RESEARCH ARTICLE

Association of Matrix Metalloproteinase (MMP)-2 and -9 Expression with Extra-gastrointestinal Stromal Tumor Metastasis

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Abstract

Matrix metalloproteinase (MMP)-2 and MMP-9 are important proteases involved in invasion and metastasis of various tumors. Extra-gastrointestinal stromal tumors (EGISTs) are rare neoplasms. This study was performed to assess MMP-2 and MMP-9 expression in EGIST tissue samples for association with clinicopathological data from the patients. Twenty-one surgical EGIST tissue specimens were collected for analysis of MMP-2 and MMP-9 expression using immunohistochemistry. MMP-2 and MMP-9 proteins were expressed in all of the epithelial cell types of EGISTs, whereas they were only expressed in 75% of the spindle cell type, although there was no statistically significant difference ($p>0.05$). Expression of MMP-2 and MMP-9 proteins was associated with tumor size, mitotic rate, tumor necrosis, and distant metastasis ($p<0.05$). MMP-2 expression was linked with MMP-9 levels ($p<0.05$). However, there was no correlation between MMP-9 expression and age, sex, primary site, or cell morphology in any of these 21 EGIST patients ($p>0.05$). Moreover, expression of MMP-2 and MMP-9 proteins increased with the degree of EGIST risk. This study provided evidence of an association of MMP-2 and MMP-9 expression with advanced EGIST behavior.

Keywords: Extra-gastrointestinal stromal tumor (EGIST) - MMP-2 - MMP-9 - tumor invasion and metastasis - biomarker

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Introduction

Extra-gastrointestinal stromal tumor (EGISTs) are rare neoplasms localized in the extra-gastrointestinal tracts (such as the omentum majus, including the mesentery, retroperitoneal space, the pancreas, spleen, vagina, and rectovaginal septum) (Yamamoto et al., 2004). Although they are similar to gastrointestinal stromal tumors (GISTs) in terms of histopathology and immunophenotypes (Reith et al., 2000), there are various differences between EGISTs and GISTs, such as tumor size, necrosis, and tumor cell mitotic rate. GISTs are one of the most common mesenchymal tumors with Kit or PDGFRA gene mutations (Wang et al., 2013). However, the risk and prognostic evaluations of primary GISTs are different due to their localization in different parts of the gastrointestinal tract. To date, EGIST metastasis only has been determined by parameters resembling those of GIST metastasis. Thus, EGIST pathogenesis and progression warrant further investigation. The molecular mechanism of EGIST progression needs to be determined in order to provide effective prevention and treatment of EGISTs in clinical applications (Agaimy et al., 2006).

To this end, the invasion and metastasis of EGISTs, like most human cancers, greatly contribute to cancer-related mortality. Tumor invasion and metastasis are a complex and multi-step continuous process involving various molecules, especially matrix metalloproteinases (MMPs) (Yadav et al., 2014). During tumor invasion and metastasis, MMPs degrade the basement membrane and extracellular matrix (ECM) so that tumor cells can invade and metastasize into adjacent or distant organs. The basement membrane and ECM, on one hand, provide substrates and nutrition for tumor growth and metastasis, but on the other hand, they are major blockades in the prevention of tumor cell invasion and metastasis (Dumont et al., 2003; Adler, 2004). During the degradation of the basement membrane and ECM, MMPs are the most important enzymes and play a key role in this degradation process (Labauge et al., 2001). The expression and activation of MMPs are also involved in various physiological and pathological events, such as inflammation, tissue fibrosis, angiogenesis, invasion, and tumor metastasis. Specifically, MMPs selectively degrade different components of the ECM (Roy et al., 2009; Hatfield et al., 2010) and thus regulate numerous biological events including cell growth, inflammation, invasion, and angiogenesis by eliminating cell surface proteins such as the cytokine receptor, cell adhesion molecules, and urokinase receptors (Backstrom et al., 1995; Cauwe et al., 2007; Kessenbrock et al., 2010; Rodriguez et al., 2010; Klein et al., 2011). Among these MMPs, MMP-2 is secreted by tumor cells and interstitial

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cells in the form of a zymogen and can specifically degrade collagen IV when it is hydrolyzed and activated. MMP-9 enhances metastasis of tumor cells by degrading collagen proteins of the ECM after being activated by extracellular proteases under different physiological and pathological conditions. Thus, MMP-2 and MMP-9 are important proteases that are involved in invasion and metastasis of various tumors. In the present study, we performed immunohistochemical analysis to detect the protein expression of MMP-2 and MMP-9 in EGISTs and then associated their expressions with clinicopathological data from EGIST patients.

Materials and Methods

Tissue specimens

In this study, we collected surgical EGIST tissue specimens from 21 patients at The First Hospital Affiliated to Bethune Medical College, Jilin University (Changchun, Jilin, China) between September 2010 and December 2012. The clinicopathological data of these 21 EGIST patients were retrieved from the patients' medical records, and all cases were confirmed by histopathological diagnosis. Tissue specimens were fixed in 10% buffered formaldehyde and embedded in paraffin for the preparation of tissue sections for hematoxylin and eosin (HE) staining and immunohistochemical analysis. The tissues were grouped according to the risk degree classification criteria of EGISTs defined by the US National Institutes of Health (NIH) (Joensuu, 2008) (Table 1). This study was approved by the Institutional Review Committee of Jilin University, and informed consent from all patients was provided according to the Declaration of Helsinki (Yuan et al., 2009).

Immunohistochemistry

To detect MMP-2 and MMP-9 protein expression, immunohistochemistry experiments were performed on the paraffin-embedded tissue sections using a two-step EnVision method according to the kit instructions (Maixin Biotechnology Co., Ltd., Fuzhou, China) (West et al., 2004; Espinosa et al., 2008). Briefly, consecutive paraffin-embedded tissue sections (4 μm-thick) were first dewaxed in xylene three times for 15 min each and rehydrated with decreasing concentrations of ethanol (100%, 95%, 90%, 80%, and 70%). The sections were then rinsed in phosphate-buffered saline (PBS) three times for 3 min each. Endogenous peroxidases of the sections were blocked for 10 min at room temperature with a blocking solution containing 3% H_2O_2 in methanol. Antigens in tissues were repaired with sodium citrate solution (pH 6.0) for 90 s, followed by washing with PBS three times. Next, the sections were incubated with the primary antibody for 90 min in humidified boxes. Rabbit monoclonal (L638) anti-MMP-2 and rabbit monoclonal (W680) anti-MMP-9 antibodies were purchased from Bioworld (Louis Park, MO, USA) at dilutions of 1:100 and 1:50, respectively. After washing with PBS three times, the sections were further incubated with the secondary antibody for 30 min. To visualize the reaction, 3,3'-diaminobenzidine (DAB) solution was added to the sections, and the sections were incubated for up to 10 min. Next, the sections were counterstained with hematoxylin for 2 min and then treated with 1% hydrochloric acid/ethanol and with aqueous ammonia before being dehydrated and mounted with coverslips in a neutral gum. Breast carcinoma tissue sections and PBS-treated sections were used as positive and negative controls, respectively. The stained sections were evaluated by three pathologists. When there was a discrepancy in the evaluation results, another evaluation was made to determine the final score of the stained sections.

Table 1. Classification of EGISTs According to USA NIH Classification Criteria for Defining the Risk of Aggressive Clinical Course of Primary EGISTs

Risk category	Tumor size in largest dimension (cm)	Mitotic count (per 50 HPFs)
Very low risk	<2.0	≤5
Low risk	2-5	≤5
Intermediate risk	≤ 5.0	5-10
	5-10	≤5
High risk	>5	>5
	>10	Any mitotic rate
	Any size	>10

HPFs, high-power microscopic fields

The MMP-2 and MMP-9 protein expression in the cell cytoplasm was reviewed and scored according to the criteria previously described (West et al., 2004; Espinosa et al., 2008). According to the percentage of positively stained tumor cells, we scored the sections as 0 (no staining), 1 (less than 10% of tumor cells stained positively), 2 (between 10-50% of cells stained positively), and 3 (more than 50% of cells stained positively). Next, the sum of these two scores was calculated to give a final score for each case so that expression could be determined to be high (score 3 or more) or low (score 0 to 2).

Statistical analysis

The data were summarized as mean±standard deviation (x±SD). Statistical significance between means was determined and analyzed by one-way analysis of variance (ANOVA) and the χ^2 test by using SPSS 18.0 software (SPSS Chicago, IL, USA). The Fisher exact test was used to analyze the correlation between expression of MMP-2 and MMP-9 proteins and metastatic EGISTs. A P-value less than 0.05 was considered significant.

Results

Patient clinicopathological data

Out of the 21 EGIST patients, 12 were males and 9 were females between 39 and 78 years of age (median age of 57 years old). Localization of the EGIST was in the abdominal cavity (7 cases, 33%), mesentery (4 cases, 19%), retroperitoneum (3 cases, 14%), liver (2 cases, 9.5%), esophagus (2 cases, 9.5%), omentum majus (1 case, 5%), prostate (1 case, 5%), and pancreas (1 case, 5%). Histologically, there were 16 cases of the spindle cell type and 5 cases of the epithelial cell type of EGISTs. The size of the tumor lesions ranged from 0.1 cm to 24 cm, with a median diameter of 8.0 cm. Specifically, there were 7 patients with tumors less than 2 cm in diameter, 3

Table 2. Association of MMP-2 and MMP-9 Protein Expression with Clinicopathological Features of EGIST Patients

		n	Precent of all EGISTs	MMP-2			MMP-9		
				n	%	*p* value	n	%	*p* value
Age (years, median age=57 years)									
	<40	1	5	1	100	0.143	1	100	0.217
	40–60	11	52	9	81.8	0.362	9	81.8	0.383
	>60	9	43	8	88.8	0.252	7	77.7	0.42
Sex	Male	12	57	11	91.6	0.191	10	83.3	0.243
	Female	9	43	7	77.7	0.158	7	77.7	0.138
Site	Abdominal cavity	7	33	5	71	0.229	5	71	0.32
	Mesentery	4	19	3	75	0.454	4	100	0.479
	Retroperitoneum	3	14	3	100	0.555	2	66	0.498
	Liver	2	9.5	2	100	0.511	2	100	0.535
	Esophagus	2	9.5	2	100	0.493	1	50	0.506
	Omentum	1	5	1	100	0.537	1	100	0.497
	Prostate	1	5	1	100	0.346	1	100	0.403
	Pancreas	1	5	1	100	0.42	1	100	0.411
Tumor size (cm, median=4.2 cm)									
	≤2	8	38	5	75	0.352	6	75	0.389
	>2 to ≤5	2	9.5	2	100	0.456	1	50	0.479
	>5	11	52.4	11*	100	0.026	10*	91	0.039
Mitotic rate (per 50 HPFs)									
	≤5	8	38	6	75	0.386	5	62.5	0.405
	6 to 10	5	24	5	10	0.544	5	100	0.528
	>10	8	38	7#	87.5	0.016	7#	87.5	0.036
Risk of malignancy									
	High risk	13	62	12★	92	0.038	12★	92	0.047
	Intermediate risk	1	5	1	100	0.369	1	100	0.389
	Low risk	4	19	3	75	0.548	3	75	0.412
	Very low risk	3	14	2	67	0.524	1	33	0.466
Tumor necrosis									
	Yes	10	47.6	10●	100	0.044	10●	100	0.039
	No	11	52.4	8	72.7	0.359	7	63.6	0.316
Distant metastasis									
	Yes	10	47.6	10★	100	0.028	9★	90	0.035
	No	11	52.4	8	72.7	0.447	8	72.7	0.454

*p<0.05 compared with the group with tumor diameter<2 cm; # p<0.05 compared with the group with mitotic rate ≤ 5 per 50 high-power microscopic fields (HPFs); ▲p<0.05 compared with the low risk degree group; ●p<0.05 compared with the group with tumor necrosis; ★p<0.05 compared with the group with distant tumor metastasis

patients with 2-5 cm tumors, and 11 patients with tumors exceeding 5 cm. The mitotic rate from 50 high-power microscopic fields (HPFs) showed that there were 8 patients with a mitotic rate less than 5, 5 patients with a mitotic rate ranging from 6 to 10, and 8 patients with a mitotic rate exceeding 10. According to the NIH consensus classification criteria, 3 (14%) patients belonged to the very low risk group, 4 (19%) patients belonged to the low risk group, 1 (5%) patient belonged to the intermediate risk group, and 13 (62%) patients belonged to the high risk group (Table 2).

Protein expression of MMP-2 and MMP-9 in EGIST tissue specimens

Immunohistochemistry experiments were performed to detect MMP-2 and MMP-9 expression in EGIST tissues. We found that the expression of these proteins was localized in the cytoplasm of tumor cells, while the negative control was similar to the background color (Figure 1).

Among these 21 EGIST patients, 18 cases were positive for anti-MMP-2 antibody staining in the cytoplasm of tumor cells and interstitial cells. Specifically, positive MMP-2 staining occurred in 71% (5/7) of EGISTs of the abdominal cavity, 75% (3/4) of EGISTs of the mesentery, 100% (3/3) of EGISTs in the retroperitoneum, 100% (2/2) of EGISTs in the liver, 100% (2/2) of EGISTs in the esophagus, and 100% (1/1) of EGISTs in the omentum majus, prostate, and pancreas. In most cases, MMP-2 staining exhibited uniform disseminated strong staining (Figure 2).

Moreover, there were 17 cases that stained positively for MMP-9 protein in the cytoplasm of tumor and interstitial cells. Specifically, positive MMP-9 staining occurred in 71% (5/7) of EGISTs of the abdominal cavity, 100% (4/4) of EGISTs in the mesentery, 66% (2/3) of EGISTs in the retroperitoneum, 100% (2/2) of EGISTs in the liver, 50% (1/2) of EGISTs in the esophagus, and 100% (1/1) of EGISTs in the omentum majus, prostate, and pancreas. In most cases, MMP-9 staining exhibited uniform disseminated strong staining (Figure 3).

Association of MMP-2 expression with EGIST clinicopathological characteristics

We found that there was a significant association between MMP-2 expression and tumor size, mitotic rate, tumor necrosis, and distant metastasis (p<0.05). In addition, there was an association between the expression

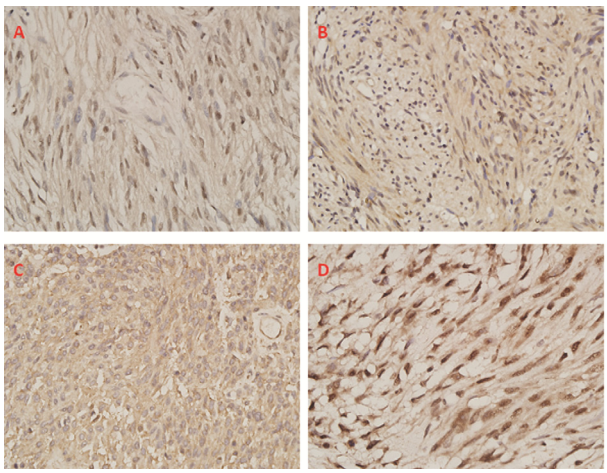

Figure 1. Expression of MMP-2 and MMP-9 proteins in EGISTs (original magnification, ×40). A) MMP-2 was expressed in the mesentery, the cytoplasm contains no tan particles and is similar in color to the background; thus, the score is 0. **B)** MMP-2 was expressed in the omentum, the color of the cytoplasm is slightly darker than that of the background and negative controls; thus, the score is 1. **C)** MMP-9 was expressed in the abdominal cavity, there are dim or clearly brown particles in the cytoplasm; thus, the score is 2. **D)** MMP-2 was expressed in the retroperitoneum, strong brown particles are clearly shown in the cytoplasm; thus, the score is 3

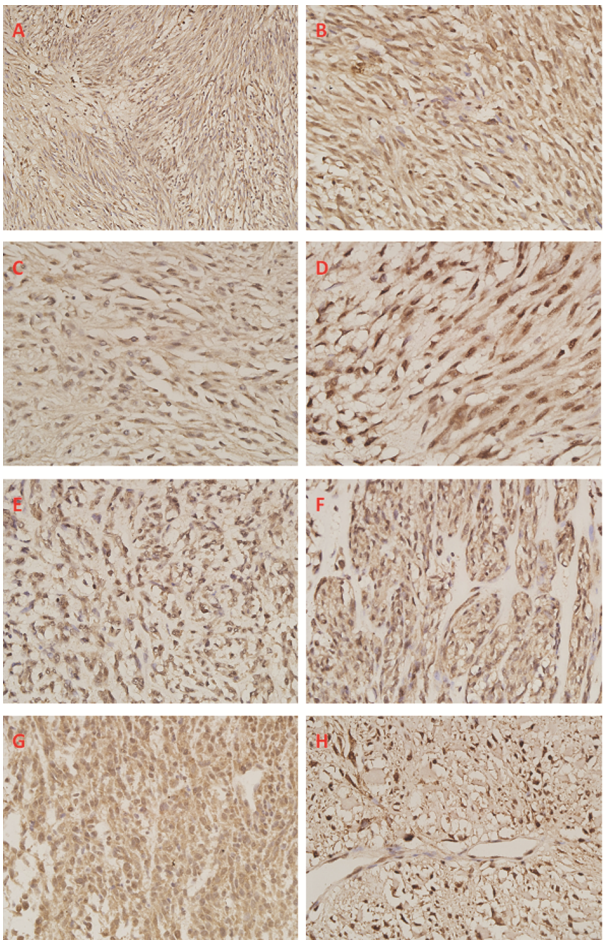

Figure 2. Expression of MMP-2 Protein in Different EGISTs (Original Magnification, ×40). A) In the abdominal cavity, the score is 1; **B)** in the mesentery, the score is 2; **C)** in the omentum, the score is 1; **D)** in the retroperitoneum, the score is 3; **E)** in the liver, the score is 2; **F)** in the esophagus, the score is 2; **G)** in the prostate, the score is 3; **H)** in the pancreas, the score is 2

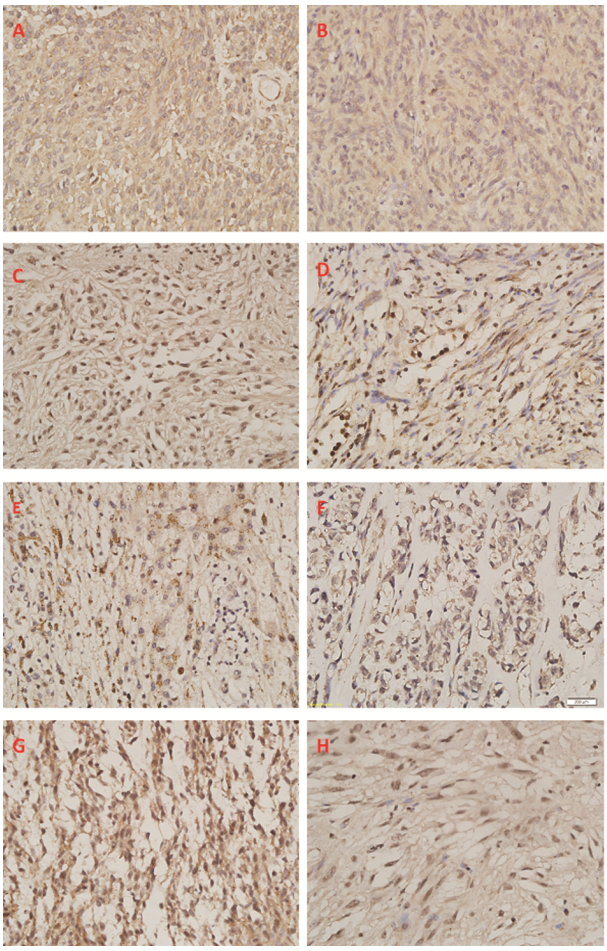

Figure 3. Expression of MMP-9 Protein in Different EGISTs (Original Magnification, ×40). A) In the abdominal cavity, the score is 2; **B)** in the mesentery, the score is 1; **C)** in the omentum, the score is 1; **D)** in the retroperitoneum, the score is 1; **E)** in the liver, the score is 1; **F)** in the esophagus, the score is 1; **G)** in the prostate, the score is 3; **H)** in the pancreas, the score is 1

of MMP-2 and MMP-9 ($p<0.05$). MMP-2 protein was expressed in all of the epithelial cell type of EGISTs and 81.25% of the spindle cell type of EGISTs, although there was no statistical difference of MMP-2 expression between these two types ($p>0.05$). Furthermore, there was no association between MMP-2 expression and age, sex, primary site, or cell morphology in these 21 EGIST patients ($p>0.05$). In addition, MMP-2 expression in the very low risk group, low risk group, intermediate risk group, and high risk group was 67% (2/3), 75% (3/4), 100% (1/1), and 92% (12/13), respectively, indicating that MMP-2 expression increased with the degree of risk.

Specifically, MMP-2 expression increased with the tumor size. If the cut-off point was set to 2 cm vs. 5 cm, there was a statistical difference in MMP-2 expression between a tumor that was less than 2 cm and one that was greater than 5 cm ($p<0.05$). As for the tumor cell mitotic rate, these 21 cases were divided into three groups (≤5 per 50 HPFs, 6-10 per 50 HPFs, and ≥10 per 50 HPFs). MMP-2 protein was expressed in 100% (21/21 cases) of tumors with a cell mitotic rate of 6-10 per 50 HPFs. MMP-2 expression was greater in tumors with a cell mitotic rate of ≥10 per 50 HPFs than in tumors with a cell mitotic rate of ≤5 per 50 HPFs, which was statistically significant

(p<0.05).

Moreover, MMP-2 protein was expressed in 100% (10/10) of EGISTs with necrosis, whereas only in 72.7% (8/11) of non-necrotic tumors; this difference was statistically significant (p<0.05). In addition, MMP-2 protein was expressed in 100% (10/10) of metastatic EGISTs, whereas it was expressed in only 72.7% (8/11) of nonmetastatic EGISTs; again, this difference was statistically significant (p<0.05).

Association of MMP-9 expression with EGIST clinicopathological characteristics

Next, we associated MMP-9 expression with clinicopathological data from EGIST patients. We found that there was an obvious association between MMP-9 expression and tumor size, mitotic rate, tumor necrosis, and metastasis (p<0.05). In addition, there was a high positive association between the expression of MMP-2 and MMP-9 proteins (p<0.05). MMP-9 protein was expressed in all of the epithelial cell type of EGISTs, whereas it was expressed in only 75% of the spindle cell type of EGISTs, although there was no statistically significant difference between these two groups in terms of MMP-9 expression (p>0.05).

However, there was no correlation between MMP-9 expression and age, sex, primary site, or cell morphology in any of these 21 EGIST patients (p>0.05). In contrast, MMP-9 protein was expressed in 33% (1/3) of patients in the very low risk group, 75% (3/4) of patients in the low risk group, 100% (1/1) of patients in the intermediate risk group, and 92% (12/13) of patients in the high risk group of EGISTs, indicating that MMP-9 expression increased with the degree of risk.

Discussion

In this study, we analyzed the protein expression of MMP-2 and MMP-9 in EGIST tissue specimens and found that they are highly expressed in EGIST tissues. The expression of MMP-2 and MMP-9 proteins was associated with increased tumor size, mitotic rate, tumor necrosis, and distant metastasis. In addition, there was an association between the expression of MMP-2 and MMP-9 proteins. Moreover, MMP-2 and MMP-9 proteins were expressed in all of the epithelial cell type of EGISTs, whereas only in 75% of the spindle cell type of EGISTs. The protein expression of MMP-2 and MMP-9 was increased with the degree of risk of EGISTs. These data indicate that the detection of MMP-2 and MMP-9 expression may be useful to predict EGIST progression.

To date, surgical removal is still the major option for EGIST treatment; however, up to 80% of EGIST patients who undergo surgery will face tumor recurrence and metastasis (Bloomston et al., 2002). Most recently, EGIST patients who underwent imatinib treatment had an extended 5 years of survival, although approximately one half of patients eventually showed drug resistance after more than six months of treatment. Therefore, more effective treatments are urgently needed. To this end, we performed the current study and explored whether MMP-2 and MMP-9 are novel targets in the effective control of EGIST progression. Previously, Bloomston et al. (Bloomston et al., 2002) transfected MMP-2 into human breast carcinoma cells and found that the invasion ability of tumor cells increased dramatically. Moreover, MMP-2 has been shown to be ubiquitously expressed in colorectal and gallbladder cancer tissues. For example, the expression of MMP-2 and its inhibitor TIMP-2 has been shown to be altered during the progression of colorectal cancer (Levy et al., 1991; Ring et al., 1997). Additionally, MMP-9 mRNA expression and early recurrence and serious prognosis of colorectal cancer have been significantly linearly correlated (Zeng et al., 1996). Furthermore, the MMP-9 inhibitor TIMP-1 has been shown to be inversely associated with colon cancer progression (Yukawa et al., 2001). In addition, the overexpression of MMP-9 induces angiogenesis and enhances tumor growth by degrading the ECM and increasing the invasion and metastasis ability of tumor cells. MMP-2 and MMP-9 also are involved in malignant behaviors of gastrointestinal tract cancers. Langers et al. (Langers et al., 2012) have found that high expression of MMP-2 and MMP-9 in the mucosa of colon cancer patients is associated with a 5-year mean survival (Grigioni et al., 1994; Sier et al., 1996). In this study, we found similar results regarding MMP-2 and MMP-9 expression in EGISTs, i.e., the expression of MMP-2 and MMP-9 was associated with tumor progression, which is consistent with previous findings (Grigioni et al., 1994; Sier et al., 1996; Zeng et al., 1996; Ring et al., 1997; Yukawa et al., 2001; Zhang et al., 2003; Liu et al., 2010; Langers et al., 2012).

However, our current study did not show the underlying molecular mechanisms responsible for upregulation of MMP-2 and MMP-9 in EGISTs or how MMP-2 and MMP-9 proteins promote EGIST progression. We only provided evidence of an association of MMP-2 and MMP-9 expression with advanced tumor behaviors, such as increased tumor size, necrosis, mitotic rate, and distant metastasis of EGISTs. Further studies will investigate whether targeting MMP-2 and MMP-9 expression or enzymatic activity could help control EGIST progression.

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