MMP13 is a potential prognostic marker for colorectal cancer

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Abstract. Matrix metalloproteinase 13 (MMP13), a member of the matrix metalloproteinase family, is considered to play a role in the tumor cell proliferation and invasion. The purpose of this study was to verify the expression of MMP13 in colorectal cancer (CRC) *in vitro* and *in vivo*, and subsequently analyze whether the MMP13 expression levels correlate with the clinicopathological features and prognosis of CRC patients. We assessed MMP13 mRNA expression profile in human colorectal adenocarcinoma cell lines by quantitative RT-PCR, and further verified if it was a secreted protein or not by Western blot analysis of cell culture medium. By immunohistochemical staining the immunoreactivity of MMP13 showed that MMP13 was localized in the cytoplasm of CRC cells. MMP13 mRNA expression of 80 cancerous tissues collected from UICC stage I to III CRC patients were examined by membrane array. The correlations between MMP13 mRNA expression and patients' clinicopathological features were analyzed. MMP13 was confirmed to be a secreted protein by Western blot analysis. The larger tumor size (P<0.0001), advanced clinical stage (P=0.002), tumor invasive depth (P=0.039), lymph node metastasis (P=0.001) and post-operative relapse (P<0.0001) were significantly correlated with the MMP13 mRNA overexpression. Patients with MMP13 mRNA overexpression have a higher risk of postoperative relapse (P<0.0001; OR=7.989; 95% CI, 2.607-24.481). The results of the present study highly suggest that MMP13 is a secreted protein with a significant correlation to development of postoperative relapse; hence it could be a potential prognostic marker for CRC patients.

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Introduction

Colorectal cancer (CRC) is a leading cause of morbidity and mortality in Europe and the United States (1,2). In Taiwan, CRC is one of the most frequent malignancies and is also the third major cause of cancer death, with more than 10,000 new cases and over 4,100 patients expiring from this disease in 2007 (3); its incidence is gradually approaching Western figures. Even with the recent advances in diagnostic and surgical techniques, the outcome remains dismal in the cases of advanced disease (4). Pathologic prognostic factors of primary tumor invasion, regional lymph node involvement, and the presence or absence of metastasis have been used for many decades as the three major prognostic determinants for CRC patients, and predict the risk of relapse of this malignancy. Several investigators have reported that approximately 40-50% of CRC patients who undergo a supposedly curative resection subsequently develop metastatic disease and die of their disease within 5 years (5,6).

As tumor invasion and metastasis ultimately affect cancer prognosis, it is important to predict the invasion potential of tumor cells. Several steps are involved in the invasion and metastasis of malignant cells including the attachment of cells to the extracellular matrix (ECM), the breakdown of matrix components, cell detachment, and migration of cells through the degraded matrix. Of these steps, the degradation of the ECM surrounding tumor cells is a key move in tumor invasion and progression (7). The main enzymes involved in matrix degradation is the matrix metalloproteinases (MMPs) (8,9). The human MMP family includes 24 members (10,11), and they mainly function as proteolytic enzymes to dissolve ECM (12,13). This family of proteins not only participates in normal physiological functions, such as wound healing and modulating growth factors and enzyme activation pathways (13-16), and it also plays an important role in cancer infiltration and metastasis (9,17-19). MMP1, MMP8 and

MMP13 are all collagenases. These three types of collagenase all have very high structural homogeneity. They can break down collagen types I, II, III, and V (16). Among them, MMP13 is most effective in breaking down type II collagen (20). Recently, several studies have investigated the relationship between MMP13 and cancers. For example, MMP13 was overexpressed in CRC tissues (21,22), and Leeman *et al* also showed that CRC patients with a high immunohistochemical MMP13 staining score in cancerous tissue had a trend towards poorer survival (23).

In the current investigation, we utilized our well-established membrane array technique and quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) technique to analyze MMP13 mRNA expression profile in human CRC adenocarcinoma cell lines SW480 (UICC stage II) and SW620 (UICC stage III). Western blot analysis of the cell culture medium was used to identify if MMP13 is a secreted protein. Using immunohistochemical (IHC) staining, immuno-reactivity of MMP13 was identified in the cytoplasm of tumor cells of CRC patients. Finally, we employed a membrane array to quantitatively analyze the expression of MMP13 mRNA markers in 80 clinical CRC tissues, and to analyze the correlation between MMP13 expression profiles and clinicopathological features, and to clarify whether it is a new prognostic marker for CRC patients.

Patients and methods

Sample collection. Enrolled in this study were 80 American Joint Commission on Cancer/International Union Against Cancer (AJCC/UICC) stage I-III CRC patients (52 males and 28 females; mean age, 63.09±10.67 years) who underwent surgical treatment in the Department of Surgery at Kaohsiung Medical University Hospital. Patients with other malignant diseases in their medical history were excluded. Clinical stage and pathological features of primary tumors were defined according to criteria of the AJCC/UICC (24). Written informed consent was obtained from all subjects and/or guardians for use of their tissue samples. Sample acquisition and subsequent use were approved by the institutional review board at the Kaohsiung Medical University Hospital. Complete medical history, physical examination, and laboratory studies, including assessing serum carcinoembryonic antigen (CEA) levels were reviewed. Computed tomography (CT) or magnetic resonance imaging (MRI) of abdomen, abdominal ultrasonography, and chest radiography, bone scans, and colonoscopy were performed before surgical intervention. The development of new post-operative recurrent or metastatic lesions was defined as postoperative relapse. All paired samples including tumor and tumor-free tissues were obtained from 80 patients. Tumor grading was carried out and confirmed by pathologists. Samples were further used for real-time PCR, Western blot and membrane array analysis.

Detection of serum CEA. A 3-ml sample of peripheral blood was obtained from enrolled patients less than one week prior to operation. Serum CEA levels were determined by means of an enzyme immunoassay test kit (DPC Diagnostic Product Co., Los Angeles, CA) with the upper limit of 5 ng/ml defined as normal according to the manufacturers of the kits that were used.

Total RNA extraction and first strand cDNA synthesis. Total RNAs were extracted from CRC patient's tissue and cell line with ISOGEN™ (Nippon Gene, Toyama, Japan) and QIAmp® RNA Blood Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions (25). The reaction mixtures with RNA were incubated at 42˚C for >2 h, heated to 95˚C for 5 min, and then stored at -80˚C until analysis.

Real-time polymerase chain reaction (RT-PCR). Two microliters of each cDNA sample were used for each reaction. Sequences of the oligonucleotide primers were designed according to a PCR primer selection program based on primer 3 at http://frodo.wi.mit.edu/cgi-bin/primer3/prime3www.cgi (Table I). PCR products were quantified by measuring the fluorescent intensity at the end of each amplification cycle. For each sample, real-time PCR analysis was repeated in three independent experiments to ensure the reproducibility of results. We used ß-actin and *Oryza sativa* sequence, respectively, as positive and negative control, and used dd water to be blank control.

Membrane array. The procedure of the membrane-array method for gene detection was performed based on our previous study (26). Visual OMP3 (Oligonucleotide Modeling Platform, DNA Software, Ann Arbor, MI) was used to design probes for target gene and ß-actin, and the latter served as an internal control (Table II). The newly synthesized oligonucleotide fragments were dissolved in distilled water to a concentration of 20 mM, applied to a BioJet Plus 3000 nl dispensing system (BioDot, Irvine, CA), which blotted the target oligonucleotide, and the ß-actin control sequentially (0.05 μl per spot and 1.5 mm between spots) on SuPerCharge nylon membrane (Schleicher and Schuell, Dassel, Germany) in triplicate (Fig. 1A). After rapid drying and cross-linking procedures, the preparation of membrane array was accomplished (27). The membrane array was used to analyze the gene expression of tumor and normal counterpart tissue in 80 CRC patients.

Preparation of digoxigenin (DIG)-labeled cDNA targets and hybridization. First-strand cDNA targets for hybridization were made by reverse transcription of the mRNA from the tumor and corresponding normal tissues of CRC patients in the presence of DIG-labeled UTP (Roche Diagnostics GmbH, Penzberg, Germany) using SuperScript II reverse transcriptase (Gibco-BRL). The hybridized arrays were then scanned with an Epson Perfection 1670 flatbed scanner (Seiko Epson Corp., Nagano-ken, Japan). Subsequent quantification analysis of each spot's intensity was carried out using AlphaEase® FC software (Alpha Innotech Corp., San Leandro, CA). Spots consistently carrying a factor of two or more were considered as differentially expressed. A deformable template extracted the gene spots and quantified their expression levels by determining the integrated intensity of each spot after background subtraction. The fold ratio for each gene was calculated as follows: spot intensity ratio = mean intensity of target gene/mean intensity of ß-actin.

Table I. Primer sequences of target genes.

Gene	Primer sequences	Product (bp)
MMP13	Forward: 5'-GTTCTTCCCTTGATGGCCGATCATAT-3' Reverse: 5'-GTGATCCCTTGAGATATGGAAGGATGC-3'	140
ß-actin[a]	Forward: 5'-GCATCCACGAAACTACCTTC-3' Reverse: 5'-CAGGAGGAGCAATGATCTTG-3'	183

[a]ß-actin primers were added as internal controls to correct for the differences in the cells.

Table II. Oligo sequences of target genes.

Gene name	Oligo sequence
MMP13	5'-AATAAGTGCCAAGCACCCTCCCCAAGTATCAATAGGCACTGTGGGAAGTG-3'

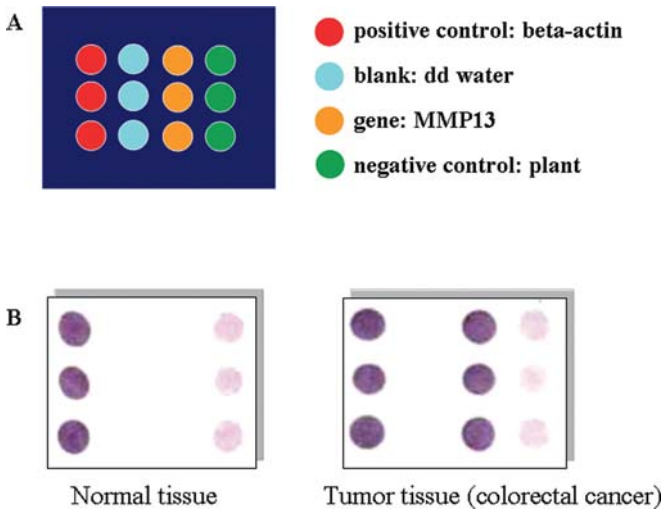

Figure 1. Results of membrane array. (A) Location of dotting on gene array nylon membranes. The arrangement of gene dotting on the nylon membranes is demonstrated. Each tested gene was dotted in triplicate on the nylon membrane. On the right side, there are three repeated dots of negative control (TB, tuberculosis gene). Three repeated dots of positive control (ß-actin) and three repeated dots of blanks (dd water) are on the left side. (B) Figure of color expression of Nylon membrane gene array. Color effects of the MMP13 gene on nylon membranes. The involved tissue samples were colorectal cancer tissue paired with normal colorectal tissue. Left picture is the genetic expression of a normal colorectal tissue sample. Right picture is the genetic expression of a colorectal cancer tissue sample.

Fig. 1A provides a representation of the membrane array with target gene (MMP13), one housekeeping gene (ß-actin), one bacterial gene (TB), and the blank control (dd water).

ATCC cell lines. In this study, to further validate whether MMP13 is indeed a secretion protein, other than cultivating two lines of ATCC human colorectal cancer cell lines, we also performed Western blot analysis for MMP13 protein detection in 1 line of ATCC human cervical cancer cell lines, 4 ATCC human breast cancer cell lines, and 2 ATCC human lung cancer cell line. Four different kinds of cell lines were purchased from ATCC (American Type Culture Collection, ATCC, Rockville, MD, USA) including two human CRC cell lines [SW480 (CCL228) and SW620 (CCL227)], one human cervical cancer cell line [CRL13011 (HeLa NR1)], four human breast cancer cell lines [CRL1500 (ZR75-1), HTB133 (T47D), HTB26 (MDA-MB231) and HTB22 (MCF7))] and two human lung cancer cell lines [CRL5800 (NCI-H23) and CRL5807 (NCI-H358)].

Western blot analysis. Cells were placed in a T75 culture dish and grown until it was 80% confluent. Next, the cell culture solution was discarded, and the cells were washed three times with PBS. Afterwards, 8 ml of FBS culture solution was poured in, and then placed into a 5% CO_2 incubator at 37˚C for cultivation. After 48 h, the top clear solution was collected, and then concentrated with an Amicon Ultra-4 PLGC Centrifugal Filter Unit (Blossom Biotechnologies Inc., Taiwan). After 4 μl of reducing dye was added into 20 μl of the concentrated clear cell solution sample on top, it was poured onto a specifically made 10% SDS electrophoresis gel for electrophoresis. Target protein antibodies were added under room temperature and shaken for 1 h for specificity linking. After washing off non-specific bound antibodies with PBS, secondary antibodies of the target HRP were then added and shaken for 1 h under temperature for immune reactions. After washing off extra antibodies, the substrate, Immobilon Western Chemiluminescent HRP Substrate (Millipore, Mississauga, Canada), was added to achieve color effects.

Immunohistochemical stain (IHC). The CRC tissues were baked on slides at 60˚C for 1 h. De-waxed biopsy samples were soaked in 10 mM of sodium citrate, and placed in

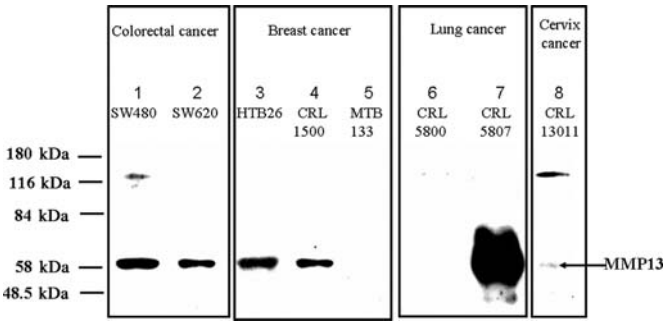

Figure 2. Results of Western blot. The top clear cell solution was collected and concentrated, and then Western blot analysis was used to confirm whether MMP13 is a secreted protein. Samples 1 and 2, ATCC human colorectal cancer cell lines: 1, SW480 cell line, 2, SW620 cell line. Samples 3-5, ATCC human breast cancer cell line: 3, HTB26 cell line, 4, CRL1500 cell line, and 5, MTB133 cell line. Samples 6 and 7, ATCC human lung cell line: 6, CRL5800 cell line and 7, CRL5807 cell line. Sample 8, ATCC human cervical cancer cell line CRL13011.

a microwave for 5 min. Three repeated antigen retrieval procedures were then performed. After washing off the antibody at the end of the specific bond, DAB substrate was added (20 ml of DAB + 1 ml substrate buffer solution; Dako, #K3468). The slides were observed at all times under the microscope to prevent over dyeing. To examine the possibility of false positive results, we used a non-immune antiserum instead of the primary antibody as negative control. When 5% or <5% was stained, the results were considered negative. Semi-quantitative scores were used for MMP13 stains according to the percentage of positively stained cells (+, 6-10%; ++, 11-25%; +++, 26-50%; ++++, >50%). Cancer tissues that expressed scores of ++ or +++ or ++++ were regarded as the MMP13 overexpression group, whereas those with scores of + or negative staining were regarded as the MMP13 non-overexpression group.

Statistical analysis. All data were analyzed using the Statistical Package for the Social Sciences Version 11.5 software (SPSS Inc., Chicago, IL). Continuous data are presented as means ± SE and P-values were determined by unpaired Student's t-test. The two-sided Pearson χ^2 test was used to analyze the differences in the overexpression of the MMP13 mRNA between different gender, age groups, tumor size, stages, invasion depth, lymph node metastasis, CEA level and postoperative relapse. A P-value of <0.05 was considered statistically significant.

Results

Confirmation of MMP13 as a secretion protein by Western blot test. MMP13 (pro form) and MMP13 (active form) protein of 60 KD of molecular weight were detected on the clear cell solution on top (Fig. 2). The test results of all cell culture solutions showed that all the different cell lines exhibited MMP13 (Table III). Interestingly, MMP13 is indeed a secreted protein and it is highly expressed in CRC cell lines. The positive rate of MMP13 protein in CRC cell lines is 100%.

Table III. Results of MMP13 secretion in 9 cell lines.

Cell lines	MMP13 protein expression	Positive result samples/ total samples	Positive rate (%)
Colorectal cancer		2/2	100
SW480	+		
SW620	+		
Cervical cancer		1/1	100
CRL13011	+		
Breast cancer		2/4	50
CRL1500	+		
HTB133	-		
HTB26	+		
HTB22	-		
Lung cancer		1/2	50
CRL5800	-		
CRL5807	+		

+, indicates the cell line secretions expressed MMP13 protein. -, indicates no MMP13 protein expression.

Localization and expression of the MMP13 protein in human CRC tissue by immunohistochemical stain. At the same time, we utilized MMP13 antibody to perform IHC stain in the collected tissue biopsies of CRC patients, to localize and analyze MMP13 expression in CRC tissues. The antibody used was the same as that in Western blot analysis. A positive yellowish-brown appearance after tissue dyeing indicated that MMP13 was overexpressed (Fig. 3), and the immunoreactivity (yellowish-brown appearance) was localized to the cytoplasm of CRC tumor cells.

MMP13 mRNA expressions in CRC tissue specimens. We spotted the well-designed oligonucleotide for MMP13 gene onto nylon membrane for further analysis (Fig. 1A). Membrane array analysis was performed on 80 cancerous tissues collected from UICC stage I-III CRC patients and paired human normal colorectal tissue samples. Based on results of membrane array hybridization analysis (Fig. 1B), if the gene presented a color response of >2-fold between the CRC and normal tissue, it was defined as MMP13 mRNA overexpression. Among the 80 patients, there were 42 CRC cases (52.5%) showing MMP13 mRNA overexpression and 38 CRC cases (47.5%) did not have MMP13 overexpression.

Correlation between MMP13 mRNA expressions with clinicopathological data. This study assessed correlations between MMP13 mRNA expression and clinicopathological features of 80 Taiwanese CRC patients (Table IV). Statistically significant correlation existed between MMP13 mRNA overexpression and tumor size, cancer stage, invasion depth, lymph node metastasis and postoperative relapse, respectively (all P<0.05). MMP13 increased their differential expression

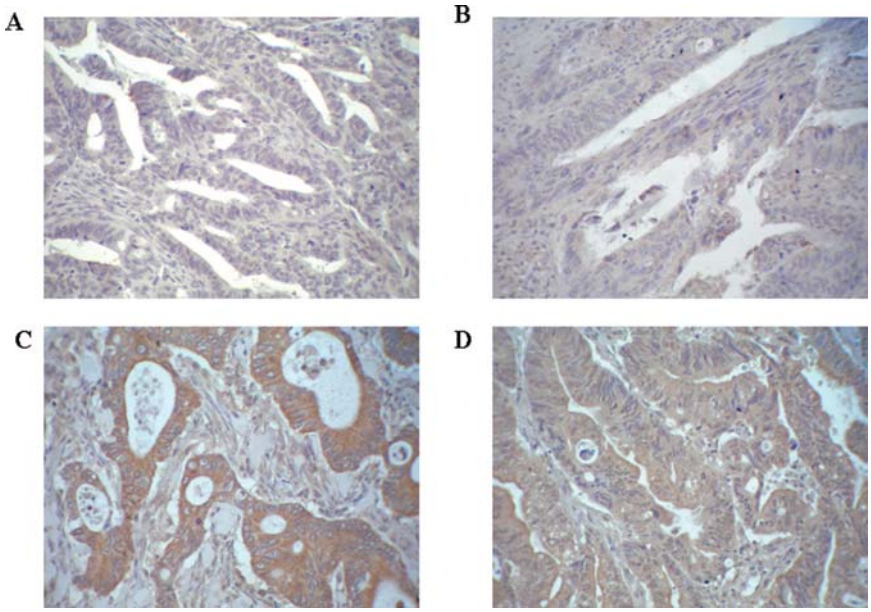

Figure 3. Immunohistochemical staining of MMP13 in the colorectal cancer tissues. (A) The staining was absent over the control section when the primary antibody was replaced with non-immune antiserum; (B) Negative MMP13 immunoreactivity; (C and D) MMP13 immunoreactivity (score +++ to ++++) was seen within the cytoplasm of colorectal cancer tissue, of which was considered as overexpression group (original magnifications, x200).

Table IV. Correlation between the clinicopathological features and MMP13 overexpression for 80 colorectal cancer patients.

		MMP13 overexpression		
Characteristic	Total case n	Positive n (%)	Negative n (%)	P-value
Gender				
Male	52	26 (50)	26 (50)	0.542
Female	28	16 (57.1)	12 (42.9)	
Age (years)				
<60	33	17 (51.5)	16 (48.5)	0.882
≥60	47	25 (53.2)	22 (46.8)	
Tumor size (cm)				
<5	43	14 (32.6)	29 (67.4)	<0.0001
≥5	37	28 (75.7)	9 (24.3)	
Stage (UICC)[a]				
I + II	38	13 (32.4)	25 (67.6)	0.002
III	42	29 (69)	13 (31)	
Invasion depth				
T1 + T2	12	3 (25)	9 (75)	0.039
T3 + T4	68	39 (57.4)	29 (42.6)	
Lymph node metastasis				
Negative	41	14 (34.1)	27 (65.9)	0.001
Positive	39	28 (71.8)	11 (28.2)	
Postoperative relapse				
Negative	51	19 (43.9)	33 (56.1)	<0.0001
Positive	29	23 (73.9)	5 (26.1)	
Serum CEA marker				
Normal	34	15 (44.1)	19 (55.9)	0.197
Abnormal	46	27 (58.7)	19 (41.3)	

[a]International Union Against Cancer.

in the advanced stage (stage III/I + II, P=0.002; OR, 4.290; 95% CI, 1.682-10.944). Patients with MMP13 overexpression have a risk of postoperative relapse of 7.989 times greater than that of those without MMP13 overexpressions (P<0.0001; 95% CI, 2.607-24.481).

Discussion

CRC is one of the most common malignant tumors and has a relatively poor prognosis. The reason for this poor prognosis is that CRC exhibits extensive local invasion, frequently regional lymph node metastasis or metastatic tumor spread, even at the time of initial diagnosis. The recent identification of gene overexpression in CRC, combined with advances in molecular biology, provides the opportunity to establish more sensitive, specific, and cost-effective ways of identifying advanced CRC. Knowing how to rapidly organize new techniques for exploring the prognosis is a key to providing physicians with the information needed for further intervention.

Tumor invasion and metastasis involve degradation of different components of the extracellular matrix and require the actions of proteolytic enzymes, such as MMPs, produced either by the tumor cells themselves or by surrounding stromal cells (28). It therefore, seems evident that MMPs play an important role in tumor invasion and metastasis (29,30). The mechanism involved in MMP13 activation is complex. It has been observed that MMP13 expression is enhanced by cytokines, such as transforming growth factor-α and -ß, and tumor necrosis factor-α (31). Leeman *et al* localized MMP13 staining to the cytoplasm of tumor cells (23), which is consistent with studies of other tumors, including breast cancer and malignant melanoma, of which they have also found that MMP13 expression is primarily present in tumor cells (32,33). In a head and neck squamous cell carcinoma study, MMP13 was found to be related to the invasiveness of cancer cells, and could be used for evaluation of patient's prognosis (34). MMP13 is overexpressed in metastatic tissues in prostate cancer patients, thus it can be a biomarker for prostate cancer diagnosis, treatment monitoring, and prognosis determination (35). Nielsen *et al* pointed out that in the process of breast cancer turning from ductal carcinoma *in situ* to invasive ductal carcinoma, MMP13 will break down basement membranes of tissues to form an invasive cancer (36). A consensual theory may be reached for this biochemical characteristic, which demonstrate that MMP13 was only expressed in the highly invasive breast cancer cell line, MDA-MB-231, but not in the less invasive MLF-7 breast cancer cell line (37-39). The above observation lends support to the hypothesis of different MMP13 expression in each type of tumor cells.

In the present study, MMP13 staining was localized to the cytoplasm of tumor by immunohistochemical staining firstly, and further verified that MMP13 was a secreted protein by Western blot analysis. We found that MMP13 was highly expressed in CRC cell lines compared to breast or lung cancer cell lines. Furthermore, we studied MMP13 mRNA expression in the cancerous tissue of CRC patients by membrane array method, and the correlations between MMP13 mRNA expression and patients' clinicopathological features were analyzed. Tumor size larger than 5 cm, the tumor invasion depth deeper than muscularis propria and positive lymph node metastasis were correlated with MMP13 overexpression with statistical significance. Advanced cancer stage was significantly related to MMP13 overexpression, the risk of which was higher in stages III than in stage I/II (P=0.002; OR, 4.290; 95% CI, 1.682-10.944). In addition, patients with MMP13 overexpression have a risk of postoperative relapse of 7.989 times greater than that of those without MMP13 overexpressions. The increase in MMP13 overexpression probably represents MMP13 up-regulation. It is logical that advanced tumors have far higher MMP13 overexpression because this molecule catalyses the breakdown of ECM for invasion. From results obtained in this study, we believe that MMP13 has the potential to become a new CRC prognostic marker, when accompanied by current clinical surveillance methods, may increase the rate of early prediction of relapse and provide benefits in establishing novel therapeutic strategies for preventing the invasion and metastasis of these tumor cells.

In summary, we found that MMP13 is highly-expressed in mRNA level with the advance of CRC. Therefore, we suggest that the secreted protein MMP13 has the potential to be a new prognostic marker for CRC.

Acknowledgements

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