miR-146a in gastric cancer

Clinical significance of miR-146a in gastric cancer cases

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miR-146a in gastric cancer

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.
miR-146a in gastric cancer

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 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...
miR-146a in gastric cancer

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.
miR-146a in gastric cancer

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,
miR-146a in gastric cancer

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 IkB (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
miR-146a in gastric cancer

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™) 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 % CO₂ 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™, Ambion). Total protein (40 µg) was electrophoresed and then electroblotted as previously described (22). Protein was
miR-146a in gastric cancer

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™) or Pre-miR negative control™ (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.
miR-146a in gastric cancer

Statistical analysis Differences between two groups were estimated with Student's $t$ test and $\chi^2$ 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).
miR-146a in gastric cancer

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; 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 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
miR-146a in gastric cancer

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.
miR-146a in gastric cancer

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

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

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.
miR-146a 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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miR-146a in gastric cancer

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miR-146a in gastric cancer


miR-146a in gastric cancer


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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
miR-146a in gastric cancer

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.
Table 1 *miR*146a level and clinicopathologic factors

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<th><em>miR</em>146a high expression (n = 45)</th>
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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)
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<tr>
<td>Lymph node metastasis (Positive / Negative)</td>
<td>3.57</td>
<td>1.96 - 8.88</td>
</tr>
<tr>
<td>Lymphatic invasion (Positive / Negative)</td>
<td>2.13</td>
<td>1.27 - 4.34</td>
</tr>
<tr>
<td>Venous invasion (Positive / Negative)</td>
<td>1.86</td>
<td>1.31 - 2.64</td>
</tr>
<tr>
<td>MiR-146a level (Low / High)</td>
<td>1.67</td>
<td>1.28 - 2.43</td>
</tr>
</tbody>
</table>

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 (Signet)
Figure 1

A

Relative miR-146a expression level

*P < 0.001

N (n = 90)

T (n = 90)

B

Probability of survival

miR-146a (T / N) high expression group (n = 45)

miR-146a (T / N) low expression group (n = 45)

*P = 0.003

Years after operation
Figure 2

A. Migration

![Bar chart showing migration results with fluorescence units.](chart)

- Parent: 25000
- Control: 20000
- miR-146a: 15000

B. Invasion

![Bar chart showing invasion results with fluorescence units.](chart)

- Parent: 6000
- Control: 5000
- miR-146a: 4000

C. Western blot analysis

- EGFR
- IRAK1
- β-actin

D. Relative luciferase activity

- EGFR -3’UTR
- IRAK1 -3’UTR

*P = 0.044
**P = 0.012

*P = 0.022
**P = 0.017

*P < 0.001
*P = 0.017
Figure 3

A

Pre-miR-146a G allele

5'-------GUAUCC CAGCU

miR-146a

C allele

3'-------CAUAGG GUC

Pre-miR-146a C allele

miR-146a

miR-146a*

5'-------GUAUCC CAGCU

miR-146a

miR-146a*

3'-------CAUAGG GUC

miR-146a*

B

Cancerous tissue

Noncancerous tissue

Relative miR-146a expression level

12

11

10

9

8

7

6

5

4

3

2

1

0

CC (n = 34)

GC (n = 34)

GG (n = 8)

CC (n = 34)

GC (n = 34)

GG (n = 8)

*P = 0.009

**no significant difference

**P = 0.023

**no significant difference
Clinical Cancer Research

Clinical significance of miR-146a in gastric cancer cases
Ryunosuke Kogo, Koshi Mimori, Fumiaki Tanaka, et al.

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