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Cancer Therapy: Preclinical

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

Naohiro Nishida[1,2], Koshi Mimori[1], Muller Fabbri[3], Takehiko Yokobori[1], Tomoya Sudo[1], Fumiaki Tanaka[1], Kohei Shibata[1], Hideshi Ishii[1,2], Yuichiro Doki[2], and Masaki Mori[1,2]

Abstract

Purpose: MicroRNA 125a-5p (*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 showed that *ERBB2* is a direct target of *miR-125a-5p*, which 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. *Clin Cancer Res; 17(9); 2725–33. ©2011 AACR.*

Introduction

MicroRNAs (miRNA) constitute a class of small (19–25 nucleotides) noncoding RNAs that function as posttranscriptional gene regulators. miRNAs 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 downregulated 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 miRNA 125a-5p (*miR-125a-5p*) is closely associated with breast cancer tumorigenesis (5). Other reports showed that epidermal growth factor (EGF) receptor signaling suppresses *miR-125a-5p* expression and leads to cancer metastasis in lung cancer (8) and ovarian cancer (10). Furthermore, in squamous cell carcinoma of the oral cavity, the levels of *miR-125a-5p* were significantly downregulated 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 homologue, *miR-125b*, regulate *ERBB2* and *ERBB3* in human breast cancer cells (12). In gastric cancer, ERBB2 overexpression 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), a monoclonal antibody directed at ERBB2 in gastric cancer (15, 16).

Authors' Affiliations: [1]Department of Surgery and Molecular Oncology, Medical Institute of Bioregulation, Kyushu University, Beppu, Oita; [2]Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, Suita City, Osaka, Japan; and [3]Department of Molecular Virology, Immunology and Medical Genetics. The Ohio State University Comprehensive Cancer Center, Columbus, Ohio

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Masaki Mori, Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita City, Osaka 565-0871, Japan. Phone: 8166-879-3251; Fax: 8166-879-3259; E-mail: mmori@gesurg.med.osaka-u.ac.jp

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Translational Relevance

Quantitative real-time reverse transcriptase 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 overexpression 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.

In this study, we showed 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.

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 3 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 (optimum cutting temperature) 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). Clinicopathologic 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 histologic grade, were obtained from the clinical and pathologic records and are summarized in Table 1.

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

For *miR-125a-5p* quantitative real-time reverse transcriptase PCR (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*, *DACH1*, and *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 2 mL in 6-well flat-bottomed microtiter plates. Total RNA from cell lines was isolated using the mirVana miRNA Isolation Kit (Ambion) after 48-hour incubation. Quantitative RT-PCR was carried out to measure *ERBB2*, dachshund homolog 1 (*DACH1*), and programmed cell death 6 (*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

Immunohistochemical studies of ERBB2 were conducted 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 5 minutes at 500 W to retrieve cell antigens. Mouse monoclonal 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 counterstained with hematoxylin.

Evaluation of ERBB2 immunohistochemical staining

The slides were examined and scored independently by 2 experienced pathologists. Evaluation of the results was done according to the criteria recommended by Hofmann and colleagues and other groups (19, 20), by assigning a score from 0 to 3+. Scores were defined as follows: 0, no reactivity or membranous reactivity in less than 10% of ≥ cells; 1+, faintbarely perceptible membranous reactivity in 10% or more of cells; cells are reactive only in part of their membrane; 2+, weak to moderate complete or basolateral membranous reactivity in ≥ 10% or more of tumor cells; 3+, moderate to strong complete or basolateral

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

Factors	Low expression group ($n = 55$)		High expression group ($n = 32$)		*P*
	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	
Histologic grade					
Well and moderately	21	38.2	17	53.1	0.18
Poorly and others	34	61.8	15	46.9	
Size					
50 mm > (small)	18	32.7	20	62.5	0.0068[a]
50 mm < (large)	37	67.3	12	37.5	
Depth of tumor invasion[b]					
m, sm, mp	12	21.8	21	14	0.031[a]
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[a]
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[a]
III–IV	33	60	11	34.4	

[a]$P < 0.05$.

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

membranous reactivity in ≥ 10% or more of tumor cells. Specimens with scores of 0 and 1+ were regarded as being negative for ERBB2 expression, whereas scores of 2+ and 3+ indicated positive expression of ERBB2.

Experimental studies

Cell lines and cell culture. The human gastric cancer cell lines AZ521, KATO, MKN1, MKN45, MKN74, NUGC3, and NUGC4 were provided by the Cell Resource Center of Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. These cell lines were maintained in RPMI 1640 containing 10% FBS with 100 units/mL penicillin and 100 units/mL streptomycin sulfates and cultured in a humidified 5% CO_2 incubator at 37°C.

Transfection of miRNA-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), was transfected at 30 nmol/L (final concentration) by using Lipofectamine RNAiMAX (Invitrogen Life Technologies) according to the manufacturer's instruction.

In vitro assays

The 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

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without addition of trastuzumab (0.1 or 1 μg/mL) and were seeded at 8.0×10^3 cells per 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% CO_2) for 24, 48, 72, and 96 hours after initiation of transfection. MTT assays were used to measure cell proliferation at each period, as described in Supplementary Data. The assay was carried out with 6 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 subcloned 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 to 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 done with 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 e100 nmol/L of either *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.

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 Web site of the Genomics and Bioinformatics Group (http://discover.nci.nih.gov). The data were analyzed by JMP 5 for Windows software (SAS Institute, Inc.).

Protein expression analysis

Western blotting was used to confirm the expression of ERBB2 and 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 1:1,000 dilution; phosphorylated AKT (p-AKT) rabbit monoclonal antibody (Cell Signaling Technology, Inc.) at a 1:2,000 dilution; BAK1 (Cell Signaling Technology, Inc.) at a 1:1,000 dilution; p53 (Dako, Inc.) at a 1:1,000 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 χ^2 test, Student's t test, repeated-measures ANOVA 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 were greater tumor size ($P = 0.0068$), tumor invasion ($P = 0.031$), liver metastasis

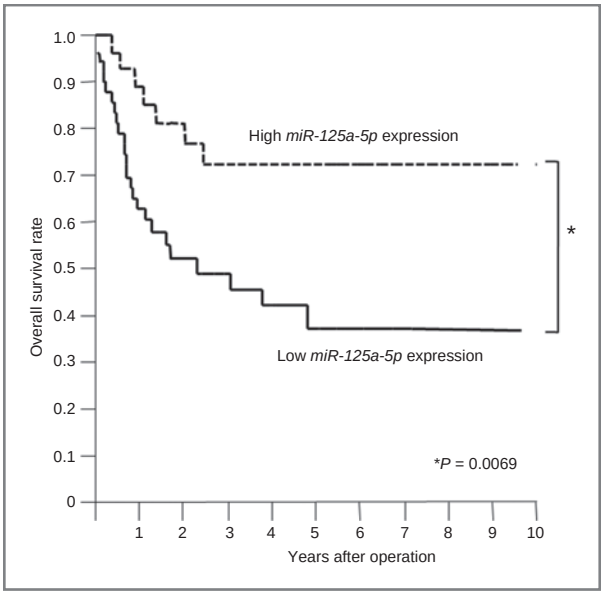

Figure 1. Kaplan–Meier overall survival curves of gastric cancer patients according to the level of *miR-125a-5p* expression. Patients in the low *miR-125a-5p* expression group had a significantly poorer prognosis than those in the high *miR-125a-5p* expression group. The high *miR-125a-5p* expression 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.

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($P = 0.029$), and clinical staging ($P = 0.02$) than the high *miR-125a-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 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 was previously reported as prognostic factors for gastric cancer patients (22). However, in univariate analysis for overall survival, the expression levels of those 2 molecules were not superior to *miR-125a-5p* expression as prognostic factors, at least in the group we investigated (Supplementary Table S1).

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

ERBB2 mRNA expression was examined in 7 gastric cancer cell lines by 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 with other cell lines, ($P < 0.0001$; Supplementary Fig. S1A) and was chosen for experiments on validation of *ERBB2* suppression by *miR-125a-5p*. MTT assays were carried out 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. S1B).

miR-125a-5p regulates ERBB2 in gastric cancer cells

Using *in silico* miRNA 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 carried out 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*, FFPE surgical sections were available in 52 cases. To explore the association between *miR-125a-5p* expression and ERBB2 protein expression status, we carried out immunohistochemical analysis with 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 with that in the high ERBB2 expression group (ERBB2 staining 2+ or 3+; $n = 7$; mean ± SEM; 4.97 ± 0.88 vs. 2.34

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

Factors	Univariate analysis			Multivariate analysis		
	RR	95% CI	*P*	RR	95% CI	*P*
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 and others)	1.309	0.913–1.946	0.146	–	–	–
Depth of tumor invasion[a] (m, sm, mp/ss, se, si)	8.387	2.531–51.86	0.0001[b]	2.415	0.650–15.87	0.208
Lymph node metastasis (negative/positive)	4.675	2.162–19.74	0.0001[b]	2.892	1.219–12.88	0.011[b]
Lymphatic invasion (negative/positive)	2.684	1.474–6.676	0.0003[b]	1.177	0.481–5.237	0.761
Venous invasion (negative/positive)	3.324	1.627–6.080	0.0012[b]	1.316	0.883–1.971	0.176
ERBB2 mRNA expression (low/high)	1.466	1.003–2.263	0.0482[b]	1.010	0.991–1.025	0.277
miR-125a-5p expression (high/low)	3.018	1.372–7.588	0.0051[b]	2.438	1.035–6.727	0.041[b]

Abbreviation: RR, Relative risk.

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

[b]$P < 0.05$.

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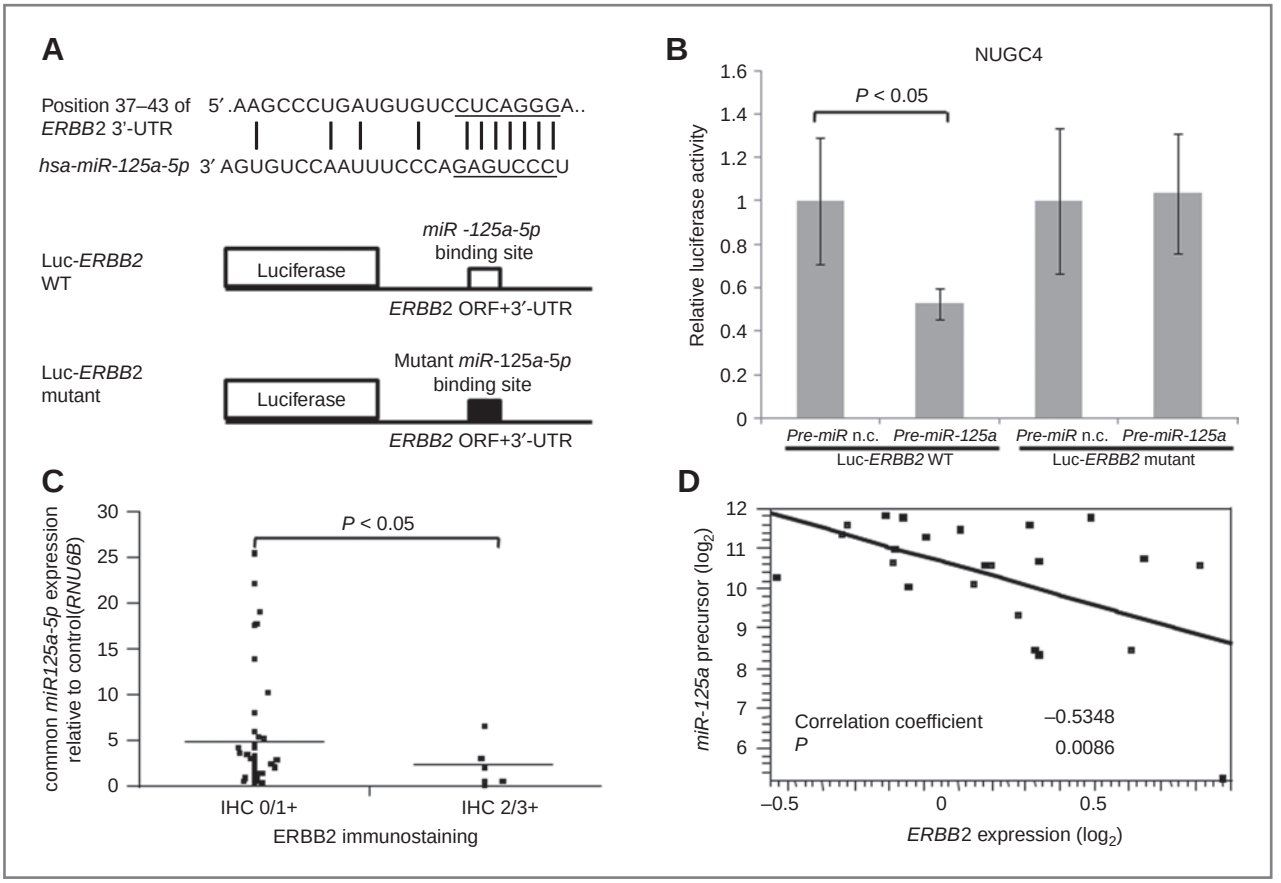

Figure 2 . *miR-125a-5p* targets *ERBB2* A, top, sequence of the *miR-125a-5p* binding sites in the 3′-UTRs of transcripts encoding *ERBB2*. Bottom, schematic diagram of the luciferase reporters in target validation. B, *miR-125a-5p* represses its target in the luciferase assay in NUGC4. Relative luciferase 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 SD from 6 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 with 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.

± 2.24; $P < 0.05$; Fig. 2C). Images of immunohistochemistry are shown in Supplementary Figure S2.

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 data set 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 ($r = -0.5348$, $P = 0.0086$; 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. S3).

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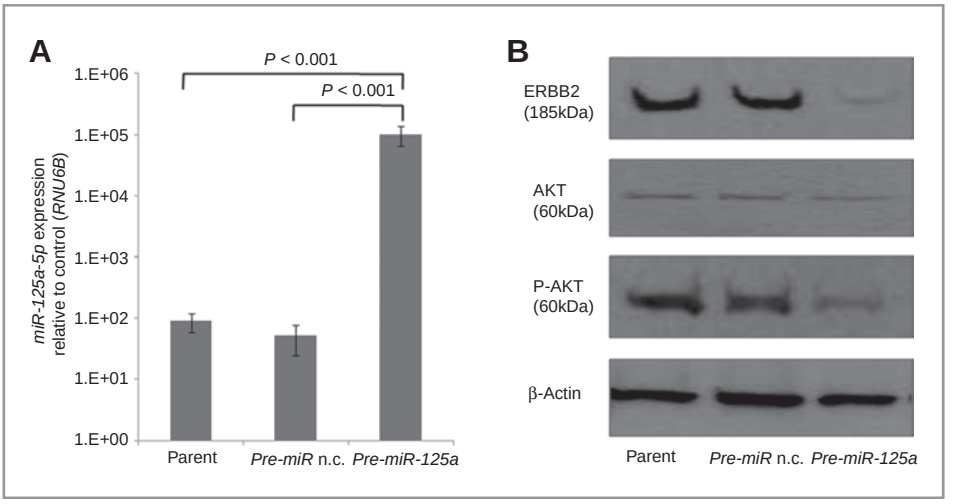

Figure 3 . *miR-125a-5p* regulates ERBB2. A, *miR-125a-5p* expression after treatment with negative control or *Pre-miR-125a* in NUGC4 (quantitative RT-PCR). *miR-125a-5p* expression in *Pre-miR-125a*–treated cells is significantly higher than in untreated cells (parent) and in *Pre-miR* negative control–treated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

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 carried out with *Pre-miR-125a*–treated cells or negative control cells by 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 carried out at 2 different concentrations of trastuzumab [0.1 μg/mL (Fig. 4A) and 1 μg/mL (Fig. 4B)]. Interestingly, *miR-125a* not only potently suppressed the proliferation of gastric cancer cells by itself but also 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 miRNAs is complex because they regulate hundreds of targets, resulting in the downregulation 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. Clinicopathologic analysis revealed that low *miR-125a-5p* expression contributes to more advanced tumor size and tumor invasion (Table 1). It

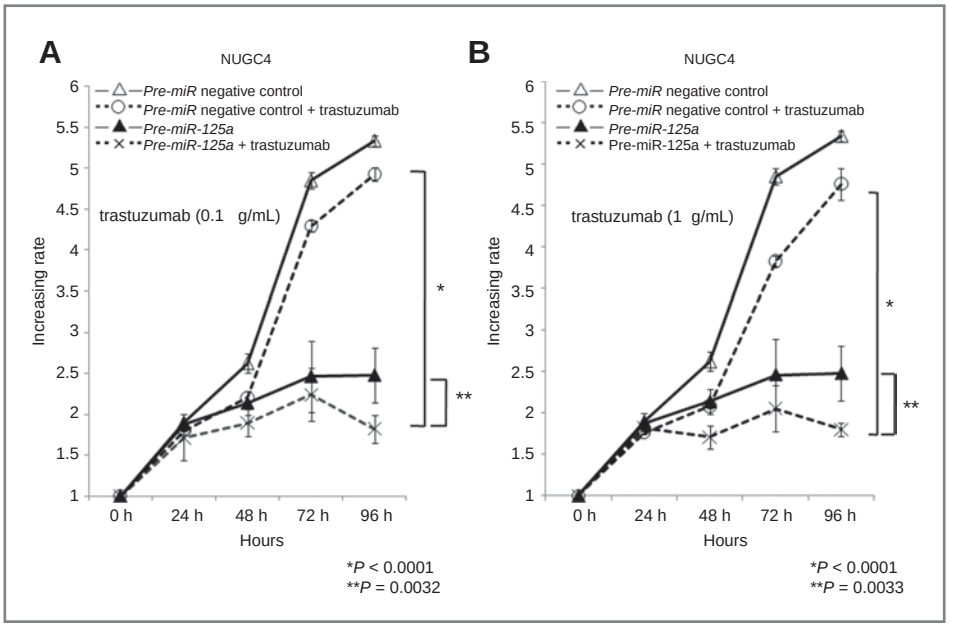

Figure 4. *miR-125a-5p* inhibits the proliferation of the gastric cancer cell line NUGC4 in combination with trastuzumab. *Pre-miR-125a* or *Pre-miR* negative control transfected with or without trastuzumab (A, 0.1 μg/mL; B, 1 μg/mL) treatment were seeded at 8.0×10^3 cells per well in 96-well plates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

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suggests that this miRNA primarily achieves its antiproliferative effect through downregulation of proliferation-related genes, including *ERBB2*, a member of the 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 signaling (28). *miR-125a-5p* is shown to be a superior biomarker to previously reported gastric cancer biomarkers such as *DACH1* and *PDCD6* (ref. 22; Supplementary Table S1). 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* (ref. 27; Supplementary Fig. S3). 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. 4A and 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 2 different mechanisms. *miR-125a-5p* suppresses the molecule at the posttranscriptional level before protein synthesis, whereas 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 with chemotherapy alone. These reliable large-scale clinical data indicate 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Naohiro Nishida, Koshi Mimori, Muller Fabbri, et al.

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