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ORIGINAL ARTICLE

Expression and Significances of MTSS1 in Pancreatic Cancer

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Abstract Thus far, expression of metastasis suppressor 1 (MTSS1), its clinicopathologic and prognostic significances in pancreatic cancer (PC) remain unknown. Expression of MTSS1 was detected by Western blotting in PC cell lines, and by tissue microarray-based immunohistochemical staining in paired tumor and non-tumor samples from 242 patients with PC. Furthermore, the correlations between MTSS1 expression and clinicopathologic variables as well as overall survival were evaluated. In PC cell lines, MTSS1 was differentially expressed. In addition, MTSS1 expression was significantly lower in tumor than in non-tumor tissues ($P<0.001$ in both McNemar and Mann–Whitney U tests). High tumoral expression of MTSS1 was closely associated with absence of lymph node metastasis ($P=0.023$). Univariate analysis found that high MTSS1 expression in tumor tissues was a strong predictor of favorable overall survival in the whole cohort ($P<0.001$). Besides, its impacts on prognosis were also observed in nine out of fourteen subgroups. Finally, MTSS1 expression was identified as an independent prognostic marker in the whole cohort ($P=0.031$) as well as in six subgroups ($P<0.05$), as shown by multivariate Cox regression test. Down-regulation of MTSS1 expression is evident in PC, and is associated with lymph node metastasis and poor prognosis.

Keywords Metastasis suppressor 1 · Pancreatic cancer · Lymph node metastasis · Prognosis

Introduction

Pancreatic cancer (PC) has been well acknowledged as a lethal malignancy. Thus far, its estimated mortality is almost equal to its estimated incidence [1]. This most dismal prognosis has been ascribed to the advanced stages when diagnosed and low resection rate [1]. Therefore, factors affecting prognosis of PC caught much attention. Previously, clinical and pathological ones, such as tumor size, lymph node status, histological grade and CA19-9 level [2–5], have been identified. During recent years, the prognostic roles of genes that have been proven to have biological effects in PC, including K-ras, CDKN2A, P53 and DPC4 [1], have been gradually valued. However, more significant molecules remain to be found.

Metastasis suppressor 1 (MTSS1), also known as MIM (missing in metastasis) [6], was previously suggested to be implicated in actin assembly, cell shape changes and cell-cell junction assembly/stability [6–9]. In cancer cells, forced overexpression of MTSS1 was shown to significantly inhibit malignant phenotypes, such as growth, migration and invasion [10–15]. Furthermore, it was established, based on human specimens, that down-regulation of the gene/protein was common in several kinds of cancers and was associated with some

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unfavorable clinical and pathological features as well as poor prognosis [10, 11, 16–18]. A microarray investigation also revealed that MTSS1 was more expressed in breast cancer samples responsive to doxorubicin-based therapy [19]. All these data support that *mtss1* is a tumor suppressor gene. However, the opposite examples were also found. In colorectal cancer (CRC), MTSS1 was discovered to be up-regulated and was positively correlated with more advanced clinical TNM stage and shorter survival [20]. The finding that MTSS1 expression was elevated in metastatic CRC sublines provided further evidence [21]. Moreover, MTSS1 was defined as a metastasis driver in a subset of human melanomas, on the basis of in vitro and in vivo experiments [22]. Thus, the expression patterns and biological roles of MTSS1 might be tissue-type specific. However, expression and significance of MTSS1 in PC remain unknown.

In the present study, the authors aimed to elucidate MTSS1 expression in PC and its clinicopathologic and prognostic roles.

Materials and Methods

Cell Culture

Eight human PC cell lines, AsPC-1 (ascites derived), BxPC-3 (primary tumor derived), Capan-1 (liver metastasis derived), Colo357 (lymph node metastasis derived), MIAPaCa-2 (primary tumor derived), PANC-1 (primary tumor derived), SU86.86 (liver metastasis derived) and T3M4 (primary tumor derived), were kind gifts of Professor Helmut Friess, Heidelberg University, Germany. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 medium (Hyclone, Thermo Fisher Scientific Inc, Waltham, MA), containing 10 % fetal bovine serum (FBS, Hyclone), respectively.

Western Blotting

Cells were washed with PBS and proteins were extracted according to protein extraction protocols. Protein concentrations were determined using a BCA protein assay kit (Thermo Scientific, Meridian Rd, Rockford). Protein extracts (80 μg/lane) were electrophoresed on 10 % polyacrylamide gels (SDS-PAGE) followed by transfer to PVDF membranes (Millipore, Billerica, MA) and blocking with 5 % non-fat dry milk for 2 h. Membranes were incubated with a primary antibody against MTSS1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4 °C. Secondary antibody (anti-rabbit IgG) was incubated at 37 °C for 1 h. Blots were washed with PBS for three times, exposed to chemiluminescence reagents (Merck, Darmstadt, Germany) and photographic films. All experiments were repeated for four times.

Patients

A total of 242 patients with PC were included. There were 155 men and 87 women. The median age was 59 (range: 34–85) years. The histological grade, perineural invasion (PNI), T and N stages were determined based on post-surgical pathologic examinations. The project was approved by the Institutional Ethics Committee.

Construction of Tissue Microarray (TMA)

Formalin-fixed paraffin-embedded blocks of PC were used in TMA construction. After re-identification of representative tumor and non-tumor areas, two cores of tumor and non-tumor tissues for each patient were sampled using a 1.5-mm punch. The TMAs were constructed by a manual tissue arrayer (Beecher Instruments, 686 Progress Way, Sun Prairie, WI).

Immunohistochemical Staining and Result Evaluation

A rabbit anti-human MTSS1 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and a two-step staining kit (EnVision^{TM} + kit, Dako, Denmark) were applied for staining. Briefly, 4 μm-thick sections were mounted, deparaffinized, rehydrated, and washed with phosphate buffered saline (PBS), followed by antigen retrieval in an autoclave, using 0.01 M citrate buffer (pH 6.0) for 10 min. Slides were then incubated with 3 % hydrogen peroxide for 10 min to block endogenous peroxidase. Then, slides were incubated overnight at 4 °C with the primary antibody at a dilution of 1:30. After PBS washing, horseradish peroxidase (HRP)-labeled secondary antibody was added for reaction of 30 min. Diaminobenzidine was used as a chromogen. Slides were finally counterstained with hematoxylin. Pre-immune rabbit serum at the same dilution was adopted as the negative control.

Two pathologists who had no prior information with the clinicopathologic and follow-up data (Z.Y. L. and W.X. Z.) independently evaluated the slides, and then performed joint re-evaluation for a consensus when they were divergent. The brown coloration in cells was defined as the positive signal. According to the criteria used in a published article [23], the positive cell proportion of MTSS1 was classified into four grades (0 %=0, 0–25 %=1, 26–50 %=2, 51–75 %=3, >75 %=4). In addition, the staining intensity was graded from 0 to 3 (no staining = 0, weak staining = 1, moderate staining = 2, strong staining = 3). Different with the final scoring method previously reported [23], the grades of positive proportion and staining intensity in one section were multiplied to get a total staining score. Finally, MTSS1 expression was determined by a simplified classification (scores 0–3 = low expression; scores 4–12 = high expression).

Follow-up

One hundred and sixty-two patients (66.9 %) accepted follow-up. The follow-up time ranged from 1 to 87 months (median, 17 months). There were 110 patients have died, 32 patients censored during the follow-up, and 20 patients have lived 10 to 87 months.

Statistical Analysis

The comparison of MTSS1 staining scores between tumor and non-tumor tissues was performed using McNemar and Mann–Whitney *U* tests, respectively. Chi-square test was used to detect the relationship between MTSS1 staining scores and clinicopathologic features. Overall survival was calculated by Kaplan-Meier method, and their differences were analyzed log-rank test. Cox regression (Proportional hazard model) was applied for multivariate analysis of prognostic factors. Statistical software package SPSS11.5 (SPSS Inc, Chicago, Ill) was employed for all the analyses. A *P* value less than 0.05 was defined as statistically significant.

Results

Expression of MTSS1 in PC Cell Lines

Western blotting showed that MTSS1 was differentially expressed in all the PC cell lines (Fig. 1). It could be found that MTSS1 expression was highest in Colo357 cell line, with significant differences compared with BxPC-3, MiaPaCa-2, PANC-1 and T3M4 (P=0.041, 0.007, 0.017 and 0.029, respectively).

MTSS1 Expression in PC Samples and its Clinicopathologic Significance

According to aforementioned criteria, low and high MTSS1 expressions in tumor and non-tumor tissues (Fig. 2a–d) were observed in 65 and 177, and in 16 and 226 patients, respectively. The high MTSS1 expression was significantly less common in tumor than in non-tumor tissues (P<0.001, McNemar test, Fig. 2e). Besides, the expression rank in tumor tissues was also statistically lower than that in non-tumor ones (P<0.001, Mann–Whitney *U* test, Fig. 2f). Chi-square analysis showed that N0 tumors carried significantly higher ratio of high MTSS1 expression in tumor tissues, compared with N1 ones (P=0.023, Table 1), but other clinicopathologic variables were not of significance (P>0.05, Table 1). No significant relationship between MTSS1 expression in non-tumor tissues and clinicopathologic parameters was found (data not shown).

Prognostic Factors for Overall Survival of PC After Resection in the Whole Cohort

In univariate analysis, high MTSS1 expression in tumor tissues was significantly associated with better overall survival (P<0.001; Fig. 3 and Table 2). Moreover, sex, histological grade, perineural invasion and N stage were also prognostic (P<0.05; Table 2). Multivariate Cox regression analysis identified histological grade, N stage and MTSS1 expression as independent prognostic markers for overall survival of PC after surgical resection (P<0.05; Table 2).

Impacts of MTSS1 Expression on Overall Survival of Subgroups of PC After Resection

Based on seven clinicopathologic variables evaluated in the present study, patients were divided into fourteen subgroups. Univariate analysis established that tumoral MTSS1 expression was a significant prognosticator for overall survival in nine ones, i.e., female patients, patients <65 years, tumors ≤4 cm, G1-2, G3-4, T1-2, T3 and N0 tumors as well as those without PNI (P<0.05; Fig. S1). Using Cox regression analysis, MTSS1 expression in tumor tissues was identified as one of independent prognostic indicators in females, patients <65 years, G3-4 tumors and those without PNI (P<0.05; Table 3), together with some clinicopathologic variables (Table 3). And, MTSS1 expression was the single significant prognosticator in tumors ≤4 cm and T3 tumors (P<0.05; Table 3).

Discussion

The biological roles of MTSS1 in skeleton-related cell machineries were previously found [6–9]. Thus, it can be easily speculated that alteration of this protein might be involved in tumor metastasis in which cell membrane remodeling is a frequent event. In fact, more and more reports have suggested the inhibitory effects of overexpressed MTSS1 on growth, migration and invasion of cancer cells [10–15]. On the other hand, it was shown that MTSS1 expression was a predictor of favorable clinical/pathologic characteristics, satisfactory prognosis and chemosensitivity [10, 11, 16–19]. Therefore, MTSS1 seems to be a tumor suppressor. Of course, controversial data were also presented in CRC and melanoma [20–22]. Thus far, expression and significance of MTSS1 in PC have not been investigated. In the present study, Western blotting that revealed differential expression of MTSS1 in PC cell lines provided the preliminary clue (Fig. 1). Then, the authors found that MTSS1 expression was quite lower in tumor tissues, compared with that in non-tumor ones, in PC, as confirmed by McNemar and Mann–Whitney *U* test (Fig. 2).

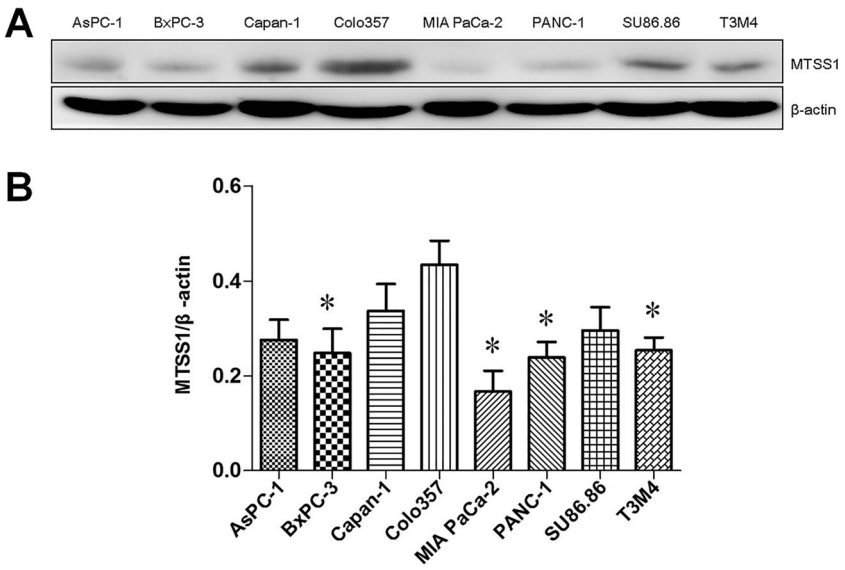

Fig. 1 Expression of MTSS1 in pancreatic cancer cell lines. **a** MTSS1 expression detected by Western blotting; **b** protein expression levels in different cell lines (*: $P<0.05$ in comparison with Colo357). MTSS1, metastasis suppressor 1

This result was similar with those in many other types of cancers, including breast cancer, esophageal squamous cell carcinoma, bladder cancer, kidney cancer, gastric cancer and hepatocellular carcinoma [10–13, 16, 17, 23]. Ubiquitination [14], methylation [24, 25] and some microRNAs (miR-135, −23 and −182) [23, 26–28] might account, at least in part, for

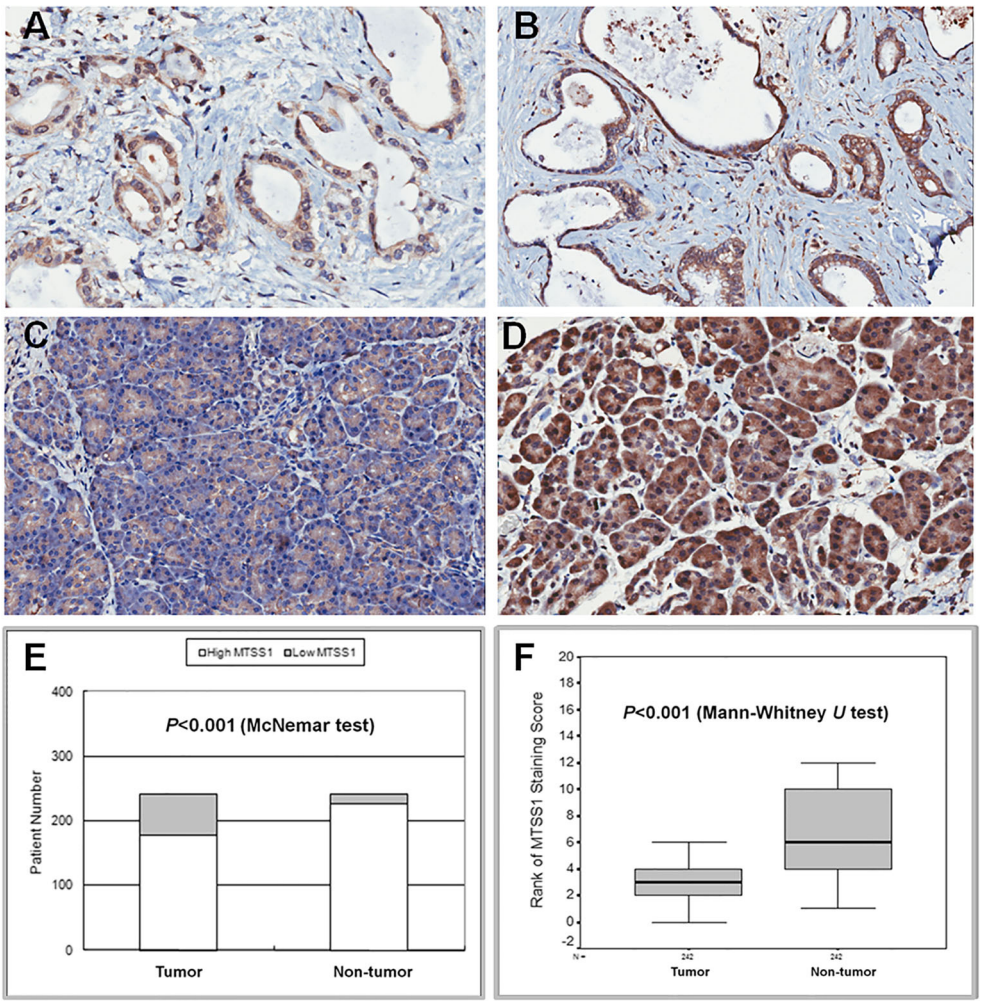

Fig. 2 Expression of MTSS1 in pancreatic cancer tissues. **a** Low expression of MTSS1 in tumor tissues (original magnification ×200); **b** high expression of MTSS1 in tumor tissues (original magnification ×200); **c** low expression of MTSS1 in non-tumor tissues (original magnification ×200); **d** high expression of MTSS1 in non-tumor tissues (original magnification ×200); **e** comparison of MTSS1 expression ratios between tumor and non-tumor tissues (compared by McNemar test); **f** comparison of rank values of MTSS1 expression between tumor and non-tumor tissues (compared by Mann–Whitney *U* test). MTSS1, metastasis suppressor 1

Table 1 MTSS1 expression and clinicopathological features of PC

Variables	Number (*n*)	MTSS1 expression in TT		
		High	Low	*P**
Sex				0.309
Male	155	110	45	
Female	87	67	20	
Age				0.510
≥65 years	85	60	25	
<65 years	157	117	40	
Tumor size				0.171
>4 cm	139	97	42	
≤4 cm	103	80	23	
Histological grade				0.820
G1-2	167	122	45	
G3-4	59	44	15	
PNI				0.877
Present	112	83	29	
Absent	116	87	29	
T stage				0.984
T1-2	175	128	47	
T3	63	46	17	
N stage				**0.023**
N0	136	109	27	
N1	97	65	32	

Bold numbers indicate the *P* value which is less than 0.05, and we defined it as statistically significant

MTSS1 metastasis suppressor 1, *PC* pancreatic cancer, *TT* tumor tissu, *G1* well differentiated, *G2* moderately differentiated, *G3* poorly differentiated, *G4* undifferentiated, *PNI* perineural invasion, *T* tumor, *N* lymph node

* Chi-square test

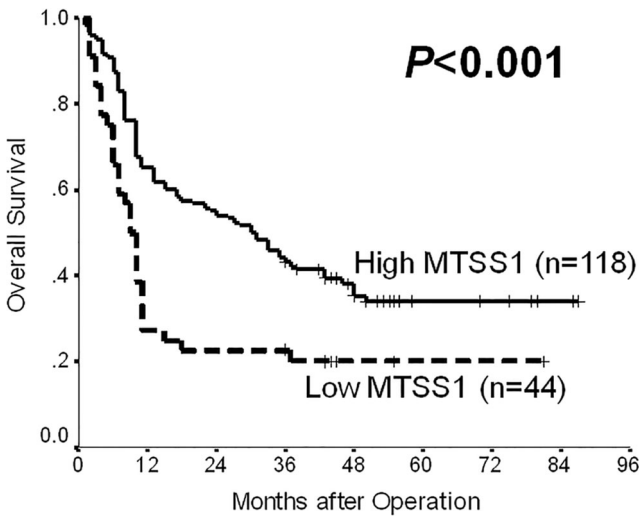

Fig. 3 Influences of tumoral MTSS1 expression on overall survival in the whole cohort of pancreatic cancer after resection. MTSS1, metastasis suppressor 1

the reduced expression of MTSS1 in malignancies. Moreover, MTSS1 expression in tumor cells was found to be adversely associated with N stage, a conventional factor that represents invasion and predicts prognosis in PC [3, 29]. These findings indicated the inhibitory effects of MTSS1 in PC, thus expanding the spectrum of malignant tumors in that MTSS1 functions as a negative modulator. Previous studies demonstrated that MTSS1 might play roles in multiple phenotypes of cancer cells [10–13]. Recently, it was shown that MTSS1 resulted in G2/M arrest in hepatocellular carcinoma cells [30]. No doubt these publications are helpful for understanding the regulation and biological effects of MTSS1 in cancer cells. In the future, detailed mechanistic investigations for MTSS1 modulation might be of particular interest.

It has been long known that the prognosis of PC remains extremely poor. Therefore, its prognostic makers have caught much attention. Many clinicopathological ones, including lymph node status, tumor grade, tumor size and CA19-9 level [2–5, 29], have been defined. Recently, the prognostic value of molecules involved in growth, apoptosis, angiogenesis, invasion and resistance to chemotherapy in PC was summarized [31, 32]. This work provided a new candidate, i.e., MTSS1. Univariate analyses found that high MTSS1 expression was associated with significantly better survival in the whole cohort (Fig. 3, Table 2). Moreover, its independent prognostic value was demonstrated in the whole cohort, in multivariate test. In addition, further analyses revealed that high MTSS1 expression was a statistically significant factor of favorable overall survival in most (nine out of fourteen) subgroups (Fig. S1). These results suggested the potential of MTSS1 expression as a strong and comprehensive predictor of long-term outcome in patients with PC. Based on the finding in this study that tumors with high MTSS1 expression were less likely to carry lymph node metastasis, this prognostic relevance might be easily understood. It is a pity that relative molecular mechanisms of MTSS1 in PC have not been discovered, although some clues have been found in other types of cancers [10–13]. Therefore, whether MTSS1 acts though independent mechanisms in PC deserves further investigations. The differential expression of MTSS1 in PC cell lines establishes a basis of cell selection for further functional investigations in vitro and in vivo.

Conclusions

To be summarized, the present study shows that MTSS1 expression is down-regulated in PC. In addition, low expression of MTSS1 is associated with lymph node metastasis and poor prognosis of PC. Therefore, MTSS1 might serve as a tumor suppressor gene, and have a potential role in gene therapy for the malignancy.

Table 2 Factors associated with overall survival of patients with PC after resection

Variables	Number (*n*)	OS (Univariate)			OS (Multivariate)		
		median±SE	95%CI	P^*	RR	95%CI	$P^\#$
Sex				**0.007**			0.075
Male	106	13±3	8–18		1.558	0.956–2.539	
Female	56	43±4	34–51		1		
Age				0.798			
≥65 years	57	13±8	0–28				
<65 years	105	18±7	5–31				
Tumor size				0.118			
>4 cm	94	13±8	0–29				
≤4 cm	68	18±7	5–31				
Histological grade				**<0.001**			**<0.001**
G1-2	104	33±6	22–44		1		
G3-4	47	10±1	7–13		2.408	1.535–3.778	
PNI				**0.032**			0.067
Present	66	11±3	5–17		1.496	0.971–2.305	
Absent	85	31±10	11–51		1		
T stage				0.981			
T1-2	113	18±6	6–30				
T3	47	15±12	0–39				
N stage				**<0.001**			**<0.001**
N0	95	33±8	17–49		1		
N1	60	11±1	10–12		1.977	1.283–3.047	
MTSS1 in TT				**<0.001**			**0.031**
High	118	30±5	20–40		0.569	0.341–0.949	
Low	44	9±1	7–11		1		

Bold numbers indicate the *P* value which is less than 0.05, and we defined it as statistically significant

PC, pancreatic cancer, *OS* overall survival, *SE* standard error, *RR* relative risk, *CI* confidence interval, *G1* well differentiated, *G2* moderately differentiated, *G3* poorly differentiated, *G4* undifferentiated; *PNI* perineural invasion, *T* tumor, *N* lymph node, *MTSS1* metastasis suppressor 1, *TT* tumor tissue

* Log-rank test

Multivariate Cox regression test

Table 3 The impacts of MTSS1 expression on overall survival in univariate log-rank test-identified subgroups of PC after resection (estimated by Cox regression test)

Subroups	RR	95%CI	*P*	Other independent prognosticators
Females	0.289	0.115–0.727	**0.008***	PNI, N stage
<65 years	0.557	0.318–0.978	**0.042***	Sex, histological grade, N stage
≤4 cm	0.229	0.113–0.461	**<0.001#**	None
G1-2	0.637	0.320–1.268	0.199*	Tumor size, PNI, N stage
G3-4	0.356	0.170–0.749	**0.006***	Age
Without PNI	0.428	0.202–0.910	**0.027***	Tumor size, Histological grade
T1-2	0.576	0.316–1.050	0.072*	Histological grade, PNI, N stage
T3	0.441	0.209–0.932	**0.032#**	None
N0	0.607	0.299–1.233	0.168*	Sex, histological grade

Bold numbers indicate the *P* value which is less than 0.05, and we defined it as statistically significant

MTSS1 metastasis suppressor 1, *PC* pancreatic cancer, *RR* relative risk, *CI* confidence interval, *PNI* perineural invasion, *N* lymph node, *G1* well differentiated, *G2* moderately differentiated, *G3* poorly differentiated, *G4* undifferentiated, *T* tumor

* Multivariate Cox regression test

Univariate Cox regression test

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