Clinical Significance of miR-146a in Gastric Cancer Cases

Ryunosuke Kogo1,2, Koshi Mimori1, Fumiaki Tanaka1, Shizuo Komune2, and Masaki Mori3

Abstract

Purpose: The profiles of microRNAs change significantly in gastric cancer. MiR-146a 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 q-real-time (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 single nucleotide polymorphism (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. In addition, G/C SNP within the pre-miR-146a seed sequence significantly reduced miR-146a levels in the GG genotype compared with the CC genotype.

Conclusions: MiR-146a contains an 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. Clin Cancer Res; 17(13); 4277–84. ©2011 AACR.

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 seem 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 noncoding RNAs which incompletely bind to the 3′ untranslated region (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 that 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 RafGDS (9, 10). IRAK1 is upstream of NF–xB and is involved in cancer progression (8, 11, 12). Moreover, EGFR activates NF–xB by phosphorylation of IxB (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
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 miR-146a expression was associated with the upregulation of both EGFR and IRAK1. Lower expression of miR-146a was significantly associated with the progression and poorer prognosis of gastric cancer cases. Besides, mature miR-146a expression was significantly related to the single nucleotide polymorphism (SNP) located within pre-miR-146a seed sequence. Genomic DNA would clearly be the best strategy for evaluation of SNP; this same stable method should be used to direct treatment of gastric cancer 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.

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 showed 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 mature 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 between 1992 and 2000. All patients had a clear histologic 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, histologic 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. Forty-seven patients received adjuvant chemotherapy after surgery. Resected cancerous tissues (T) and paired noncancerous tissues (N) were immediately cut and stored in RNAlater (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)

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 μg/mL streptomycin sulfate and cultured in a humidified 5% CO2 incubator at 37°C. Using 2 × 106 MKN45 cells, either Pre-miR-146a or Pre-miR negative control (Pre-miR, Ambion) was transfected at 60 pmol using Nucleofector kit V (Amaxa) according to the manufacturer’s instruction.

Real-time quantitative real-time 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 hours after transfection of miR-146a (Pre-miR-146a, 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 horseradish peroxidase (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′-ATTITTA-CAGGCGTGGGACAG- 3′ and 5′-TCCTCCAAGTCTT-CAGCGG- 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 conducted 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 × 10⁴ cells/well) with serum-free medium were seeded in the top chamber, and the bottom chamber was filled with medium with 10% FBS as a chemoattractant. After 48 hours, 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.

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™) or Pre-miR negative control (Ambion). Assays were conducted 48 hours after transfection by using the Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase signals were normalized to the Renilla luciferase signals. Transfections were conducted three times in independent experiments.

Statistical analysis

Differences between two groups were estimated with Student's t test and 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 (q-real time) 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; Fig. 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; χ² test). T factor, peritoneal dissemination, and clinical stage are associated with miR-146a expression with tendency (P < 0.1; χ² test). However, no significant differences were observed about 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; Fig. 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; Fig. 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 (Fig. 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, Fig. 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 (Fig. 3A). We investigated pre-miR-146a G/C polymorphism in 76 of the 90 cases from which we were able to isolate RNA. Pre-miR-146a G/C polymorphism was significantly associated with mature miR-146a levels (P = 0.004; Student's t test, Table 3A).
Table 2. Univariate and multivariate analysis for overall survival (Cox proportional hazards regression model)

<table>
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<tr>
<th>Factors</th>
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<th>Multivariate analysis</th>
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<td></td>
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<td>MiR-146a level (Low/High)</td>
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<td>1.28–2.43</td>
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*Well differentiated adenocarcinoma (Well), Moderately differentiated adenocarcinoma (Moderate), Poorly differentiated adenocarcinoma (Poor), Signet ring cell carcinoma (Signet).

bP < 0.05.

Abbreviations: RR, Relative risk; CI, Confidence interval.

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. A, B, and D, error bar represents the SD from six replicates.
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; Fig. 3B, 3C).

**Discussion**

This study showed 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 miR-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 (6, 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 with 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 incidence of lymph node recurrence or peritoneal recurrence, not distant recurrence, compared with 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 incidence of lymph node recurrence or peritoneal recurrence, not distant recurrence, compared with high expression group (Supplementary Table 1). In general, most gastric cancer develops more lymphatic metastasis than hematogenous metastasis. 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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 and colleagues 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 and colleagues 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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