells.

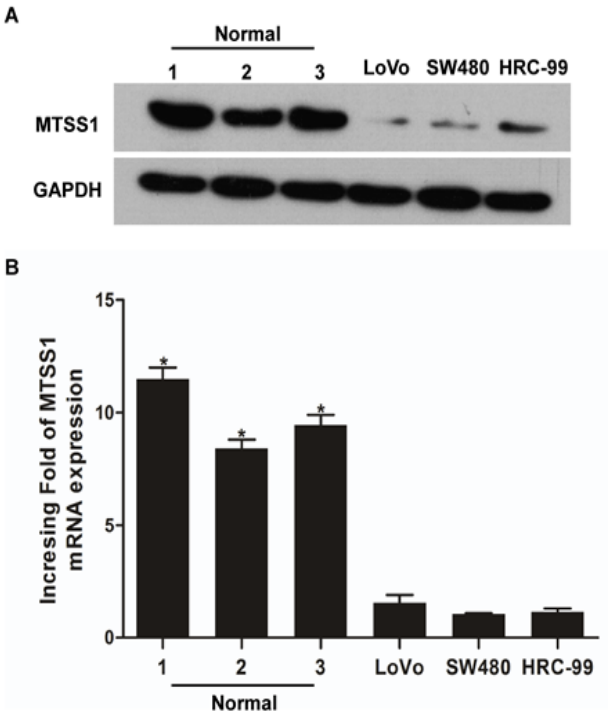

Figure 1: Downregulation of *MTSS1* expression in rectal adenocarcinoma cells. **A)** qPCR and **B)** Western blotting were performed to examine the transcription and protein levels of *MTSS1* in rectal adenocarcinoma cell lines (LoVo, SW480 and HRC-99) and normal rectal tissues, and respectively. Expression levels were normalized to GAPDH. Error bars represent standard deviation of the mean (SD) calculated from three independent experiments. *p<0.05.

II *MTSS1* Expression was Downregulated in Rectal Adenocarcinoma Tissues

Next, we employed the same methods as mentioned above to analyse the *MTSS1* mRNA and protein expression in four matched pairs of rectal adenocarcinoma specimens (T) and adjacent noncancerous tissue samples (ANT). The transcription of *MTSS1* was significantly lower in all rectal adenocarcinoma tissues compared to that of the adjacent noncancerous tissues, with the differential expression levels ranging from 4.4-11.8 fold (Figure 2A). In line with this finding, the *MTSS1* protein levels were also downregulated in rectal adenocarcinoma tissues compared with that of ANT (Figures 2B & 3). We conclude that rectal adenocarcinoma tissues have lower expression levels of *MTSS1* compared with the ANT.

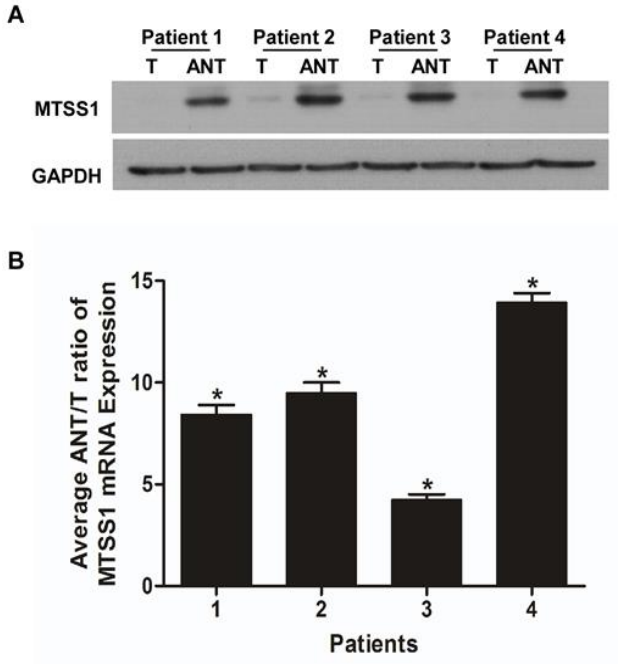

Figure 2: Downregulation of *MTSS1* expression in rectal adenocarcinoma tissues. **A)** ANT/T ratios of *MTSS1* mRNA expression in each of the four patients analysed. Experiments were performed in triplicate and data were normalized to the GAPDH expression. **B)** Representative images of Western blotting showing the *MTSS1* expression in four matched pairs of rectal adenocarcinomas (T) and adjacent noncancerous tissues (ANT). GAPDH serves as a loading control. Error bars represent the standard deviation of the mean (SD) calculated from three independent experiments. *P<0.05.

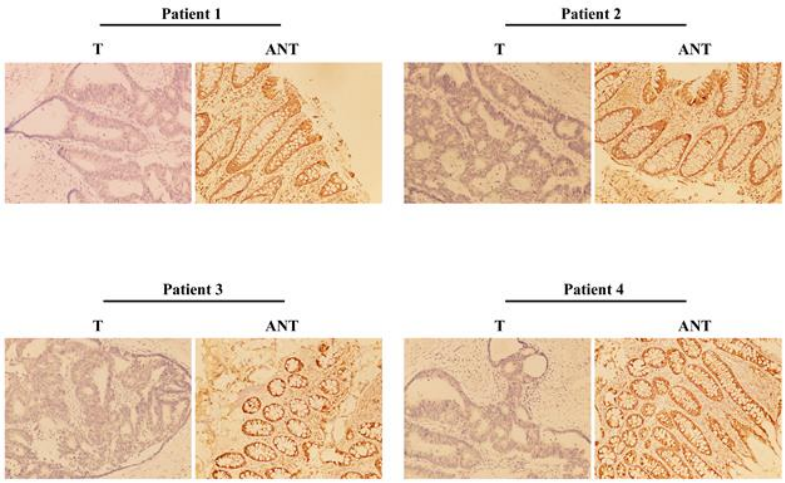

Figure 3: Representative images of immunohistochemistry showing the *MTSS1* expression in four pairs of matched rectal adenocarcinoma and normal tissues.

III Downregulation of *MTSS1* was Associated with Rectal Adenocarcinoma Clinical Features

Out of 180 patients with rectal adenocarcinoma, 37, 50, 84 and 9 had stage I to IV tumors with a median survival age of 58 years (range, 13 ~ 84 years), respectively. All 180 patients received initial treatments, including surgery. There was a statistically significant correlation between the expression levels of *MTSS1* and the clinicopathological characteristics of rectal adenocarcinoma ($p<0.001$ for the T stage, Dukes stage, UICC stage, recurrence and vital status at follow-up, and $p=0.002$ for the N stage). However, there was no significant correlation of *MTSS1* expression with age, pathological differentiation, M stage and other clinicopathological features (Table 1). Logistic multivariate analysis indicated that the *MTSS1* expression was significantly correlated with tumor stage ($P=0.049$), but not with peritoneal cytology and the ascites volume ($P>0.05$). In addition, the *MTSS1* expression levels in patients with an advanced tumor were significantly lower than that in patients with an early stage tumor (Table 1). Thus, *MTSS1* expression levels are associated with the clinical manifestations of rectal adenocarcinoma.

Table 1: Clinicopathological characteristics of patients with rectal adenocarcinoma and their correlations with *MTSS1* expression.

Characteristics	Number of cases (%)	*MTSS1* expression (%)		*P* value
		Low or no expression	High expression	
Age (years, median 59 years)				*P=0.528*
＜median	88(48.9)	61(50.8)	27(45)	
≥median	92(51.1)	59(49.2)	33(55)	
Gender				*P=0.749*
Male	104(57.8)	68(56.7)	36(43.3)	
Female	76(42.2)	52(60.0)	24(40.0)	
Pathologic differentiation				*P=0.255*
Well	10(5.6)	7(5.8)	3(5.0)	
Moderately	148(82.2)	95(79.2)	53(88.3)	
Poorly	22(12.2)	18(15)	4(6.7)	
T stage				***P<0.001***
T 1	5(2.7)	0(0)	5(8.4)	
T 2	46 (25.6)	19(15.8)	27(45.0)	
T 3	46(25.6)	35(29.2)	11(18.3)	
T 4	83(46.1)	66(55.0)	17(28.3)	
N stage				***P=0.002***
N0	91(50.6)	50(41.7)	41(68.3)	
N1	54(330)	48(40.0)	6(10.0)	
N2	35(19.4)	22(18.3)	13(21.7)	
M stage				*P=0.275*
M0	171(95)	112 (93.3)	59(98.3)	
M1	9(5)	8(6.7)	1(1.7)	
Dukes stage				***P<0.001***
A	37(15.0)	8(6.7)	29(48.30)	
B	50(16.4)	38(31.7)	12(20.0)	
C	84(68.6)	66(55.0)	18(30.0)	
D	9(76.4)	8(6.7)	1(1.7)	
UICC stage				***P<0.001***
I	37 (20.6)	8(6.7)	29(48.3)	
II	50(27.8)	38(31.7)	12(20.0)	
III	84(46.7)	66(59.2)	18(30.0)	
IV	9(5)	8(6.7)	1(1.7)	
Surgical method				*P=0.134*
Harfmann's	8(4.4)	7(5.8)	1(1.7)	
Miles'	41(22.8)	31(25.8)	10(16.7)	
Dixon's	131(72.8)	82(68.3)	49(81.7)	
Recurrence				***P<0.001***
No	141(78.3)	82(68.3)	59(98.3)	
Yes	39(21.7)	38(37.1)	1(1.7)	
Vital status (at follow-up)				***P<0.001***
Alive	114(63.3)	59(49.2)	55(91.7)	
Dead	66(36.7)	61(50.8)	5(8.3)	

IV Low Expression Levels of *MTSS1* were Correlated with Poor Prognosis of Patients with Rectal Adenocarcinoma

We next examined the correlation between the expression levels of *MTSS1* and the survival of patients with rectal adenocarcinoma. We followed these patients for 1-108 months and found 115 death cases and 65 survival cases during this follow-up period. The medians for PFS and OS were 70 and 75 months, respectively. Kaplan-Meier analysis showed that there was a significant positive correlation between the *MTSS1* expression levels and OS and between the *MTSS1* expression levels and PFS in rectal adenocarcinoma patients, respectively (P<0.001, Figures 4A & 4B). We also used the Cox proportional hazards model to determine whether the *MTSS1* expression levels could be used as an independent prognostic factor for OS and PFS (Table 2). We examined several potential prognostic factors including differentiation stages such as T, N, Dukes, and UICC stage, recurrence and *MTSS1* expression, and found that only tumor UICC stage (P=0.009), recurrence (P<0.001) and *MTSS1* down-regulated expression (P=0.002) were independent prognostic factors for poor OS. Similarly, Cox regression analysis revealed that UICC stage (P=0.017), recurrence (P<0.001) and downregulated expression of *MTSS1* (P=0.003) were also independent prognostic factors for poor PFS (Table 2).

Table 2: Multivariate analysis of prognostic factors for rectal adenocarcinoma patients.

Outcomes	Variable	HR	P	95%CI
OS	T Stage	0.012	0.911	0.492-1.884
	N Stage	2.171	0.141	0.855-3.028
	M Stage	2.861	0.091	0.191-1.130
	UICC Stage	6.797	***0.009***	1.231-4.341
	MTSS1 expression	9.770	***0.002***	0.075-0.553
	Recurrence	39.596	***<0.001***	3.932-13.573
	Variable	HR	P	95%CI
PFS	T Stage	0.222	0.637	0.436-1.662
	N Stage	1.367	0.242	0.773-2.770 0.462-2.739
	M Stage	0.067	0.796	1.150-4.079
	UICC Stage	5.718	***0.017***	0.081-0.595
	MTSS1 expression	8.909	***0.003***	5.886-21.595
	Recurrence	53.361	***<0.001***	

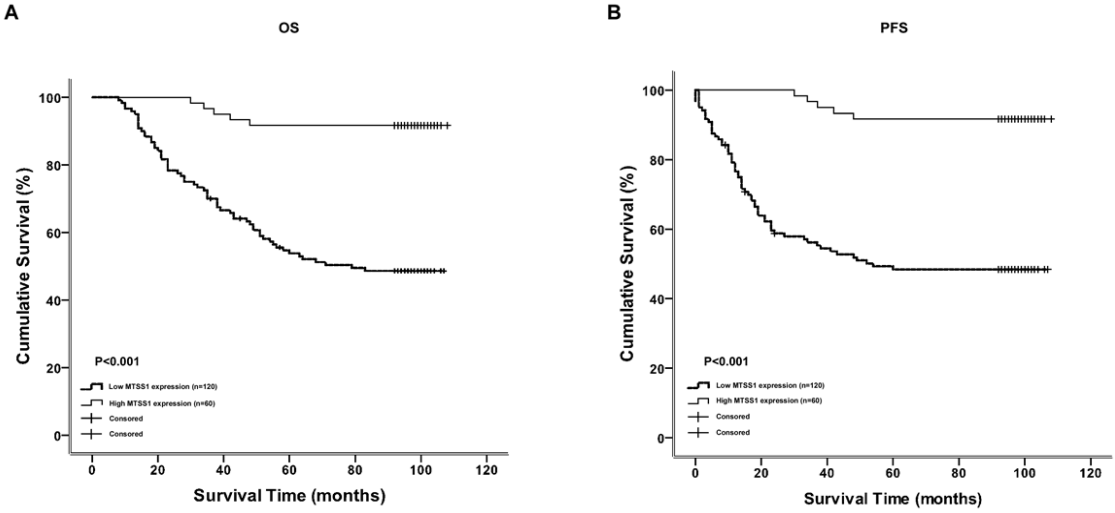

Figure 4: The expression level of *MTSS1* is correlated with the overall survival and progression free survival. Kaplan-Meier curves with univariate analysis (log-rank) are established for rectal adenocarcinoma patients with low *MTSS1* expression (n=120) versus high *MTSS1* expression (n=60) for **A)** OS and **B)** PFS.

Discussion

In the present study, we showed that both mRNA and protein levels of *MTSS1* were downregulated in rectal adenocarcinoma cells and tissues, and that this downregulation was significantly correlated with the advanced stages of this disease. In addition, the *MTSS1* expression level was an independent prognostic factor for patients with rectal adenocarcinoma. In human, the *MTSS1* gene is located on chromosome 8q24.1, and was first identified in bladder cancer, suggesting that *MTSS1* may be implicated in invasion and metastasis in bladder cancer [7]. Previous studies have shown that *MTSS1* was a significant cancer-related gene and that had important functions in many human cancers. If expressed compulsively, *MTSS1* significantly inhibits the occurrence of malignant phenotypes such as tumor cell growth, migration and invasion.

Previously, the *MTSS1* expression was shown to be downregulated in a variety of human cancer samples, which was correlated to unfavourable

clinical outcomes. For example, decreased expression of *MTSS1* is associated with poor prognosis in patients with esophageal squamous cell carcinoma [13]. Previous studies on the expression levels of *MTSS1* in rectal adenocarcinoma revealed discrepant observations. Compared with the normal tissues, MTSS1 protein levels were shown to be significantly lower in colorectal carcinoma in one study, but higher in another [15, 16]. The exact mechanisms leading to these discrepant findings were not clear, but probably involved multiple factors such as regional and ethnic differences, environmental cues. In the present study, we found that the *MTSS1* expression was downregulated in rectal adenocarcinoma cells and human rectal cancerous tissues compared with controls.

In the present investigation, we also revealed that the decreased expression of *MTSS1* was associated with the disease progression and poor outcomes of patients with rectal adenocarcinoma, and we believe that *MTSS1* may be regarded as a potential prognostic factor. Surgery is the mainstay of treatment for rectal adenocarcinoma. Despite advances in surgical technologies and new chemotherapy regimens, the OS rate of patients with rectal adenocarcinoma was still poor [3-5]. In the present study, we not only showed that the reduced expression level of *MTSS1* was associated with the tumor stages, but also revealed that the lower protein expression levels of MTSS1 were significantly correlated with the shorter PFS time and poorer OS of patients with rectal adenocarcinoma. Multivariate analysis further indicated that the expression level of *MTSS1* was an independent prognostic factor for the outcomes of patients with rectal adenocarcinoma. Collectively, these findings suggested that *MTSS1* was involved in the progression of rectal adenocarcinoma, and that the diminished expression level of *MTSS1* was predictive of poor patients' outcomes. Hence, *MTSS1* may serve as a prognostic factor for patients with rectal adenocarcinoma.

Some limitations of this study needed to be acknowledged. For example, in the current study, our patients were solely from the Chinese population. Thus, whether our conclusion may be extrapolated to other populations needs to be corroborated in the future studies. Also, the mechanisms by which *MTSS1* promotes the development, progression and metastasis of rectal adenocarcinoma merits investigation in the future.

Conclusion

We demonstrate that the expression levels of *MTSS1* are downregulated in rectal adenocarcinoma and that this downregulation is associated with the late stages of this disease. Patients with lower *MTSS1* expression have shorter PFS and poor OS. Therefore, the *MTSS1* expression level may serve as an independent prognostic factor for patients with rectal adenocarcinoma.

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Ethical Approval

Our study was approved by the Institutional Research Ethics Committee of the First Affiliated Hospital of Hainan Medical University.

Consent

Prior to the initiation of this study, all patients had signed written consent forms and were followed up until 1/1/2015.

Conflicts of Interest

None.

Abbreviation

***MTSS1*/MIM:** Metastasis Suppressor 1
UICC: Union for International Cancer Control
PFS: Progress-Free Survival
ANT: Adjacent Noncancerous Tissue
T: Tissue
OS: Overall Survival
IHC: Immunohistochemical
qPCR: Quantitative PCR
PVDF: Polyvinylidene Fluoride
TBST: Tris-Buffered Saline with 0.1% Tween 20
PBS: Phosphate-Buffered Saline
RIPA: Radio Immunoprecipitation Assay
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

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