MiR-125a-5p *regulates* ERBB2 *in gastric cancer cells*

Using *in silico* microRNA target prediction tools, such as miRanda (23), PicTar (24) and TargetScan (25), we identified the sequence of the *miR-125a-5p* binding sites in the 3' UTRs of transcripts encoding *ERBB2* (Fig. 2A) . To investigate binding and repression, a luciferase reporter assay was performed with a vector which included the ORF sequence and 3'UTR of *ERBB2* downstream from the luciferase reporter gene (Luc-*ERBB2*-WT). Transient cotransfection of NUGC4 cells with the reporter plasmid and *Pre-miR-125a* significantly reduced luciferase activity in comparison with the negative control ($P < 0.05$). However, the activity of the reporter construct with mutant sequence (Luc-*ERBB2*-mutant) was unaffected by simultaneous transfection with *Pre-miR-125a* (Fig. 2B). These data suggest that *ERBB2* mRNA is a direct functional target of *miR-125a-5p*.

MiR-125a-5p *expression and ERBB2 protein expression in clinical samples*

Of the 87 gastric cancer patients we examined for the expression of *miR-125a-5p*, formalin-fixed, paraffin-embedded (FFPE) surgical sections were available in 52 cases. To explore the association between *miR-125a-5p* expression and ERBB2 protein expression status, we performed immunohistochemical analysis using these samples. The results showed that in the low-ERBB2 expression group (ERBB2 staining 0 or 1+; n = 45), the expression of *miR-125a-5p* was significantly increased compared to that in the high-ERBB2 expression group (ERBB2 staining 2+ or 3+; n =

7) (mean ± SEM; 4.97 ± 0.88 vs 2.34 ± 2.24; $P < 0.05$) (Fig.2C). Images of IHC are shown in Supplementary Fig.2.

MiR-125a-5p *and* ERBB2 *expression levels in carcinoma cells of different origin from the NCI60 tumor cell panel*

To evaluate whether the *miR-125a -ERBB2* pathway functions in cells of different origin, we exploited the cDNA array and the miRNA array dataset of NCI60, as described in Material and Methods. *ERBB2* expression was inversely correlated with the expression of *miR-125a-5p* in 23 cell lines including colon, lung, prostate and renal cancer (correlation coefficient -0.5348, $P = 0.0086$; Fig. 2D).

***Both ERBB2 and its primary downstream signal through AKT were suppressed by* miR-125a-5p**

Using RT-PCR, we confirmed that *miR-125a-5p* expression in *Pre-miR-125a*-treated cells was significantly higher than that in untreated cells (parent) and in *Pre-miR negative control* treated cells ($p < 0.05$, Fig.3A).To determine whether *miR-125a-5p* suppresses *ERBB2* and its downstream signaling in the gastric cancer cell line NUGC4, cell lysates of transfected cells were analyzed by Western blotting. Remarkable suppression of ERBB2 and phosphorylated AKT (p-AKT) was observed in *Pre- miR-125a*-treated cells, in comparison with untreated cells (parent) or Pre-miR negative control treated cells (Fig. 3B). However, AKT itself did not show significant

reduction.We also investigated the inhibition of previously reported *miR-125a-5p* targets, including apoptosis related gene, BAK1 (26) and tumor suppressor gene, p53 (27). Western blot analysis showed that protein expression of BAK1 and p53 was moderately suppressed in *Pre- miR-125a*-treated cells; however, the reduction was not as significant as that of ERBB2 (Supplementary Fig.3).

miR-125a-5p *inhibited the proliferation of gastric cancer cells in combination with trastuzumab.*

To explain the antitumor efficacy of *miR-125a-5p* in gastric cancer cells, a proliferation assay was performed with *Pre- miR-125a*-treated cells or negative control cells using NUGC4. Furthermore, we investigated whether the additional administration of trastuzumab, the ERBB2-targeting antibody, enhanced the antitumor efficacy of *miR-125a-5p.* These experiments were performed at two different concentrations of trastuzumab (0.1μg/mL [Fig. 4A] and 1 μg/mL [Fig4B]). Interestingly, *miR-125a* not only potently suppressed the proliferation of gastric cancer cells by itself, but inhibited growth more potently when combined with trastuzumab ($P = 0.0032$, Fig. 4A; $P = 0.0033$, Fig. 4B) . The combined growth inhibitory effect was more robust at the higher concentration of trastuzumab.

Discussion

Recent evidence has shown that altered patterns of miRNA expression correlate with various human cancers. The behavior of microRNAs is complex because they regulate hundreds of targets, resulting in the down-regulation of numerous target genes, including oncogenes and tumor suppressors. Therefore, exploring their clinical potential is especially worthwhile.

In the current study, we show that altered *miR-125a-5p* expression significantly affected cancer progression and prognosis in human gastric cancer. Multivariate analysis revealed that *miR-125a-5p* is an independent prognostic factor for survival. Clinicopathological analysis revealed that low *miR-125a-5p* expression contributes to more advanced tumor size and tumor invasion (Table 1). It suggests that this microRNA primarily achieves its antiproliferative effect through down-regulation of proliferation-related genes, including *ERBB2*, a member of the epidermal growth factor (EGF) receptor family of receptor tyrosine kinases which regulate a key initiator of phosphoinositide-3 kinase (PI3K)-AKT and RAS/RAF/Mitogen-Activated Protein Kinase (MAPK) signaling (28). *MiR-125a-5p* is shown to be a superior biomarker to previously reported gastric cancer biomarkers such as *DACH1* and *PDCD6* (22)

(Supplementary Table 1). However, because of the differences in patient backgrounds such as clinical stage and the presence or absence of chemotherapy, further investigation is required for adequate use of these biomarkers.

We confirmed *miR-125a-ERBB2* interaction in the human gastric cancer cell line, NUGC4. *MiR-125a- 5p* significantly repressed ERBB2 expression and the phosphorylation of its downstream molecule, AKT (Fig.3B). In addition, *ERBB2* expression was shown to be inversely correlated with expression of *miR-125a-5p* both *in vitro* and in clinical samples. Overexpression of *Pre- miR-125a* also led to the inhibition of previously reported *miR-125a-5p* targets, such as apoptosis related gene, BAK1 (26) and tumor suppressor gene, p53 (27) (Supplementary Fig.3). However, the inhibition of these tumor suppressor genes was modest compared with that of ERBB2, suggesting ERBB2 is a crucial target of *miR-125a-5p,* at least in the gastric cancer cell line NUGC4.

It is noteworthy that the growth inhibitory effect of *miR-125a-5p* was enhanced when combined with trastuzumab (Fig.4 A.B). This could be partly due to the fact that *miR-125a-5p* and trastuzumab share the same target, *ERBB2*. *MiR-125a-5p* and trastuzumab silence the *ERBB2* pathway through two different mechanisms. *MiR-125a-5p* suppresses the molecule at the post-transcriptional level before protein synthesis, while trastuzumab is a monoclonal antibody targeted against completed ERBB2 protein. In other words, *miR-125a-5p* blocks the synthesis of the oncoprotein at an earlier phase than does trastuzumab. These considerations suggest that *miR-125a-5p* mimic and trastuzumab have the potential to be highly effective against ERBB2 when

used together.

ERBB2-positive gastric cancer patients constitute about 19.0% (8.2 - 53.4%) of all gastric cancer patients (19, 29-30). A recent phase III study (the ToGA trial) combining treatment of trastuzumab and conventional chemotherapy against ERBB2-positive gastric cancer showed a statistically significant advantage in overall survival for patients who received combined therapy compared to chemotherapy alone. This reliable large scale clinical data indicates that ERBB2 is a crucial therapeutic target in gastric cancer (31).

In conclusion, our data suggest that *miR-125a-5p* functions as a powerful tumor suppressor and could be a *bona fide* prognostic marker for gastric cancer patients. Furthermore, *miR-125a-5p* mimic alone or in combination with trastuzumab could be a novel therapeutic approach against gastric cancer.

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Table 1. *miR-125a-5p* expression and clinicopathological factors

Factors	expression low group (n = 55)		expression high group (n = 32)		p value
	n	%	n	%	
Age (mean ± SD)	63.5 ± 1.60		67.2 ± 2.09		0.16
Sex					
Male	36	65.5	20	62.5	0.78
Female	19	34.5	12	37.5	
Histological grade					
Well ·Moderately	21	38.2	17	53.1	0.18
Poorly ·others	34	61.8	15	46.9	
Size					
50 mm> (small)	18	32.7	20	62.5	0.0068*
50 mm< (large)	37	67.3	12	37.5	
Depth of tumor invasion †					
m, sm,mp	12	21.8	21	14	0.031*
ss,se, si	43	78.2	11	18	
Lymph node metastasis					
Absent	15	27.3	13	40.6	0.20
Present	40	72.7	19	59.4	
Lymphatic invasion					
Absent	14	25.5	12	37.5	0.24
Present	41	74.5	20	62.5	
Venous invasion					
Absent	41	74.5	21	65.6	0.38
Present	14	25.5	11	34.4	
Liver metastasis					
Absent	50	90.9	32	91.0	0.029*
Present	5	9.1	0	9.0	
Peritoneal dissemination					
Absent	45	81.8	27	84.4	0.76
Present	10	18.2	5	15.6	
clinical stage					
I ·II	22	40	21	65.6	0.020*
III ·IV	33	60	11	34.4	

†Tumor invasion of mucosa (m), submucosa (sm), muscularis propria (mp), subserosa (ss), penetration of serosa (se), and invasion of adjacent strucures (si)

P < 0.05*

Table 2. Univariate and multivariate analysis for overall survival (Cox proportional regression model)

	Univariate analysis			Multivariate analysis		
Factors	RR	95% CI	p value	RR	95% CI	p value
Age (≦65 / >66)	0.928	0.648 - 1.314	0.673	-	-	-
Sex (male / female)	0.834	0.552 - 1.121	0.345	-	-	-
Histology grade (well,moderately/poorly & others)	1.309	0.913 - 1.946	0.146	-	-	-
Depth of tumor invasion† (m, sm, mp/ss, se, si)	8.387	2.531 51.86	0.0001*	2.415	0.650 – 15.87	0.208
Lymph node metastasis(negative / positive)	4.675	2.162 – 19.74	0.0001*	2.892	1.219 – 12.88	0.011*
Lymphatic invasion (negative / positive)	2.684	1.474 – 6.676	0.0003*	1.177	0.481 – 5.237	0.761
Venous invasion (negative / positive)	3.324	1.627 – 6.080	0.0012*	1.316	0.883 – 1.971	0.176
ERBB2 mRNA expression (low/ high)	1.466	1.003 – 2.263	0.0482*	1.010	0.991 – 1.025	0.277
MiR-125a-5p expression (high/ low)	3.018	1.372 – 7.588	0.0051*	2.438	1.035 – 6.727	0.041*

†Tumor invasion of mucosa (m), submucosa (sm), muscularis propria (mp), subserosa (ss), penetration of serosa (se), and invasion of adjacent strucures (si) RR; Relative risk CI; Confidence interval *P < 0.05

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Figure Legends

Figure 1. Kaplan-Meier overall survival curves of gastric cancer patients according to the level of *miR-125a-5p* expression. Patients in the low *miR-125a-5p* expression group had a significantly poorer prognosis than those in the high *miR-125a-5p* expression group. The high *miR-125a-5p* expression group (n = 32) was composed of patients with higher than average expression levels (8.66, normalized to RNU6B); the low *miR-125a-5p* expression group (n = 55) had lower than average expression levels.

Figure 2. *MiR-125a-5p* targets *ERBB2* **A:** (Upper) Sequence of the *miR-125a-5p* binding sites in the 3'UTRs of transcripts encoding *ERBB2*. (Lower) schematic diagram of the luciferase reporters in target validation. **B:** *miR-125a-5p* represses its target in the luciferase assay in NUGC4. Relative luciferase level = (Sample luc/Sample *renilla*)/(Control luc/Control *renilla*). Luc, raw firefly luciferase activity; *Renilla*, internal transfection control *renilla* activity; *Pre-miR* n.c., *Pre-miR* ™ negative control. The error bar represents the standard deviation (SD) from six replicates. **C:** Association of *miR-125a-5p* expression with ERBB2 protein expression status determined by immunohistochemical analysis in 52 gastric cancer cases. In the low-ERBB2 expression group (ERBB2 staining 0 or 1+; n = 45), the expression of *miR-125a-5p* was significantly increased compared to that in the high-ERBB2 expression group (ERBB2 staining 2+ or 3+; n = 7) ($P < 0.05$). Dots represent the *miR-125a -5p* expression of each sample. Horizontal lines indicate mean value of each group. **D:** *miR-125a-5p* and

ERBB2 expression levels of carcinoma cells of different origin from the NCI60 tumor cell panel. *ERBB2* expression is inversely correlated with expression of *miR-125a-5p* in 23 cell lines including colon, lung, prostate and renal cancer.

Figure 3. *MiR-125a-5p* regulates ERBB2. **A:** *MiR-125a-5p* expression after treatment with negative control or *Pre-miR-125a* in NUGC4 (Quantitative RT-PCR). *MiR-125a-5p* expression in *Pre miR-125a*-treated cells is significantly higher than in untreated cells (parent) and in Pre-miR negative control treated cells. The results are the mean ± S.D. of triplicates. **B:** Western blotting analysis of ERBB2 and phosphorylated AKT (p-AKT) in NUGC4 cells transfected with *Pre-miR-125a* or negative control. These proteins were normalized to the level of beta-actin.

Figure 4. *MiR-125a-5p* inhibits the proliferation of the gastric cancer cell line NUGC4 in combination with trastuzumab. *Pre-miR-125a*™ or *Pre-miR* negative control ™ transfected with or without trastuzumab (**A,** 0.1 μg/mL; **B,** 1 μg/mL) treatment were seeded at 8.0×10^3 cells/well in 96 well plates and cell growth was monitored every 24 h using the MTT assay. Absorbance at 0 h was assigned a value of 1. The results are the mean ± S.D. of three replicates.

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Fig.1

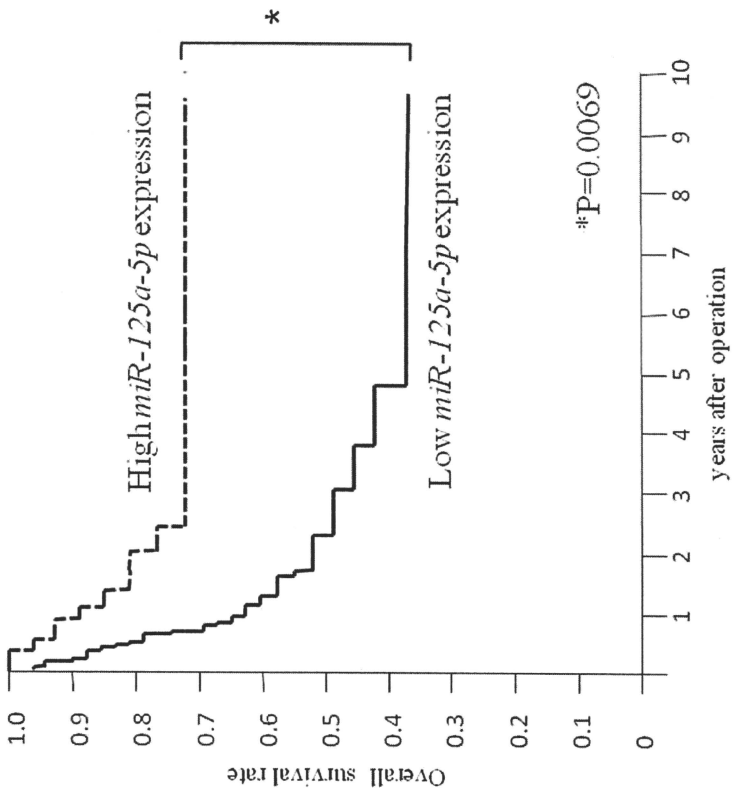

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Fig.2

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Fig.3

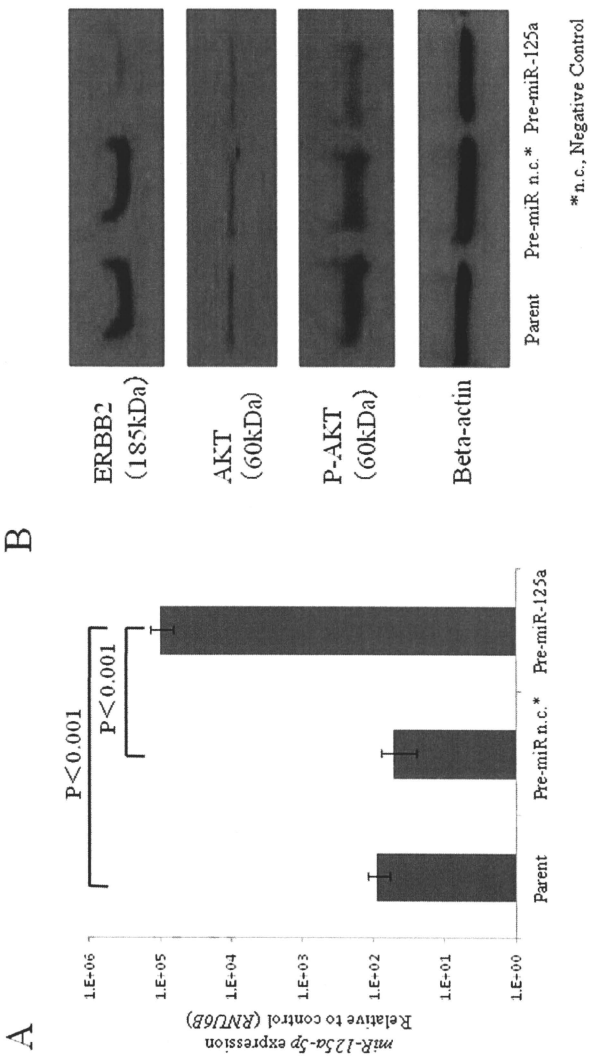

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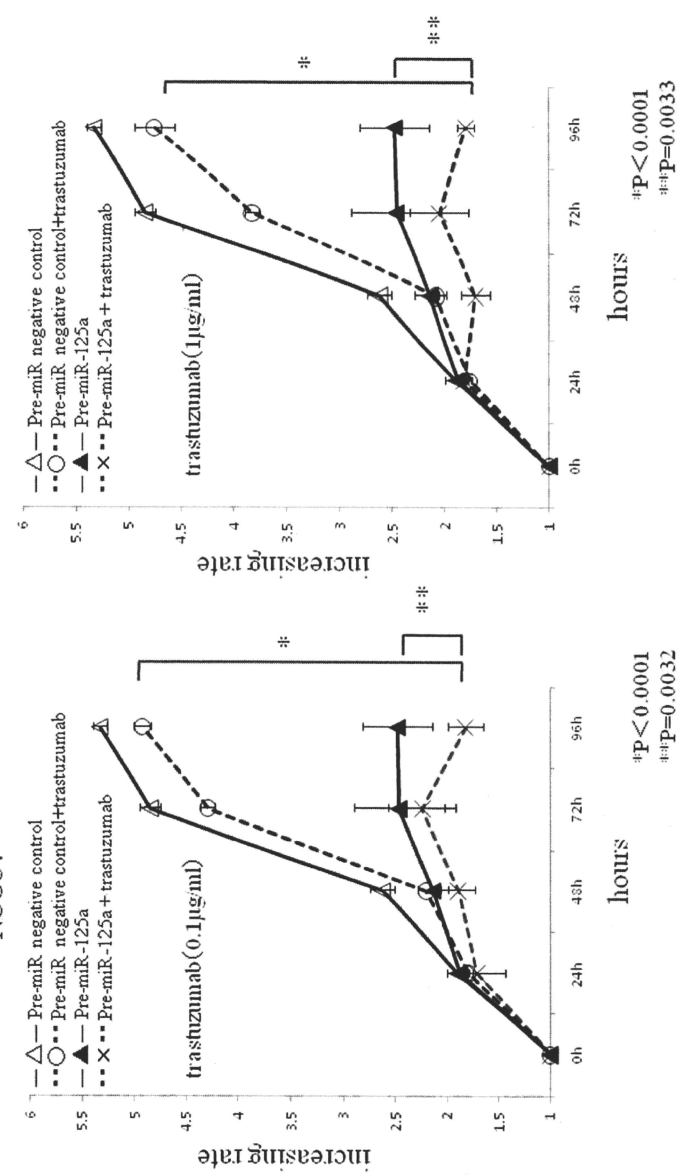
Fig.4



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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Serum Matrix-Metalloproteinase-1 is a Bona Fide Prognostic Marker for Colorectal Cancer

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ABSTRACT

Background. Matrix metalloproteinases (MMPs) are involved in the degradation of extracellular matrix components and are associated with invasion and metastasis. MMP proteins could be serum tumor markers or molecular targets in the treatment of malignancy. The purpose of the current study was to identify a prognostic serum marker in cases of colorectal cancer (CRC) prior to surgical intervention.

Materials and Methods. Laser microdissection and microarray analysis were used to characterize gene expression in 73 cases of CRC. We then focused on expression of MMP-1. We examined serum MMP-1 activity before resection in another subset of 75 cases of CRC to validate the clinical significance of MMP-1 as a prognostic marker in CRC after surgically curative operation.

Results. Disease-free survival was 51% in the MMP-1 high expression group and 81% in the low-expression group ($P < .05$). Survival was 52% in the MMP-1 high expression group and 90% in the low group ($P < .05$). In multivariate analysis for disease-free survival, MMP-1 and lymph node metastasis were significant independent prognostic indicators. In multivariate analysis of overall survival, serum MMP-1 level was the only significant independent indicator among factors.

Conclusions. Within the MMP family of proteins, MMP-1 is not a cancer-specific protease. However, MMP-1 activity does predict the future course of progression of malignant cells. Thus, MMP-1, which is activated at the primary lesion and is found in serum, assists in the clinical diagnosis of CRC. It is also an important molecule for understanding the underlying mechanism of invasion and metastasis of CRC.

In colorectal cancer (CRC), several prognostic factors have been identified, including clinicopathologic status, and gene and protein expression at primary tumor sites. Unfortunately, there are few indicators that reliably and specifically predict recurrence, metastasis, and long-term prognosis in CRC. It would be advantageous if it were possible to repeatedly assess patients' tumor markers on an outpatient basis without use of invasive techniques. In that regard, serum tumor markers would be particularly useful. Over the past half century, serum levels of carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9 have become well-established prognostic indicators in CRC.[1,2] However, no tumor marker is generally accepted as optimal in its prognostic power. Therefore, we attempted to identify novel and reliable prognostic markers within serum that could predict both tumor recurrence and metastasis.

Matrix metalloproteases (MMPs) are the most important members of a multigene family comprising more than 25 related zinc-dependent enzymes involved in the degradation of extracellular matrix components in CRC. MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-12, MMP-13, and MT1-MMP have been studied extensively in CRC.[3] In particular, MMP-2 and MMP-9 show high expression at cancer sites,

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and their expression is correlated with Dukes staging.[4,5] Moreover, MMP-7 is expressed at almost all CRC sites.[6,7] There has been unanimous agreement that enhanced expression of MMP-7 in CRC correlates with the presence of nodal or distant metastasis.[3] MMP-1, MMP-2, MMP-7, MMP-9, and MMP-11 may play roles in both conversion from adenoma to carcinoma and in the initiation of invasion and metastasis.[8] Thus, these MMPs have been regarded as particularly important in cancer progression.

Several MMPs might constitute good prognostic markers in CRC because they are secreted as inactive zymogens, are activated extracellularly, and can be measured in the serum.[9,10] Serum MMP-2, MMP-9, and MMP-7 in CRC patients have already been analyzed, and there was a clear correlation between serum MMP-7 level and poor prognosis.[11] On the other hand, matrilysin, specifically degrades collagen types I, II, and III, which are the main components of the interstitial stroma.[11,12] Increased expression of MMP-1 has been observed in CRC, and increased collagenolytic activity correlates with poor differentiation of the tumors. Therefore, we anticipated that serum levels of MMP-1 would constitute an important prognostic marker.

MATERIALS AND METHODS

Microarray Analysis of Patients' Tissue

We examined gene expression profiles of CRC-specific genes in 73 CRC patients from the Department of Surgery, Medical Institute of Bioregulation, Kyushu University. We used laser microdissection (LMD) for collection of cancer cells, stromal cells, and normal cells to perform microarray analysis. Based on microarray analyses, we selected the best MMP as a candidate tumor marker. We excluded MMP-2, MMP-9, and MMP-7 from further consideration because those MMPs had already been studied in earlier reports.

Serum Analysis of Patients

MMP-1was examined in the serum of another subset of CRC patients. This study used serial collection of serum samples from 100 CRC patients who underwent primary surgery from July 2000 to December 2004 in the Department of Surgery, Teikyo University. The patients were in good condition (performance status (PS) $\leq$ 2) and were suitable for resection of their primary tumor. All patients had a medical history, clinical examination, full blood count, and a biochemical screen of renal and liver function. Staging was done by abdominal computed tomography (CT) and chest radiography. Additional techniques such as abdominal ultrasound, chest CT, or magnetic resonance imaging (MRI) were used if needed for further refinement of staging. Serum samples were obtained before surgery, after documented informed consent.

Collection of Target Cells by LMD from Frozen Sections

For LMD, frozen section slides were fixed in 70% ethanol for 30 s, stained with hematoxylin and eosin, and the following dehydration steps were performed: 5 s each in 70, 95, and 100% ethanol and air-dried. Once dry, the sections were laser microdissected with the LMD system. Target cells were excised, at least 500–1,500 cells per section, and bound to the transfer film. A total of 15 sections were collected from every sample, equivalent to approximately 10,000–15,000 cells per sample.

RNA Extraction and the Oligonucleotide Microarray

The cells collected with LMD were placed in a microcentrifuge tube with 350 µl RLT buffer containing 1% 2-mercaptoethanol. Total RNA was extracted using the RNeasy Micro Kit (QIAGEN, Valencia) according to the manufacturer's protocol. The concentration and purity of the RNA were determined with the Nano Drop (Nano Drop Technologies, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), respectively, as described previously. We used the commercially available Human Whole Genome Oligo Microarray Kit (Agilent Technologies) that contained more than 41,000 features, including 36,866 characterized human genes. Cyanine-labeled cRNA was prepared using T7 linear amplification as described in the Agilent Low RNA Input Fluorescent Linear Amplification Kit Manual (Agilent Technologies). Briefly, 100 ng of purified total RNA was reverse transcribed to generate double-stranded cDNA using an oligo dT T7 promoter primer and MMLV reverse transcriptase. Next, cRNA was synthesized using T7 RNA polymerase, which simultaneously incorporated Cy 3 or Cy 5 labeled CTP. During this process, the samples of cancer cells were labeled with Cy 5, whereas the Human Universal Reference Total RNA (BD Clontech, Palo Alto) was labeled with Cy 3 as a control. Quality of the cRNA was verified with the Agilent 2100 Bioanalyzer. One µg aliquots each of Cy 3 labeled cRNA and Cy 5 labeled cRNA were combined and fragmented in a hybridization cocktail (Agilent Technologies). The labeled cRNAs were then hybridized to a 60-mer probe oligonucleotide microarray and incubated for 17 h at 60°C. The fluorescent intensities were determined by an Agilent DNA Microarray Scanner and were analyzed by G2567AA Feature Extraction Software Version A.7.5.1 (Agilent Technologies). This software uses the LOWESS (locally weighted linear regression curve fit) normalization method. This microarray study followed