Clinical Significance of miR-146a in Gastric Cancer Cases

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Clinical significance of *miR-146a* in gastric cancer cases

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Statement of translational relevance

Considering treatment of gastric cancer cases, epidermal growth factor receptor (EGFR) and interleukin-1 receptor-associated kinase (IRAK1) should be consecutive molecular targets of all. In the current study, we disclosed that the reduction of *miR146a* expression was associated with the up-regulation of both EGFR and IRAK1. Lower expression of *miR146a* was significantly associated with the progression and poorer prognosis of gastric cancer cases. Besides, mature *miR146a* expression was significantly related to the single nucleotide polymorphism (SNP) located within *pre-miR-146a* seed sequence. As the SNP should be evaluated certainly by using of genomic DNA extracted from peripheral bloods, therefore, this stable and reliable methodology should be applied to the practical clinical diagnosis for gastric cancer to be treated with anti-EGFR or anti-IRAK1 therapy. We might predict the robust expression of EGFR or IRAK1 in gastric cancer cases by the SNP status from patient peripheral bloods.

Abstract

Purpose: The profiles of microRNAs change significantly in gastric cancer. *MiR-146*a is reported to be a tumor suppressor in pancreatic cancer, breast cancer and prostate cancer. We investigated the clinical significance of *miR-146a* in gastric cancer, in particular focusing on hypothetical *miR-146a* target genes, such as epidermal growth factor receptor (*EGFR*) and interleukin-1 receptor-associated kinase (*IRAK1*).

Experimental design: We examined *miR-146a* levels in 90 gastric cancer samples by qRT-PCR and analyzed the association between *miR-146a* levels and clinicopathologic factors and prognosis. The regulation of *EGFR* and *IRAK1* by *miR-146a* was examined with *miR-146a*-transfected gastric cancer cells. Moreover, we analyzed the association between *miR-146a* levels and the G/C SNP within *pre-miR-146a* seed sequences in 76 gastric cancer samples, using direct sequencing of genomic DNA.

Results: In 90 clinical samples of gastric cancer, *miR-146a* levels in cancer tissues were significantly lower than those in the corresponding noncancerous tissue ($P < 0.001$). Lower levels of *miR-146a* were associated with lymph node metastasis, and venous invasion ($P < 0.05$). Moreover, a lower level of *miR-146a* was an independent prognostic factor for overall survival ($P = 0.003$). Ectopic expression of *miR-146a* inhibited migration and invasion and downregulated *EGFR* and *IRAK1* expression in

gastric cancer cells. Additionally, G/C SNP within the *pre-miR-146a* seed sequence significantly reduced *miR-146a* levels in the GG genotype compared to the CC genotype.

Conclusions: *MiR-146a* contains a SNP which is associated with mature *miR-146a* expression. *MiR-146a* targeting of *EGFR* and *IRAK1* is an independent prognostic factor in gastric cancer cases.

Introduction

Gastric cancer is one of the most common malignant tumors in Japan. The development of adjuvant chemotherapies has improved clinical outcome to a certain extent; however, advanced gastric cancer with lymph node metastasis still has a poor prognosis (1, 2). A number of genes appear to contribute to the malignant potential of gastric cancer (3, 4). However, the identification of the precise factors which predict the prognosis and recurrence of gastric cancer remains extremely important.

MiRNAs are 20-to-25 mer non-coding RNAs which incompletely bind to the 3'UTR of multiple target mRNAs, enhancing their degradation and inhibiting their translation. MiRNAs possess normal biological functions, such as regulation of proliferation, differentiation and apoptosis. Moreover, dysregulated of miRNAs play critical roles during carcinogenesis and cancer progression (5, 6). The levels of many miRNAs in cancer tissue are lower than those in normal tissue, a state which contributes to cancer progression (7).

MiR-146a reportedly suppresses the invasion of pancreatic cancer cells by downregulation of epidermal growth factor receptor (EGFR) and interleukin-1 receptor-associated kinase 1 (IRAK1) (8). EGFR plays critical roles in tumor development and its downstream signaling is important, as it includes Raf-MEK-ERK,

PI3K-PDK1-Akt, and RalGDS (9, 10). IRAK1 is upstream of NF-κB and is involved in cancer progression (8, 11, 12). Moreover, EGFR activates NF-κB by phosphorylation of IκB (13). Therefore, we have focused on the relationship between *miR-146a* and its target genes, both *EGFR* and *IRAK1*.

Previous reports indicated that *miR-146a* inhibits progression of solid tumors derived from cancer cell lines, but there are no reports about the function and significance of *miR-146a* at the clinical level (8, 14-16).

The level of *miR-146a* is regulated by a single nucleotide polymorphism (SNP). This G/C SNP (rs2910164) is located within the seed sequence of *pre-miR-146a*, which is the *miR-146a* precursor. It resides in the passenger strand of *miR-146a* (*miR-146a**). G/C SNP regulates the level of mature *miR-146a* in thyroid cancer, prostate cancer, hepatocellular carcinoma and familial breast / ovarian cancer (12, 16-19). Furthermore, G/C SNP is associated with the risk of carcinogenesis in these cancers.

In the current study, we demonstrated the clinical significance of *miR-146a* as a tumor suppressor in gastric cancer cases and analyzed the function of *miR-146a* in gastric cancer cells. Moreover, we examined the G/C SNP by direct sequencing of genomic DNA from 76 patients. We then compared the expression levels of *miR-146a* in gastric cancer tissue (T) and corresponding noncancerous tissue (N) to determine

whether or not the G/C SNP within *pre-miR-146a* seed sequence might regulate mature *miR-146a* levels in gastric cancer cases.

Materials and Methods

Clinical samples Ninety gastric cancer samples were obtained during surgery and used after obtaining informed consent. All patients underwent curative resection of the primary tumor at Kyushu University Hospital at Beppu between 1992 and 2000. All patients had a clear histological diagnosis of gastric cancer, based on the clinicopathologic criteria described by the Japanese gastric cancer association (20). All patients were closely followed after surgery at regular three-month intervals. The follow-up periods ranged from two months to 11 years, with a mean of three years. All data, including age, sex, histological grade, tumor size, depth (T factor), lymph node metastasis (N factor), lymphatic invasion, venous invasion, liver metastasis, and peritoneal dissemination were obtained from clinical and pathologic records. No patients received neoadjuvant chemotherapy or radiotherapy before surgery and adjuvant radiotherapy after surgery. 47 patients received adjuvant chemotherapy after surgery. Resected cancerous tissues (T) and paired noncancerous tissues (N) were immediately cut and stored in RNA*later* (Ambion), frozen in liquid nitrogen, and kept at –80 °C until RNA extraction. RNA was extracted using ISOGEN (NipponGene) according to the manufacturer's protocol.

Cell lines and transfection of *miR-146a* (Pre-miR-146a^{TM}) Human gastric cancer cell line MKN45 was provided by the Cell Resource Center of Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. MKN45 cells were maintained in RPMI 1640 containing 10 % fetal bovine serum with 100 units / mL penicillin and 100 ug / mL streptomycin sulfate and cultured in a humidified 5 % CO_2 incubator at 37 °C. Using 2×10^6 MKN45 cells, either Pre–miR-146a or Pre-miR negative control (Pre-miR™, Ambion) was transfected at 60pmol using Nucleofector kit V (Amaxa) according to the manufacturer's instruction.

Real-Time Quantitative RT-PCR *MiR-146a* and *RNU6B* expression levels were quantified by TaqMan miRNA assays protocol (Applied Biosystems), as previously described (21). Relative quantification of miRNA expression was calculated by using the 2-ΔΔCt method. The raw data were presented as the relative quantity of target miRNA, normalized with respect to *RNU6B*, and relative to a calibrator sample.

Immunoblot analysis Total cell protein was extracted from MKN45 cells 48 h after transfection of *miR-146a* (Pre-miR-146a^{TM}, Ambion). Total protein (40 μg) was electrophoresed and then electroblotted as previously described (22). Protein was

detected using primary antibodies, EGFR and IRAK1 antibody (Santa Cruz Biotechnology) diluted 1:500 and then primary antibodies were detected using HRP-conjugated secondary antibodies (GE Healthcare). EGFR and IRAK1 proteins were normalized to the level of β-actin protein (Cytoskeleton, Inc.) diluted 1:1000.

DNA isolation and genotyping Genomic DNAs were extracted from 76 gastric cancer tissues using the QIAamp DNA mini kit according to the manufacturer's protocol (Qiagen), followed by direct DNA sequencing. A 227 bp fragment containing the *pre-miR-146a* region and polymorphism site (rs2910164) was amplified using the following primers: 5' –ATTTTACAGGGCTGGGACAG- 3' and 5' –TCTTCCAAGCTCTTCAGCAG- 3'. The PCR products were electrophoresed on agarose gels and purified with ethanol precipitation. Purified PCR products were sequenced using a Big-Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI3130x Genetic Analyzer (Applied Biosystems).

Invasion and migration assay Invasion and migration assays were performed using the BD BioCoat Tumor Invasion Assay System and the BD Falcon HTS Fluoro Block Insert (BD Biosciences) ,as described previously (23). Briefly, cells (5.0×10^4 cells /

well) with serum-free medium were seeded in the upper chamber, and the lower chamber was filled with medium with 10% FBS as a chemoattractant. After 48 h, membranes were labeled with Calcein-AM. The invaded and migrated cells were evaluated in a fluorescence plate reader at excitation / emission wavelengths of 485/530 nm. Transfections were conducted three times in independent experiments.

Construction of reporter plasmids and luciferase reporter assay

To construct a luciferase reporter plasmid, an *EGFR* or *IRAK1* -3'UTR full length fragment was subcloned into pmirGlo Dual-luciferase miRNA Target Expression Vector (Promega) located 5' to the firefly luciferase. The nucleotide sequences of the constructed plasmids were confirmed by DNA sequencing analysis. For luciferase reporter assays, MKN45 cells were seeded in a 96-well plate and then cotransfected with the pmirGlo-*EGFR* or *IRAK1* -3'UTR construct and *miR-146a* (Pre-miR-146a^{TM}) or Pre-miR negative control ^{TM} (Ambion). Assays were performed 48 hr after transfection by using the Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase signals were normalized to the Renilla luciferase signals. Transfections were done three times in independent experiments.

Statistical analysis Differences between two groups were estimated with Student's *t* test and χ^2 test. Overall survival curves were plotted according to the Kaplan-Meier method, with the log-rank test applied for comparison. Survival was measured from the day of the surgery. Variables with a value of $P < 0.05$ by univariate analysis were used in subsequent multivariate analysis based on the Cox proportional hazards model. All differences were statistically significant at the level of $P < 0.05$. Statistical analyses were done using the JMP 5 for Windows software package (SAS Institute).

Results

Clinical significance of *miR-146a* in gastric cancer cases

MiR-146a levels in 90 cancerous and corresponding noncancerous tissues were examined by qRT-PCR. *MiR-146a* levels in cancerous tissues (T) (mean ± SD, 2.00 ± 2.28) were significantly lower than those in the corresponding noncancerous tissues (N) (mean ± SD, 4.30 ± 5.09, $P < 0.001$; Student's *t* test; Figure. 1A). We divided 90 gastric cancer patients into two groups, the *miR-146a* high expression group ($T / N > 0.5$, $n = 45$) and the low expression group ($T / N < 0.5$, $n = 45$), according to the median cancer (T) / noncancerous (N) tissue ratio of *miR-146a* expression. Clinicopathologic factors were analyzed in relation to *miR-146a* levels (Table. 1). The *miR-146a* low expression group showed more extensive lymph node metastasis (N factor) and venous invasion than the high expression group ($P < 0.05$; χ^2 test). T factor, peritoneal dissemination, and clinical stage are associated with *miR-146a* expression with tendency ($P < 0.1$; χ^2 test). However, no significant differences were observed regarding age, gender, histology, lymphatic invasion, liver metastasis, or adjuvant chemotherapy. In the overall survival curve, patients in the *miR-146a* low expression group (median survival time,1.1 years) had a significantly poorer prognosis than those in the *miR-146a* high expression group (3.1 years, $P = 0.003$; log-rank test; Figure. 1B). Univariate analysis of overall

survival revealed that the relative level of *miR-146a* expression, T factor, lymph node metastasis (N factor), lymphatic invasion and venous invasion were prognostic predictors. Variables with a P value < 0.05 were selected for multivariate analysis. Multivariate analysis showed that the level of *miR-146a* expression was an independent prognostic predictor (RR: 1.53, 95% CI: 1.06 - 2.26, P = 0.022; Cox hazard proportional model, Table 2).

MiR-146a inhibits the migration and invasion of gastric cancer cells

Because lower *miR-146a* levels were associated with the T factor, lymph node metastasis (N factor) and venous invasion, we evaluated *miR-146a* function in gastric cancer cells. We transfected *miR-146a* into the gastric cancer cell line, MKN45, followed by assays conducted under conditions of serum starvation. Expression of *miR-146a* significantly inhibited the cell's capability for migration and invasion compared with control cells (P = 0.012, P = 0.017; Student's *t* test; Figure. 2A, 2B), but did not reduce the cell's capacity for proliferation (data not shown). Moreover, *miR-146a* expression suppressed EGFR and IRAK1 levels relative to control cells (Figure. 2C). To identify whether the *EGFR* and *IRAK1* genes were direct targets of *miR-146a*, we generated an *EGFR* or *IRAK1* 3'UTR luciferase construct.

Cotransfectants expressing both *miR-146a* and *EGFR / IRAK1* 3'UTR showed a significant reduction of luciferase activity compared with control cells ($P < 0.001$; Student's *t* test, Figure. 2D).

Association of the *pre-miR-146a* G/C polymorphism with mature *miR-146a* levels in gastric cancer cases.

Pre-miR-146a, stem-loop formation, includes a G/C SNP (Figure. 3A). We investigated *pre-miR-146a* G/C polymorphism in 76 of the 90 cases from which we were able to obtain genomic DNA. The data showed the following: CC, 34 cases (44.7 %), GC, 34 cases (44.7 %), and GG, 8 cases (10.5 %). Intriguingly, the patients with a GG genotype showed lower *miR-146a* levels than those with a CC genotype in both cancerous tissues (T) and noncancerous tissues (N) ($P = 0.009$, $P = 0.023$; Student's *t* test; Figure. 3B, 3C).

Discussion

This study demonstrated that *miR-146a* levels in cancerous tissue (T) were significantly lower than those in noncancerous tissue (N) in gastric cancer patients. Moreover, the *miR-146a* level was associated with the lymph node metastasis (N factor) and venous invasion. In addition, a lower level of *miR-146a* expression was a strong independent prognostic factor. Based on array data, it was previously reported that a combination of several miRNAs may be useful as prognostic markers in gastric cancer (24, 25). Moreover, a single-miRNA, such as m*iR-451* or *miR-218* can be a prognostic factor. However these miRNAs have been investigated in just a few gastric cancer patients (24, 25). *MiR-146a*, studied here, may be useful as a prognostic marker. Our results indicate that *miR-146a* functions as a tumor suppressor in gastric cancer. Most studies support our results. For example, *miR-146a* inhibits tumor progression by targeting *EGFR*, *CXCR4*, *IRAK1*, and *ROCK1* in pancreatic, breast and prostate cancers (8, 11, 14, 15). However, *miR-146a* is reportedly oncogenic-miRNA in hepatocellular carcinoma (19). It is possible that the discrepancies in *miR-146a*'s functions in different types of cancer may reflect differences in target genes.

This study showed that the ectopic expression of *miR-146* in gastric cancer cells impaired both migration and invasion. These *in vitro* data do not contravene the

correlation between *miR-146a* levels and clinicopathologic factors, such as lymph node metastasis (N factor), and venous invasion. Moreover, we analyzed the recurrent pattern according to *miR-146a levels* in gastric cancer patients. *MiR-146a* low expression group showed the higher incidence of lymph node recurrence or peritoneal recurrence, not distant recurrence, compared to high expression group (Supplementary Table 1). In general, most gastric cancer develops more lymphatic metastasis than hematogenous metastasis. This study indicated that the reduced expression of *miR-146a* might play a role in gastric cancer progression through lymph node metastasis and peritoneal dissemination by inhibition of EGFR and IRAK1. Next, we validated that *miR-146a* binds to the *EGFR* or *IRAK1* 3'UTR and suppresses expression of these genes. In particular, molecular therapies targeted against EGFR increase the impact of treatment in breast and colorectal cancer patients (26, 27). Recently it was shown that therapy targeted against EGFR had a beneficial effect on gastric cancer patients in clinical trials (28, 29). IRAK1 and subsequent NF-κB activation is associated with poor prognosis and invasion in gastric cancer (30, 31). Because EGFR activates not only Raf-MEK-ERK and PI3K-PDK1-Akt signaling but also NF-κB by phosphorylation of IκB (13), EGFR-targeted therapy using miRNA could be a promising treatment in gastric cancer.

It is well known that the G/C SNP within the *pre-miR-146a* seed sequence changes *miR-146a* expression levels in several cancers (12, 16-19). We analyzed the G/C SNP of 76 gastric cancer patients by direct sequencing and found that *miR-146a* expression levels in patients with GG genotypes were significantly lower than those with CC genotypes, in both cancerous and noncancerous tissues. Therefore, this SNP may be associated with *miR-146a* levels in gastric cancer tissue. Shen *et al.* reported that the G allele was associated with lower *miR-146a* levels than was the C allele in the breast cancer cell line MCF-7 (18). In contrast, Xu *et al.* reported that the C allele was associated with lower *miR-146a* levels than the G allele in prostate cancer patients (16). These allele-dependent differences in *miR-146a* levels have been explained by differences in the splicing mechanism between U-G and U-C pairs in the stem region of *pre-miR-146a* and the subsequent impact on the generation of miRNA (32). However, the detailed molecular mechanisms are not clearly clarified.

This is the first report to analyze the significance of *miR-146a* in gastric cancer cases. Moreover, we showed that the G/C SNP of the *pre-miR-146a* seed sequence regulates mature *miR-146a* levels. For this reason, we hypothesize that *miR-146a* levels could be estimated by analysis of the G/C SNP in peripheral blood. *MiR-146a* may play a critical role and prove useful as a novel prognostic marker and therapeutic tool.

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Figure Legends

Figure 1

MiR-146a expression and prognosis in 90 gastric cancer cases

A. *MiR-146a* levels (normalized to *RNU6B*) assessed by qRT-PCR in cancerous (T) and noncancerous tissues (N) from gastric cancer cases (n = 90). *MiR-146a* levels in cancerous tissues (T) were significantly lower than those in noncancerous tissues (N) (P = 0.001). Horizontal line, mean value of each sample.

B. Kaplan -Meier overall survival curves according to *miR-146a* level (T / N; cancerous / noncancerous tissue). The overall survival rate of the *miR-146a* high expression group (n = 45) was significantly higher than that of the low expression group (n = 45; P = 0.003). Figure 1A, 1B: qRT-PCR data were confirmed in duplicate trials.

Figure 2

MiR-146a inhibited migration and invasion of gastric cancer cells and downregulated EGFR and IRAK1 expression.

A. Migration assay showed that ectopic *miR-146a* expression significantly inhibited the capability for migration compared with control cells (P = 0.012). The graphs show

the value of fluorescence in migrating MKN45 cells. Left, parent; middle, Pre-miR-negative control™; right, Pre-miR-146a™

B. Invasion assay showed that ectopic *miR-146a* expression significantly inhibited the capability of invasion compared with control cells ($P = 0.017$). The graphs show the value of fluorescence from the invading MKN45 cells. Left, parent; middle, Pre-miR-negative control™; right, Pre-miR-146a™

C. EGFR and IRAK1 protein expression is decreased by the ectopic expression of *miR-146a*. Left, parent; middle, Pre-miR-negative control™; right, Pre-miR-146a™ Proteins were normalized to the level of β-actin.

D. Luciferase analysis. *EGFR* or *IRAK1* 3'UTR luciferase vector + *miR-146a* transfectants showed lower luciferase activities than did control cells ($P < 0.001$). Relative luciferase activity = (Sample Luc / Sample *Renilla*) / (Control Luc / Control *Renilla*). Luc, raw Firefly luciferase activity; *Renilla*, internal transfection control *Renilla* activity. Left, target 3'UTR luciferase vector only; middle, target 3'UTR luciferase vector +Pre-miR-negative control™; right, target 3'UTR luciferase vector +Pre-miR-146a™.

Figure 2A, 2B, 2D: The error bar represents the standard deviation (SD) from six replicates.

Figure 3

Association of G/C SNP within the *pre-miR-146a* seed sequence with mature *miR-146a* levels in gastric cancer cases (n=76).

A. Schema of hairpin loop structure of *pre-miR-146a* sequence. G/C SNP within *pre-miR-146a* is underlined. Mature *miR-146a* sequence is indicated by black face. *MiR-146a** is a complementary sequence of mature *miR-146a*. Upper, C allele; lower, G allele.

B. *MiR-146a* levels in cancerous tissue (T) according to genotypes. The patients with GG genotypes showed significantly lower *miR-146a* levels relative to those with CC genotypes (P = 0.009). Horizontal line, mean value of each sample.

C. *MiR-146a* levels in noncancerous tissue (N) according to genotypes. The patients with GG genotypes showed significantly lower *miR-146a* levels relative to those with CC genotypes (P = 0.023). Horizontal line, mean value of each sample.

Figure 1

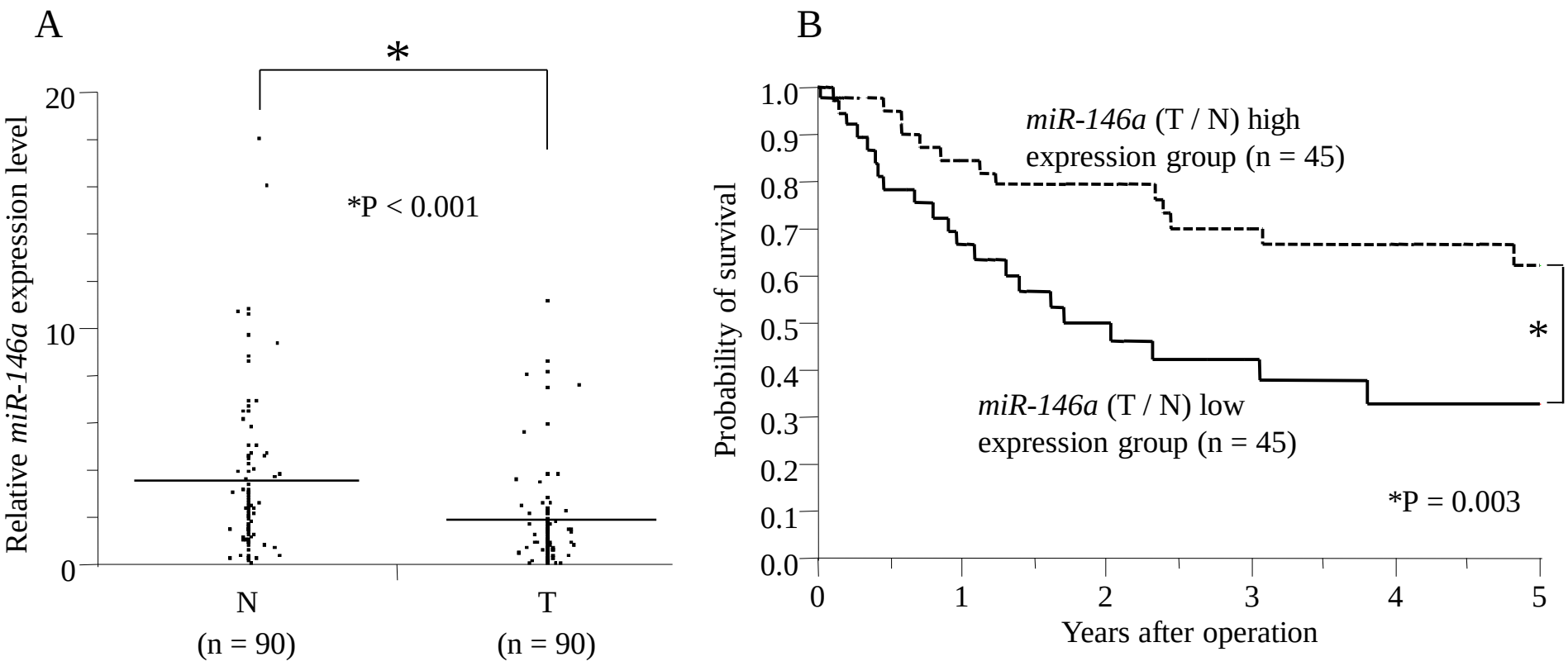
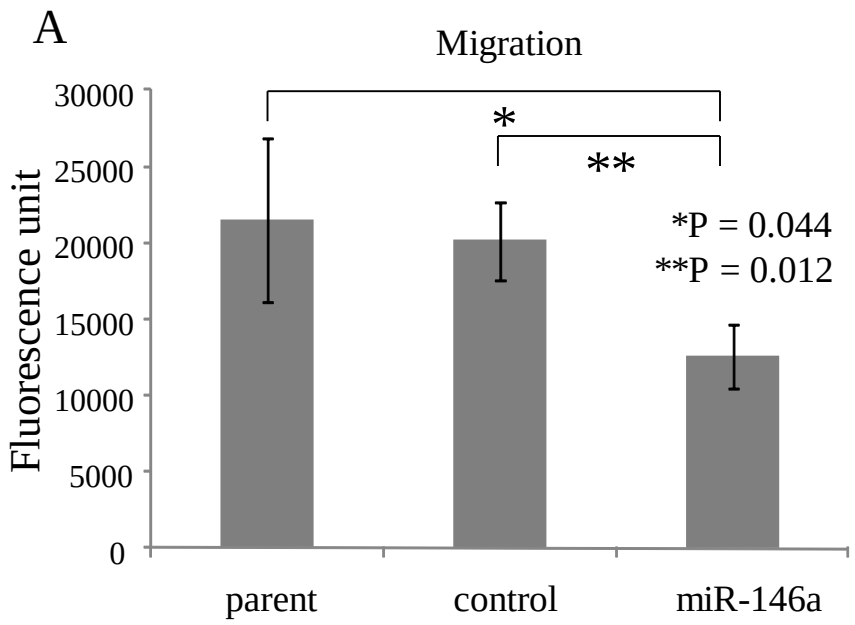
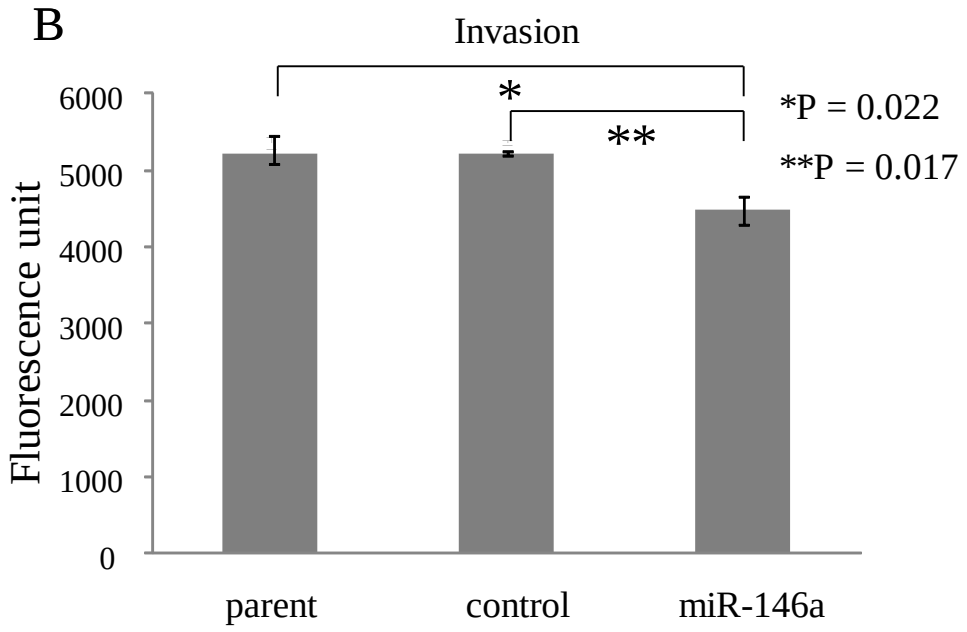
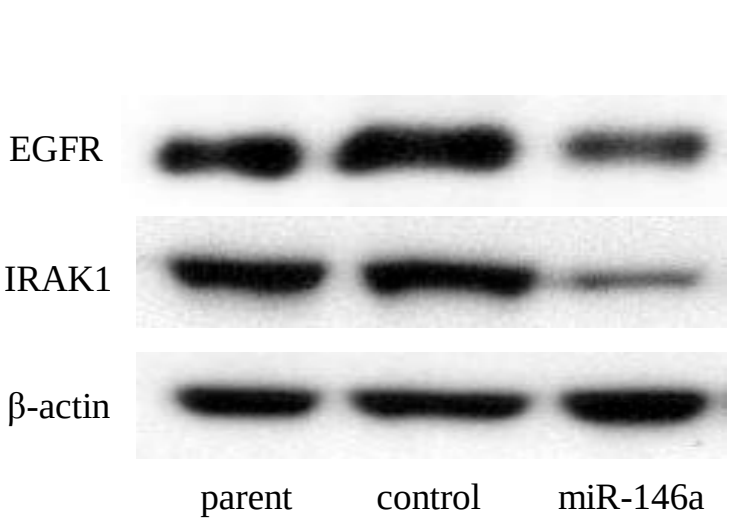

A
*
20
10
0
Relative miR-146a expression level
*P < 0.001
N
(n = 90)
T
(n = 90)
B
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Probability of survival
miR-146a (T / N) high
expression group (n = 45)
*
miR-146a (T / N) low
expression group (n = 45)
*P = 0.003
0
1
2
3
4
5
Years after operation

Figure 2


A
Migration
30000
25000
20000
15000
10000
5000
0
Fluorescence unit
*
**
*P = 0.044
**P = 0.012
parent
control
miR-146a


B
Invasion
6000
5000
4000
3000
2000
1000
0
Fluorescence unit
*
**
*P = 0.022
**P = 0.017
parent
control
miR-146a


C
EGFR
IRAK1
β-actin
parent
control
miR-146a

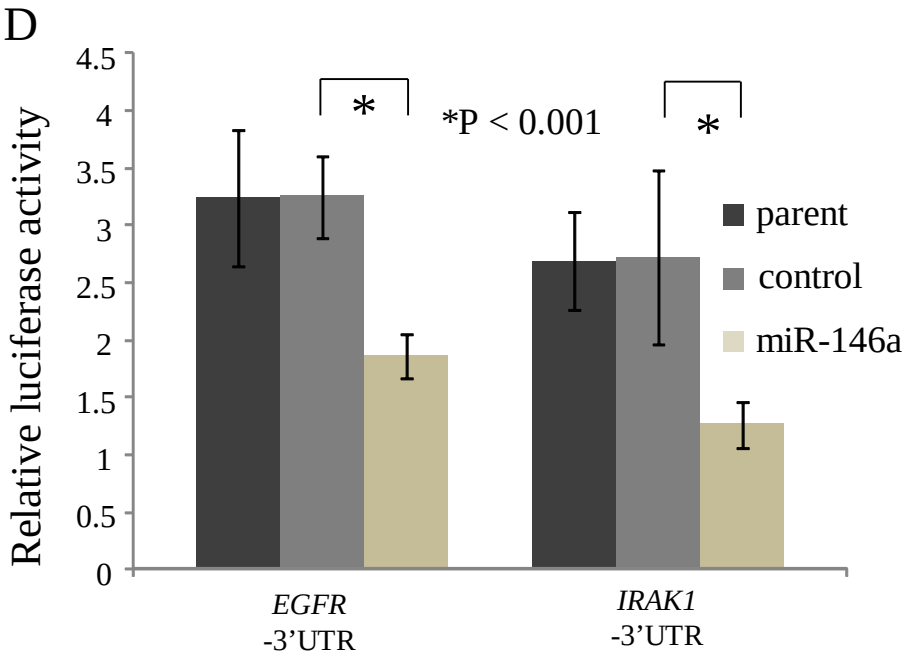
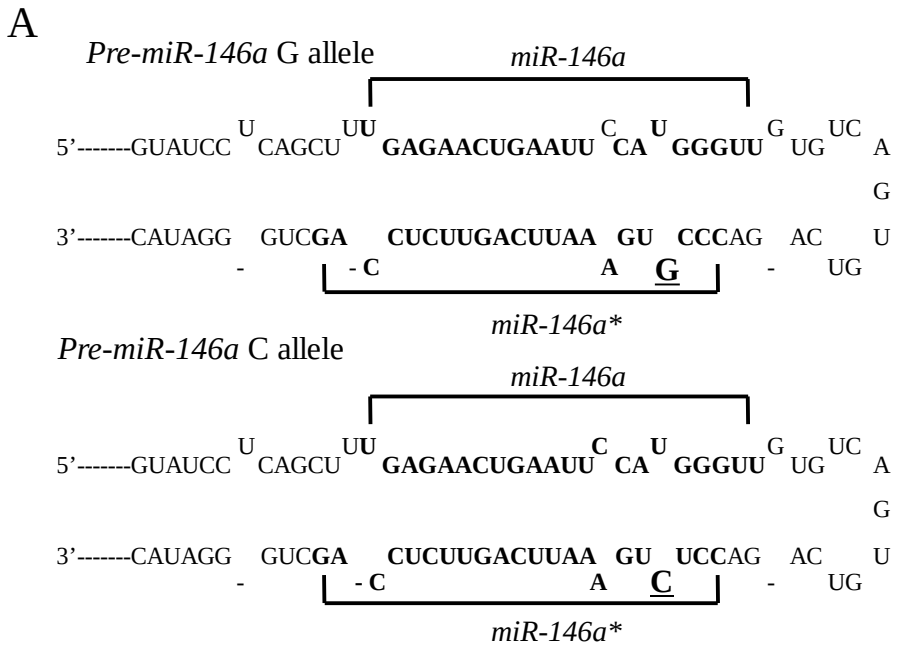
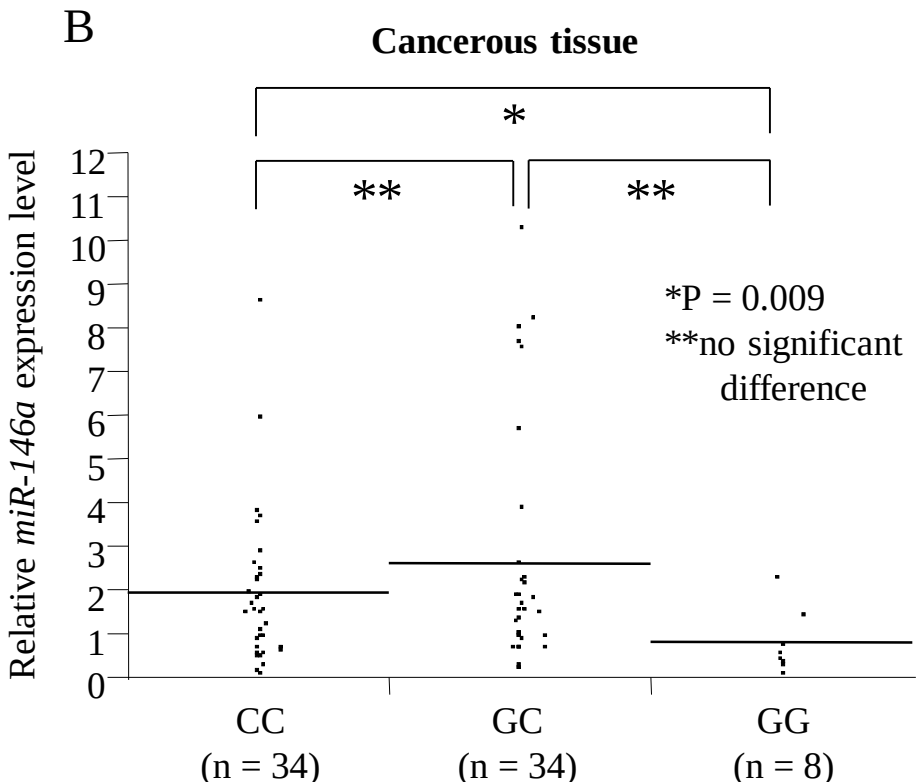

D
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Relative luciferase activity
*
*P < 0.001
*
parent
control
miR-146a
EGFR
-3'UTR
IRAK1
-3'UTR

Figure 3


A
Pre-miR-146a G allele
miR-146a
5'-------GUAUCC U CAGCU UU GAGAACUGAAUU C CA U GGGUU G UG UC A
G
3'-------CAUAGG GUCGA CUCUUGACUUAA GU CCCAG AC U
- - C A G - UG
miR-146a*
Pre-miR-146a C allele
miR-146a
5'-------GUAUCC U CAGCU UU GAGAACUGAAUU C CA U GGGUU G UG UC A
G
3'-------CAUAGG GUCGA CUCUUGACUUAA GU UCCAG AC U
- - C A C - UG
miR-146a*


B
Cancerous tissue
Relative miR-146a expression level
*
**
**
*P = 0.009
**no significant difference
CC (n = 34)
GC (n = 34)
GG (n = 8)

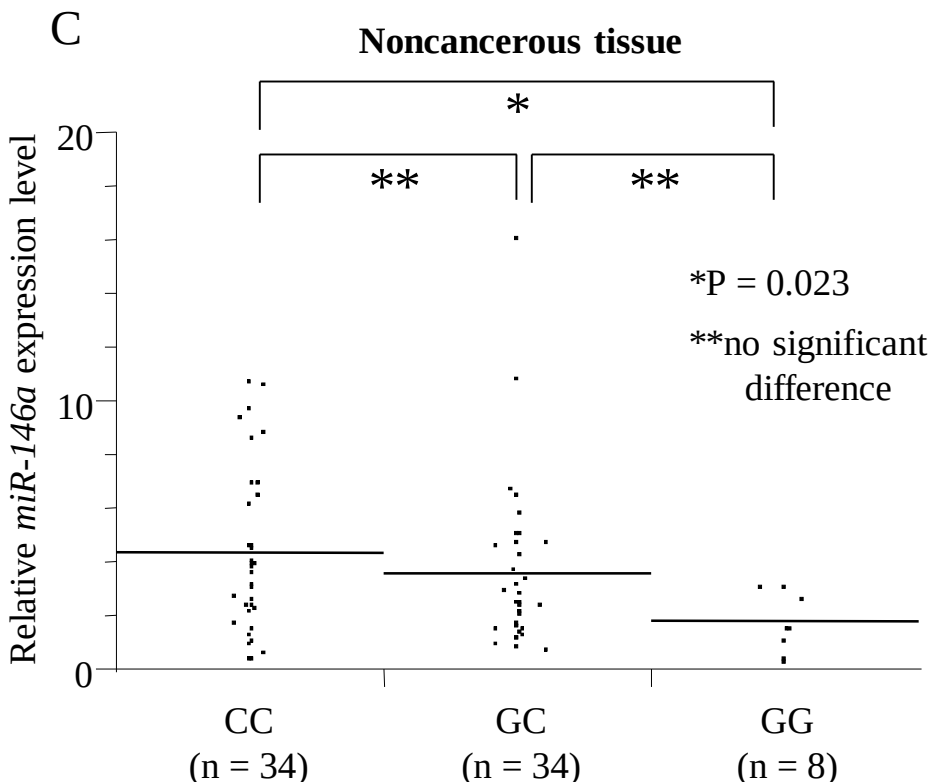

C
Noncancerous tissue
Relative miR-146a expression level
*
**
**
*P = 0.023
**no significant difference
CC (n = 34)
GC (n = 34)
GG (n = 8)

Table 1 *miR146a* level and clinocopathologic factors

Factors	*miR-146a* low expression (n = 45) number	%	*miR-146a* high expression (n = 45) number	%	P value
Age (mean ± SD)	64.7 ± 1.79		65.2 ± 1.79		0.84
Sex					
Male	25	55.6	31	68.9	0.191
Female	20	44.4	14	31.1	
Histological grade†					
Well & Moderate	18	40.0	22	48.9	0.396
Poor & Signet	27	60.0	23	51.1	
Tumor size					
< 3cm	7	15.6	12	26.7	0.176
3cm <	38	84.4	33	73.3	
T factor					
T1	5	11.1	14	31.1	0.073**
T2	18	40.0	16	35.6	
T3	16	35.6	13	28.9	
T4	6	13.3	2	4.4	
Lymph node metastasis (N factor)					
Absent (N0)	10	22.2	20	44.4	0.024*
Present (N1 - N3)	35	77.8	25	55.6	
Lymphatic invasion					
Absent	10	22.2	16	35.6	0.162
Present	35	77.8	29	64.4	
Venous invasion					
Absent	27	60.0	36	80.0	0.037*
Present	18	40.0	9	20.0	
Liver metastasis					
Absent	42	93.3	43	95.6	0.644
Present	3	6.7	2	4.4	
Peritoneal dissemination					
Absent	34	75.6	40	88.9	0.095**
Present	11	24.4	5	11.1	
Adjuvant chemotherapy					
No	16	35.6	23	51.1	0.253
Yes	28	62.2	19	42.2	
Unknown	1	2.2	3	6.7	
Clinical stage					
Stage I	9	20.0	18	40.0	0.05**
Stage II	9	20.0	9	20.0	
Stage III	11	24.4	12	26.7	
Stage IV	16	35.6	6	13.3	

SD; Standard deviation, *P < 0.05, **P < 0.1, †Well differentiated adenocarcinoma (Well), Moderately differentiated adenocarcinoma (Moderate), Poorly differentiated adenocarcinoma (Poor), Signet ring cell carcinoma (Signet)

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

	Univariate analysis			Multivariate analysis		
Factors	RR	95% CI	P value	RR	95% CI	P value
Age (<64 / 65<)	0.95	0.68 - 1.34	0.76	-	-	-
Sex (Male / Female)	0.77	0.51 - 1.10	0.153	-	-	-
Histological grade† (Poor & Signet / Well & Mc	1.24	0.88 - 1.79	0.214	-	-	-
T factor (T2 - T4 / T1)	3.72	1.73 - 15.7	< 0.001*	2.22	0.79 - 10.2	0.14
Lymph node metastasis (Positive / Negative)	3.57	1.96 - 8.88	< 0.001*	2.76	1.45 - 7.01	< 0.001*
Lymphatic invasion (Positive / Negative)	2.13	1.27 - 4.34	0.002*	0.79	0.41 - 1.85	0.555
Venous invasion(Positive / Negative)	1.86	1.31 - 2.64	< 0.001*	1.48	1.03 - 2.15	0.036*
MiR-146a level (Low /High)	1.67	1.28 - 2.43	0.003*	1.53	1.06 - 2.26	0.022*

RR; Relative risk, CI; Confidence interval *P < 0.05 †Well differentiated adenocarcinoma (Well), Moderately differentiated adenocarcinoma (Moderate), Poorly differentiated adenocarcinoma (Poor), Signet ring cell carcinoma

Supplementary Table 1 *miR-146a* level and recurrent patter

Recurrent pattern	*miR-146a* low expression group		*miR-146a* high expression group	
	number	%	number	%
LN or P*	16	72.7	6	50
Distant**	3	13.6	4	33.3
unknown	3	13.6	2	16.7
	22	100	12	100

*LN or P: lymph node metastasis + Peritoneal dissemination

**Distant: Distal (hematogenous) metastasis (liver, lung, bone)