Table I. Clinicopathological factors and ASB9 mRNA expression in 125 colorectal cancers.

Factors	High expression (%)	Low expression (%)	P-value
Age (years)			
<68	34 (54.8)	28 (44.4)	0.245
≥68	28 (45.2)	35 (55.6)	
Gender			
Male	24 (38.7)	21 (33.3)	0.531
Female	38 (61.3)	42 (66.7)	
Histological grade[a]			
Well	21 (33.9)	24 (38.1)	0.628
Others[b]	41 (66.1)	39 (61.9)	
Tumor size (mm)			
<50	30 (48.4)	37 (58.7)	0.246
≥50	32 (51.6)	26 (41.3)	
Depth			
T0-3	43 (69.4)	40 (63.5)	0.488
T4	19 (30.6)	23 (36.5)	
Lymph node metastasis			
Absent	35 (56.5)	36 (57.1)	0.938
Present	27 (43.5)	27 (42.9)	
Lymphatic invasion			
Absent	37 (59.7)	42 (66.7)	0.418
Present	25 (40.3)	21 (33.3)	
Venous invasion			
Absent	51 (82.3)	52 (82.5)	0.967
Present	11 (17.7)	11 (17.5)	
Metastasis			
M0	56 (90.3)	52 (82.5)	0.204
M1	6 (9.7)	11 (17.5)	

[a]Well, well-differentiated adenocarcinoma; [b]Others: moderately-differentiated adenocarcinoma, poorly-differentiated adenocarcinoma and mucinous carcinoma.

Results

Relationship between ASB9 mRNA expression and clinicopathological characteristics. For the clinicopathological evaluation, the experimental samples were divided into 2 groups according to expression status. Patients with a tumor ASB9/ GAPDH ratio of expression above the median were assigned to the high expression group (n=62); others were assigned to the low expression group (n=63). Clinicopathological factors related to ASB9 expression status are summarized in Table I. The number of cases with histological grades of well, moderate, poor and mucinous adenocarcinoma was 45, 71, 6 and 3, respectively. The number of cases with UICC stages 0, I, IIA, IIB, IIIA, IIIB, IIIC and IV was 6, 21, 31, 10, 5, 24, 6 and 22, respectively. The data indicate that the expression of ASB9 was not correlated with clinicopathological factors.

Relationship between ASB9 expression and prognosis. The data show that the overall survival rate was significantly higher in patients expressing elevated levels of ASB9 (Fig. 1). The median follow up was 9.98±2.94 years. Table II presents the univariate and multivariate analyses of factors related to patient prognosis. Univariate analysis showed that the following factors were significantly related to overall survival: tumor depth (P<0.001), lymph node metastasis (P=0.001), lymphatic invasion (P=0.001), venous invasion (P=0.004) and ASB9 mRNA expression (P=0.013). Multivariate analysis indicated that inclusion in the high expression group [relative risk (RR) 4.09; 95% confidence interval (CI) 1.47-11.88; P=0.007], tumor invasion (RR 5.03; 95% CI 1.86-13.58; P=0.001), and lymph node invasion (RR 4.31; 95% CI 1.60-11.63; P=0.004) were independent predictors of overall survival.

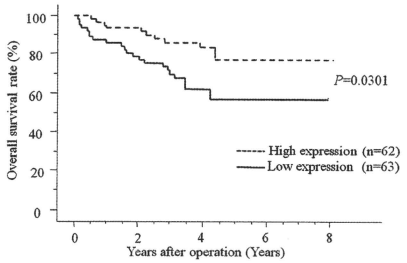

Figure 1. Survival curves of patients with colorectal cancer according to the status of ASB9 mRNA expression. Patients with low ASB9 expression had a significantly poorer prognosis than those with high ASB9 expression. P<0.0301 using the log-rank test.

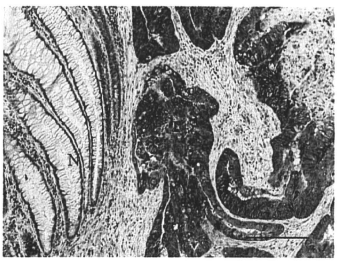

Figure 2. Positive staining observed in the cytoplasm of cancer cells, but not the stromal cells, indicating that the cancer cells are positive for ASB9 expression. A representative negative stain for ASB9 in a normal colorectal specimen shows normal glandular cells. ASB9 protein expression was evaluated in tumors and corresponding normal tissues from 23 colorectal cancer cases. Original magnification x40. T, tumor cells; N, normal glandular cells. Scale bar = 200 μm.

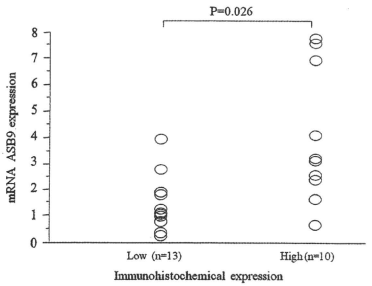

Figure 3. Relationship between ASB9 mRNA and immunohistochemistry in tissue samples from 23 colorectal cancer cases. Higher ASB9 mRNA expression was observed in the group with a higher immunohistochemistry expression (n=10) compared to the low expression group (n=13). ASB9 mRNA expression was significantly associated with protein expression (P=0.026 with the Student's t-test). The expression ratio of ASB9 mRNA was calculated and normalized against GAPDH.

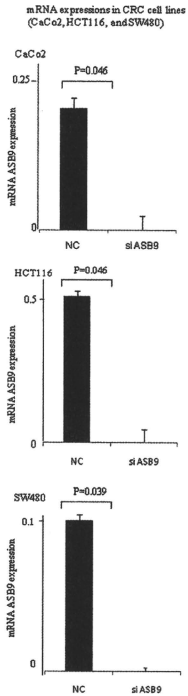

Figure 4. ASB9 mRNA knockdown by siRNA in CaCo2, HCT116 and SW480 cells. A significant reduction in ASB9 mRNA expression by siRNA was confirmed by quantitative real-time RT-PCR in CaCo2, HCT116 and SW480 cells. The data are presented as mean ± SD of four independent experiments. The expression levels were normalized to GAPDH mRNA expression.

Immunohistochemistry. ASB9 protein staining was observed predominantly in the cytoplasm of cancer cells and undetectable in the stromal cells of colorectal tumors (Fig. 2). Of 125 total cases, we examined ASB9 protein expression status in 23 available patient samples that analyzed by RT-PCR. Significantly higher ASB9 mRNA expression was observed in the group with high immunohistochemistry expression (n=10) compared to the low expression group (n=13) (P=0.026, Student's t-test; Fig. 3). Thus, the level of ASB9 mRNA expression was associated with the level of protein expression.

In vitro assessment of the knockdown of ASB9 expression. Three CRC cell lines were chosen for the proliferation and invasion studies. A significant reduction in ASB9 using siRNA was confirmed by quantitative real-time RT-PCR (Fig. 4). No significant differences in CaCo2 and SW480 cell numbers were measured in the proliferation assay between NC and ASB9 siRNA-transfected cells, whereas significant

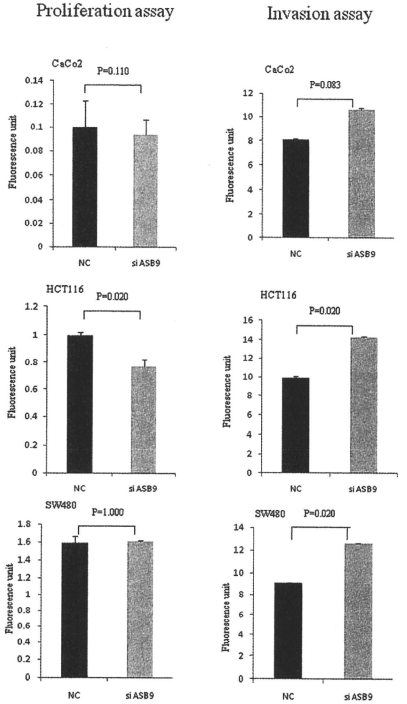

Figure 5. Cellular proliferation and invasion after ASB9 knockdown by siRNA. Top, no significant differences were measured in CaCo2 and SW480 between negative control (NC) and ASB9 siRNA-transfected cells. Bottom, ASB9 siRNA-transfected cells exhibited significantly high penetration of cells through the Matrigel-coated membrane compared to NC in HCT116 and SW480 cells. The data are presented as mean ± SD of three independent experiments.

differences were noted in HCT116 (n=4, Fig. 5). In the invasion assay, the results showed significant differences in HCT116 and SW480 cells between NC and ASB9 siRNA-transfected cells but no significant differences in CaCo2 cells (n=4, Fig. 5).

Discussion

ASB proteins constitute the largest family of SOCS box-containing proteins with 18 identified murine and human ASBs, but their biological and biochemical functions are largely unknown. The ASBs contain a protein interaction motif composed of a variable number of ankyrin repeats upstream from the SOCS box that is found in eukaryotic, bacterial and viral proteins, including receptors, proteins involved in cell cycle regulation, secreted proteins and transcription factors (14,15). The ASBs have been implicated in different biological processes. ASB-1 was studied by utilizing genetically modified mice; although ASB-1 knock-out mice displayed some testicular anomalies, the deletion and over-expression of ASB-1 had no obvious effect on mouse development (16). On the other hand, ASB-2 may regulate myeloid cell proliferation and/or differentiation (17,18), ASB-5 possibly plays a role in the initiation of arteriogenesis (2), and ASB-11 may regulate the proliferation and differentiation of the developing nervous system (3). Also, ASB-15 has been reported to regulate muscle growth by acting as a negative regulator of proliferating muscle cells and by increasing the rate of protein synthesis in differentiated myoblasts (19,20) ASB-8 expression is undetectable in normal adult lung tissue but present in several lung carcinoma cell lines. The transfection of a possible dominant negative form of ASB-8 suppressed the growth of lung adenocarcinoma cells *in vitro*, implying an association of ASB-8 with the development of lung cancer (20). In the present study, high ASB9 mRNA expression correlated with a good prognosis (Fig. 1). In addition, multivariate analysis identified ASB9 mRNA expression as a significant prognostic factor (Table II). In a previous report, high ASB9 expression was shown to reduce endogenous CKB (4). CKB, also known as the brain-type cytosolic enzyme of creatine kinase, plays a major role in cellular energy metabolism in non-muscle cells (5). Over-expression of CKB has been observed in a number of tumors, including neuroblastoma, small cell lung carcinoma, colon and rectal adenocarcinoma, and breast and prostate carcinoma, as well as some tumor cell lines (5,6). Furthermore, wild-type p53 represses the CKB promoter (7). In fact, many human small cell lung carcinomas, which exhibit elevated CKB expression, contain mutations in the p53 alleles (5). Therefore, elevated ASB9 may result in a good prognosis for CRC through reduced CKB. The interaction of ASB9 with CKB and the implied roles of ASB2 and ASB8 in other cancers (17,18,20) indicate that ASB9 may also have a role in tumor development or progression with CKB. However, no study has been conducted previously regarding the role of ABS9 in CRC.

Recently, the necessity of intensive follow-up and adjuvant therapy for CRC was proposed for predicting recurrence and metastasis in curative surgically resected cases (21-23). For these cases, prognostic markers of tumor invasion and metastasis play a very important role in cancer treatment. The expression profile of ASB9 may contribute to a certain type of prognostic factor.

Multivariate analysis indicated that inclusion in the high expression group, tumor depth and lymph node invasion were independent predictors of overall survival (Table II). We investigated the malignant potentials in proliferation and invasion assay (Fig. 5). The present *in vitro* study showed that the down-regulation of ASB9 by siRNA increased cell invasion in HCT116 and SW480 cells but was not related to tumor growth in CaCo2 and SW480 cells. Tumor size was not significantly different between the high and low ASB9 expression groups, supporting the data from the proliferation assay. We also found that the down-regulation of ASB9 significantly induced cell invasiveness. The low expression group had more T4 and M1 cases compared to the high expression group. In addition, multivariate analysis showed

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

Factors	Univariate analysis			Multivariate analysis		
	RR	95% CI	P-value	RR	95% CI	P-value
Age (years) (<68/≥68)	2.12	0.95-4.72	0.067			
Gender (Male/female)	1.59	0.68-3.71	0.282			
Histological grade[a] (Others[b]/well)	2.07	0.49-8.69	0.319			
Tumor size (mm) (≥50/<50)	1.43	0.64-3.17	0.381			
Depth (T4/T1-3)	7.16	3.02-16.97	<0.001	5.03	1.86-13.58	0.001
Lymph node metastasis (Present/absent)	5.26	2.23-12.35	0.0001	4.31	1.60-11.63	0.004
Lymphatic invasion (Present/absent)	5.26	2.23-12.35	0.0001	1.60	0.58-4.42	0.378
Venous invasion (Present/absent)	4.17	1.60-10.87	0.004	1.82	0.56-5.75	0.319
ASB9 mRNA expression (Low/high)	2.85	1.25-6.52	0.013	4.09	1.47-11.88	0.007

[a]Well, well-differentiated adenocarcinoma; [b]Others: moderately-differentiated adenocarcinoma, poorly-differentiated adenocarcinoma, and mucinous carcinoma. RR, relative risk; CI, confidence interval.

that ASB9 mRNA expression is a significant prognostic factor for overall survival. Thus, low ASB9 expression may have higher malignant potential, such as cell invasiveness and liver metastasis, than high expression, resulting in a poor prognosis for CRC. However, an analysis of up-regulated ASB9 expression and further in-depth studies are needed to investigate the precise molecular mechanism underlying the cause and effect between ASB9 and CRC. To our knowledge, this is the first report showing the role of ASB9 in cancer and its usefulness as a novel marker of CRC prognosis, implying that it may contribute to prediction of prognosis and to treatment.

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Clinical Cancer Research

MicroRNA-125a-5p is an independent prognostic factor in gastric cancer, and inhibits the proliferation of human gastric cancer cells in combination with trastuzumab

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Research Articles

MicroRNA-125a-5p is an independent prognostic factor in gastric cancer, and inhibits the proliferation of human gastric cancer cells in combination with trastuzumab

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Running Title: MicroRNA-125a IN GASTRIC CANCER

Key Words: miR-125a-5p, tumor suppressor, gastric cancer, trastuzumab, combination therapy

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Statement of Translational Relevance

Quantitative RT-PCR analysis of microRNA 125a-5p (*miR-125a-5p*) in 87 cases of gastric cancer revealed that low expression levels of *miR-125a-5p* were associated with enhanced malignant potential such as tumor size, tumor invasion, liver metastasis, and poor prognosis. To evaluate the function of *miR-125a-5p*, we focused on the *miR-125a-ERBB2* (*HER2, HER-2/neu)* pathway. In gastric cancer, ERBB2 over-expression has been increasingly recognized as an important therapeutic target similar to that in breast cancer . Our data suggested that *miR-125a-5p* directly targets *ERBB2*. *MiR-125a-5p* powerfully suppressed the proliferation of gastric cancer cells, and moreover, the growth inhibitory effect was enhanced in combination with trastuzumab, a monoclonal antibody against ERBB2. *MiR-125a-5p* is a meaningful prognostic indicator. Furthermore, miR-125a-5p mimic alone or in combination with trastuzumab could be a novel therapeutic approach against gastric cancer.

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Abstract

Purpose *miR-125a-5p* has been reported to be a tumor suppressor in malignancies of the breast, ovary, lung, and central nervous system. However, the clinical significance of *miR-125a-5p* in human gastrointestinal cancer has not been explored. We investigated a tumor inhibitory effect of *miR-125a-5p* in gastric cancer, focusing in particular on the *miR-125a-ERBB2* (*HER2, HER-2/neu)* pathway.

Experimental Design Quantitative RT-PCR was used to evaluate *miR-125a-5p* expression in 87 gastric cancer cases to determine the clinicopathologic significance of *miR-125a-5p* expression. The regulation of *ERBB2* by *miR-125a-5p* was examined with precursor *miR-125a*-transfected cells. Furthermore, we investigated whether *miR-125a-5p* suppresses proliferation of gastric cancer cells in combination with trastuzumab, a monoclonal antibody against ERBB2.

Results Low expression levels of *miR-125a-5p* were associated with enhanced malignant potential such as tumor size (P = 0.0068), tumor invasion (P = 0.031), liver metastasis (P = 0.029) and poor prognosis (P = 0.0069). Multivariate analysis indicated that low *miR-125a-5p* expression was an independent prognostic factor for survival. *In vitro* assays demonstrated that *ERBB2* is a direct target of *miR-125a-5p. MiR-125a-5p* potently suppressed the proliferation of gastric cancer cells, and interestingly, the growth inhibitory effect was enhanced in combination with trastuzumab.

Conclusions *MiR-125a-5p* is a meaningful prognostic marker. Furthermore, *miR-125a-5p* mimic alone or in combination with trastuzumab could be a novel therapeutic approach against gastric cancer.

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Introduction

MicroRNAs (miRNAs) constitute a class of small (19－25 nucleotides) noncoding RNAs that function as post-transcriptional gene regulators. MicroRNAs regulate gene expression by binding to their mRNAs (1). Alterations in miRNA expression are involved in the initiation, progression, and metastasis of human cancer and it is believed that miRNAs function both as tumor suppressors and oncogenes in cancer development (2-3).

Recent studies have shown that the expression of *miR125a-5p* is down-regulated in several human cancers such as breast cancer (4-6), ovarian cancer (7), lung cancer (8), and medulloblastoma (9). Li and colleagues reported that a germline mutation in mature *miR-125a-5p* is closely associated with breast cancer tumorigenesis (5). Other reports demonstrated that epidermal growth factor receptor signaling suppresses *miR-125a-5p* expression and leads to cancer metastasis in lung (8) and ovarian cancer (10). Furthermore, in squamous cell carcinoma of the oral cavity, the levels of *miR-125a-5p* were significantly down-regulated in the saliva of patients (11).These findings strongly suggest that the function of *miR-125a-5p* as a tumor suppressor is not organ-specific.

Scott and colleagues revealed that *miR-125a-5p* and its homolog, *miR-125b*, regulate *ERBB2* and *ERBB3* in human breast cancer cells (12). In gastric cancer, ERBB2 over-expression has been increasingly recognized as a frequent molecular abnormality and as an important therapeutic target similar to breast cancer (13-14). Preclinical and clinical data have revealed significant efficacy of anti-ERBB2 therapies, especially trastuzumab (Herceptin^{TM}), a monoclonal antibody directed at ERBB2 in

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gastric cancer (15-16).

In this study, we demonstrated that *miR-125a-5p* functions as a crucial tumor suppressor in human gastric cancer. Low *miR-125a-5p* expression was correlated with more aggressive disease and poorer prognosis, and was an independent prognostic factor. Of the numerous target genes of *miR-125a-5p*, we focused on *ERBB2* and discovered that *miR-125a-5p* regulates *ERBB2* in human gastric cancer cells. *MiR-125a-5p* potently suppressed the proliferation of gastric cancer cells. Moreover, the growth inhibitory effect was enhanced in combination with trastuzumab. This is the first report describing the clinical significance of *miR-125a-5p* and its growth inhibitory effect in human gastric cancer.

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Materials and Methods

Clinical cases

Patients and sample collection

Eighty-seven gastric cancer samples were obtained during surgery and used after obtaining informed consent. All patients underwent resection of the primary tumor at Kyushu University Hospital at Beppu and affiliated hospitals between 1992 and 2000. Written informed consent was obtained from all patients. All patients had a clear histologic diagnosis of gastric cancer, based on the clinicopathologic criteria described by the Japanese gastric cancer association (17). All patients were closely followed every three months. The follow-up periods ranged from 0.2 months to 12.3 years, with a mean of 2.6 years. Resected cancer tissues were immediately cut and embedded in Tissue-Tek OCT medium (Sakura), frozen in liquid nitrogen, and kept at -80°C until RNA and DNA extraction. Frozen tissue specimens were homogenized in guanidium thiocyanate, and total RNA was obtained by ultracentrifugation through a cesium chloride cushion. cDNA was synthesized from 8.0 μg of total RNA as previously described (18). Clinicopathological factors and clinical stage were classified by the criteria of the Japanese gastric cancer association (17). All sample data, including age, gender, tumor size and depth, lymphatic invasion, lymph node metastasis, vascular invasion, liver metastasis, peritoneal dissemination, distant metastasis, clinical stage and histological grade were obtained from the clinical and pathologic records, and are summarized in Table 1.

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Evaluation of* miR-125a-5p *expression in clinical samples

For *miR-125a-5p* quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR), cDNA was synthesized from 10 ng of total RNA using TaqMan™ MicroRNA hsa-*miR-125a-5p* specific primers (Applied Biosystems) and a TaqMan™MicroRNA Reverse Transcription Kit (Applied Biosystems). RT-PCR protocols are described in Supplementary Data.

Evaluation of* ERBB2, *dachshund homolog 1* (DACH1) and programmed cell death 6 (PDCD6) *mRNA expression in gastric cancer cells

For RNA analysis, each cell line was seeded at 2×10^5 cells per well in a volume of two mL in six well flat-bottomed microtiter plates. Total RNA from cell lines was isolated using the mirVana™ miRNA Isolation Kit (Ambion) after 48 hr incubation. Quantitative RT-PCR was performed to measure *ERBB2*, *DACH1* and *PDCD6* mRNA expression with the Universal Probe Library probe (UPL) (Roche Diagnostics). Primer sequences corresponding to UPL and RT-PCR protocols are described in Supplementary Data.

Immunohistochemistry (IHC)

Immunohistochemical studies of ERBB2 were performed on formalin-fixed, paraffin embedded (FFPE) surgical sections obtained from patients with gastric cancer. Tissue sections were deparaffinized, soaked in 0.01 mol/L sodium citrate buffer, and boiled in a microwave oven for five min at 500 W to retrieve cell antigens. Mouse monoclonal

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antibody against ERBB2 (Epitomics, Inc) diluted 1:400 was used as the primary antibody. All tissue sections were immunohistochemically stained with the avidin–biotin-peroxidase method (LSAB+ System HRP; Dako, Inc) and were counterstained with hematoxylin.

Evaluation of ERBB2 IHC staining

The slides were examined and scored independently by two experienced pathologists. Evaluation of the results was performed according to the criteria recommended by Hoffman and colleagues and other groups (19-20), by assigning a score of 0 to 3+. Scores were defined as follows: 0, no reactivity or membranous reactivity in <10% of cells; 1+, faint/barely perceptible membranous reactivity in ≧10% of cells; cells are reactive only in part of their membrane; 2+, weak to moderate complete or basolateral membranous reactivity in ≧10% of tumor cells; 3+, moderate to strong complete or basolateral membranous reactivity in ≧10% of tumor cells. Specimens with scores of 0 and 1+ were regarded as being ERBB2-negative, while scores of 2+ and 3+ indicated positive expression of ERBB2.

Experimental studies

Cell lines and cell culture

The human gastric cancer cell lines AZ521,KATOIII, MKN1, MKN45, MKN74, NUGC3 and NUGC4 were provided by the Cell Resource Center of Biomedical

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Research, Institute of Development, Aging and Cancer, Tohoku University. These cell lines were maintained in RPMI 1640 containing 10% fetal bovine serum with 100 units/mL penicillin and 100 units/mL streptomycin sulfates and cultured in a humidified 5% CO2 incubator at 37°C.

***Transfection of* microRNA-125a precursor (Pre-miR™-125a)**

Using NUGC4, a gastric cancer cell line that expresses a high level of *ERBB2* mRNA, either *pre–miR-125a* or pre-miR negative control (Ambion® Pre-miR™ miRNA Precursors Applied Biosystems Japan Ltd.) was transfected at 30 nM (final concentration) using Lipofectamine™ RNAiMAX (Invitrogen Life Technologies) according to the manufacturer's instruction.

In vitro *assays*

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay for gastric cancer cell growth after transfection with* Pre- miR-125a *with/without trastuzumab treatment

Logarithmically growing NUGC4 cells were transfected with Pre-*miR-125a* or Pre-*miR-negative control* with or without addition of trastuzumab (0.1 μg/mL or 1 μg/mL) and were seeded at 8.0×10^3 cells/well in 96 well flat-bottomed microtiter plates, in a final volume of 100 μL of culture medium per well. Cells were incubated in a humidified atmosphere (37°C and 5% CO2) for 24, 48, 72 and 96 h after initiation of

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transfection. MTT assays were used to measure cell proliferation at each period, as described in supplementary data. The assay was performed using six replicates.

Plasmid Construction

The 3' untranslated region (3′UTR) and open reading frame (ORF) of *ERBB2* was amplified by RT-PCR. The amplified product was sub-cloned and ligated into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega). The resultant reporter vector position was confirmed by sequencing and termed Luc-*ERBB2*-WT. To make *miR-125a-5p* binding site mutants, positions 37 - 43 of *ERBB2* 3'UTR (the sequence; CTCAGGG) were mutated to the sequence; CACTGCG (mutated nucleotides are underlined) using the QuikChange ® Lightning Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol. The resultant reporter vector position was confirmed by sequencing and termed Luc-*ERBB2*-mutant.

Luciferase assay

Luciferase assays were conducted using 1×10^4 NUGC4 cells plated in a 96 well plate. Transfections were performed using Lipofectamine™ 2000 (Invitrogen) in OptiMEM reduced serum media (GIBCO). Cells were transfected with 30 ng of Luc-*ERBB2*-WT vector or Luc-*ERBB2*-mutant vector and either 100 nM of pre-miR negative control or pre-*miR-125a*. Twenty-four hours following transfection, cells were assayed for both firefly and *renilla* luciferase using Dual-Glo™ Luciferase Assay System (Promega). All transfection experiments were conducted in triplicate.

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ERBB2 *and* miR-125a-5p *expression in the NCI60 panel*

For analysis of the correlation between *ERBB2* and *miR-125a-5p* expression in the NCI60 panel (21), the normalized expression levels of the cDNA array and the miRNA array were obtained from the website of the Genomics and Bioinformatics Group (http://discover.nci.nih.gov). The data were analysed by JMP 5 for Windows software (SAS Institute, Inc.).

Protein expression analysis

Western blotting was used to confirm the expression of ERBB2, phosphorylated AKT, BAK1 and p53 in pre-miR-125a transfected cells. Primary antibodies and dilutions were as follows: ERBB2 rabbit monoclonal antibody (Epitomics, Inc) at a 1:500 dilution; AKT rabbit monoclonal antibody (Cell Signaling Technology, Inc) at a 1:1000 dilution; phosphorylated AKT (p-AKT) rabbit monoclonal antibody (Cell Signaling Technology, Inc) at a 1:2000 dilution; BAK1(Cell Signaling Technology, Inc) at a 1:1000 dilution; p53 (Dako, Inc) at a 1:1000 dilution. Detailed protocols are described in Supplementary data.

Statistical analysis.

Data from RT-PCR analysis and *in vitro* transfected cell assays were analyzed with JMP 5. Overall survival rates were calculated actuarially according to the Kaplan-Meier method and were measured from the day of surgery. Differences between groups were estimated using the x^2 test, Student's t-test, repeated-measures ANOVA

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test, and the log-rank test. Variables with a value of $P < 0.05$ in univariate analysis were used in a subsequent multivariate analysis based on the Cox proportional hazards model. A probability level of 0.05 was chosen for statistical significance.

Results

The clinicopathologic significance of* miR-125a-5p *mRNA expression in gastric cancer

In this study, patients with values less than the average expression level of *miR-125a -5p* (8.66, normalized to RNU6B) were assigned to a low expression group (n = 55), whereas those with expression values above average were assigned to a high expression group (n = 32). Patients in the low *miR-125a-5p* expression group had a significantly poorer prognosis than those in the high *miR-125a-5p* expression group (P = 0.0069; Fig. 1). Clinicopathologic factors were significantly different in the low *miR-125a-5p* expression group. There was greater tumor size (P = 0.0068), tumor invasion (P = 0.031), liver metastasis (P = 0.029), and clinical staging (P = 0.02) compared to the high *miR125a-5p* expression group (Table.1). However, no significant differences were observed regarding age, gender, histology, lymphatic invasion, venous invasion, lymph node metastasis, peritoneal dissemination and distant metastasis (Table 1). The results of univariate and multivariate Cox proportional hazards regression analyses for overall survival are shown in Table 2. Multivariate analysis indicated that the high expression level of *miR-125a-5p* was an independent and significant prognostic factor for survival (OR, 2.44; CI, 1.04 – 6.73; P = 0.041; Table 2). Expression of *ERBB2*, which is a putative *miR-125a-5p* target, is shown to be an indicator of patient

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prognosis by univariate analysis (P = 0.048), although it is not an independent prognostic factor. For the comparison, using the same RNA samples, we also investigated *DACH1 and PDCD6* mRNA expression, which were previously reported as prognostic factors for gastric cancer patients (22). However, in univariate analysis for overall survival, the expression levels of those two molecules were not superior to *miR-125a-5p* expression as prognostic factors, at least in the group we investigated (Supplementary Table 1).

ERBB2 *mRNA expression in gastric cancer cell lines and the effect of trastuzumab*

ERBB2 mRNA expression was examined in seven gastric cancer cell lines using RT-PCR. NUGC4, a human cell line derived from a signet ring cell carcinoma of the stomach, showed a remarkably high level of *ERBB2* mRNA compared to other cell lines, ($p < 0.0001$; Supplementary Fig. 1A) and was chosen for experiments on validation of *ERBB2* suppression by *miR-125a-5p*. MTT assays were performed to evaluate the growth inhibitory effect of trastuzumab in gastric cancer cell lines, NUGC4, AZ521 and NUGC3. AZ521 and NUGC3 were chosen as representative low *ERBB2* expression cell lines. The results indicated that trastuzumab exerted its activity selectively on NUGC4, the *ERBB2* high expression gastric cancer cell line. At the maximum concentration of 100 μg/mL, the cell viability of NUGC4 was reduced by 28.3 ± 3.98%, whereas the viability of NUGC3 and AZ521 remained above 90% (Supplementary Fig. 1B).

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