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Expression Level of MicroRNA-449a Predicts the Prognosis of Patients With Gastric Cancer

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Abstract. *Background: In previous studies, we demonstrated the significant role of microRNA-449a (miR-449a) in colorectal cancer with in vivo and clinical samples. The importance of miR-449a in gastric cancer is still to be elucidated. This study examined the impact of miR-449a expression in tumor tissue and serum and investigated its potential as a prognostic marker in gastric cancer. Materials and Methods: Sixty-six patients with gastric cancer who underwent surgery were included in the study. miR-449a expression in tumor tissue and serum were investigated by real-time polymerase chain reaction analysis. The association of miR-449a expression with clinicopathological factors and patient prognosis were also investigated. Results: miR-449a expression was lower in tumor tissue than non-tumor tissue. miR-449a in tumor tissue negatively correlated with the malignancy of tumor and clinical stage. Increased carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) levels were seen at significantly higher frequencies in patients with low miR-449a expression. Patients with low miR-449a expression had poorer cancer-specific survival compared to those with high miR-449a expression. The univariate analysis showed that lymphovascular invasion, increased CEA and CA19-9 and a low expression of miR-449a were associated with a poorer 5-year cancer-specific survival. miR-449a expression level in serum correlated to that in tumor tissue and was also associated with tumor malignancy. Conclusion: The miR-449a level in tumor tissue might be useful as a prognostic indicator for patients with gastric cancer and miR-449a in serum appears to reflect its expression in tumor tissue.*

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MicroRNAs (miRNAs) are a class of noncoding RNAs of 21-25 nucleotides. miRNAs regulate expression of genes by targeting the 3-untranslated regions of mRNAs, which results in miRNA degradation or inhibition of miRNA translation (1, 2). miRNAs are considered to regulate various aspects of cell physiology, including development, proliferation, differentiation and apoptosis (3). Abnormally expressed miRNAs were reported to act as oncogenes or tumor suppressors and to be associated with tumorigenesis (4, 5). In the gastroenterological field, *miRNA-34a-5p* was shown to suppress gastric metastasis, and its expression predicted recurrence in patients with stages II/III gastric cancer (6). Thus, miRNAs may be used as prognostic indicators and potential targets for cancer therapy.

miR-449a is a member of the *miR-449* family. The *miR-449* cluster contains sequences and secondary structures similar to those of the *miR-34* family. Therefore, they have been classified as a single family of miRNAs. The expression of *miR-449a* is reduced in several cancer types, such as prostate and bladder cancer (3, 7). Furthermore, *miR-449a* regulates several genes associated with tumorigenesis, including the gene encoding c-MYC, histone deacetylase (HDAC) (8-11) and cell-division cycle 25 homolog A (CDC25A), suggesting that *miR-449a* may have oncogenic effects. Our previous study succeeded in the creation of a *miR-449a*-deficient mouse and demonstrated that the deficiency of *miR-449a* promoted azoxymethane and dextran sodium sulfate-induced colorectal tumorigenesis (12). The *miR-449a*-deficient mouse showed higher expression of Ki-67 compared to the wild-type mouse, suggesting that the normal colorectal mucosa has a more proliferative phenotype. We also reported the significance of *miR-449a* and its potential target HDAC1 in patients with colorectal cancer (13). However, the impact of *miR-449a* expression in gastric cancer is to date not well studied and still to be elucidated.

Various types of molecules, including microRNAs, found in liquid biopsy are reported to be possible new biomarkers (14). The greatest advantage of liquid biopsy is that it is less

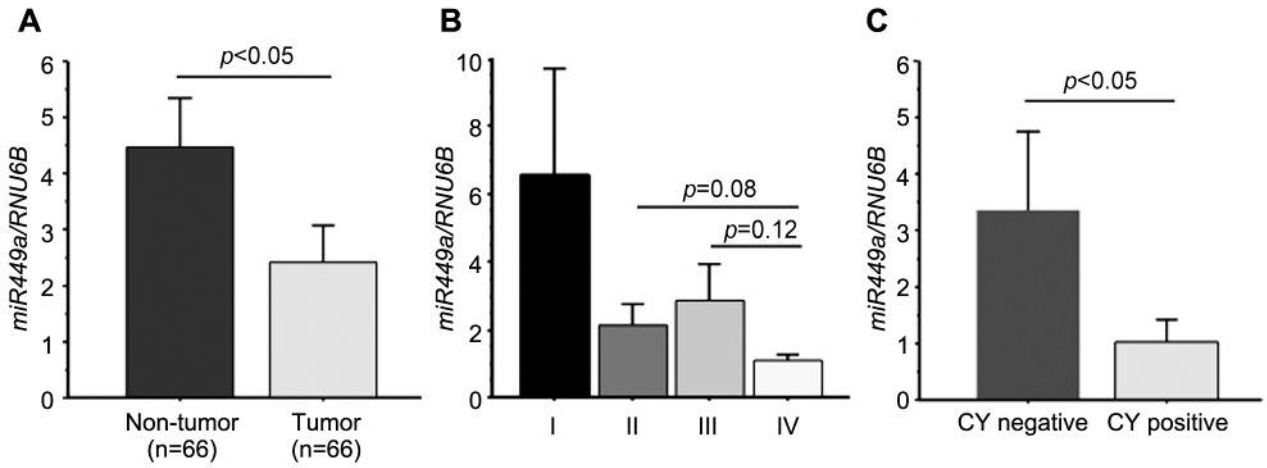

Figure 1. *Expression level of miR-449a in gastric cancer tissue. A: Comparison of miR-449a expression between tumor and non-tumor tissue. In the tumor tissue, the expression of miR-449a was significantly lower than that of non-tumor tissue. B: Comparison of miR-449a expression according to stage. Patients with stage IV gastric cancer had relatively lower expression of miR-449a compared to those with stage II or III gastric cancer. C: Comparison of miR-449a expression according to ascites cytology (CY). The expression of miR-449a in patients with cytology-positive ascites was significantly lower.*

invasive for patients. Whether serum *miR-449a* has clinical significance in gastric cancer is not yet known. The aim of this study was to investigate the significance of *miR-449a* in tumor tissue and serum from patients with gastric cancer.

Materials and Methods

Patients and tissue samples. This study was approved by the Ethics Committee (approval number: 29001) of the Tokushima University Hospital and the patient information was obtained from their medical records. Sixty-six samples of gastric cancer were used to investigate tissue expression of *miR-449a*. For 18 patients, blood samples were collected preoperatively and serum was preserved for the investigation of *miR-449a* in serum samples. The patients with gastric cancer underwent surgical resection at the Tokushima University Hospital from 2008 to 2010. Postoperatively, the patients were followed up using the level of tumor markers carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9), and computed tomography every 3 months to diagnose recurrence of the disease. The 5-year overall survival (OS), cancer-specific survival (*i.e.* death as a result of gastric cancer), and disease-free survival (DFS) were investigated. OS, cancer-specific survival and DFS were measured from the day of surgery. The prognostic factors were univariately analyzed. The staging of gastric cancer was performed according to the eighth edition of the American Joint Committee on Cancer Cancer Staging Manual (15).

miRNA expression analyses. Total RNA was isolated from resected specimen using miReasy kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Reverse transcription was carried out with the TaqMan® MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). For single miRNA assays, 25 ng of total RNA including the was reverse transcribed with sequence-specific primers. For each quantitative polymerase chain reaction, 5-10 ng of the initial total RNA was used from cDNA samples. Every sample was amplified in triplicate using specific primer pairs using TaqMan® Gene Expression Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions. miRNA gene expression assay used Hs06632500_s1, hsa-*miR-449a* as a primer. Primer *hsa-RNU6B* for RNA, U6 small nuclear 6, pseudogene was used as an internal control for tissue and serum expression. All reactions were performed on a ViiA7 Real-Time PCR system (Thermo Fisher Scientific). The standard curve method was used to evaluate the relative expression.

Statistical analysis. Statistical analyses were carried out using the JMP 10 statistical software package (SAS Institute Inc, Tokyo, Japan). Student's *t*-test was used for comparison of continuous variables. The chi-squared test was used to analyze the relationship between *miR-449a* expression and clinicopathological characteristics. The patients' survival data were plotted by the Kaplan–Meier method and analyzed by the log-rank test to calculate differences between the curves. A *p*-value of less than 0.05 was considered to be statistically significant.

Results

Expression level of *miR-449a* in gastric cancer tissue. Expression of *miR-449a* was compared between tumor and non-tumor tissue. In the tumor tissue, the expression of *miR-449a* was significantly lower than that of non-tumor tissue (Figure 1A). In respect to the *miR-449a* expression according to stage, patients with stage IV gastric cancer had relatively lower expression level of *miR-449a* compared to those with stage II or III (Figure 1B). Furthermore *miR-449a* expression was significantly lower in patients with cytology-positive ascites (Figure 1C).

Table I. *Clinicopathological characteristics of patients according to expression of miR-449a.*

Factor		*miR-449a* expression		
	Subgroup	Low (n=33)	High (n=33)	*p*-Value
Age, years	Mean±SD	71.4±11.6	68.8±15.0	0.42
Gender, n	Male/Female	26/7	28/5	0.66
Location, n	Upper/Middle/Lower	8/14/11	5/17/11	0.61
Size, cm	Mean±SD	60.5±33.7	57.9±35.1	0.76
Depth, n	M,SM/MP,SS/SE,SI	2/13/18	8/12/13	0.12
Differentiation	tub1,2/poor, sig	14/19	22/11	0.09
Lymphatic invasion, n	No/Yes	7/26	12/21	0.22
Vascular invasion, n	No/Yes	9/24	14/19	0.26
LN metastasis, n	No/Yes	7/26	12/21	0.33
Stage, n	I/II/III/IV	4/7/12/10	7/12/7/7	0.80
CEA, n	<5/≥5 ng/ml	23/10	30/3	<0.05
CA19-9, n	</≥38 ng/ml	17/16	26/7	<0.05

LN: Lymph node; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9; M: mucosa; SM: submucosa; SS: subserosa; SE: serosa; SI: serosal invasion; tub1: well-differentiated tubular adenocarcinoma; tub2: moderately differentiated tubular adenocarcinoma; poor: poorly differentiated adenocarcinoma; sig: signet-ring cell carcinoma.

Patients were divided into two groups by the median value of *miR-449a* expression (Table I). There was no difference in age, gender, tumor location, size, depth, lymphovascular invasion and lymph node metastasis. The frequency of less differentiated tumors tended to be higher in the group with low *miR449a* expression (p=0.09). Increased CEA and CA19-9 levels were seen at significantly higher frequencies in the group with low *miR-449a* expression (p<0.05).

Cancer-specific survival according to expression level of miR-449a in tumor tissue. Figure 2 shows the cancer-specific survival according to expression of *miR-449a* in tumor tissue. Patients with low expression of *miR-449a* had worse cancer-specific survival than those with high expression of *miR-449a*. The univariate analysis showed that lymph node metastasis, lymphovascular invasion, increased levels of CEA and CA19-9 and a low expression of *miR-449a* were significantly associated with poorer cancer-specific survival (Table II). Taken together, these results suggested that a low expression of *miR-449a* in gastric cancer was correlated with higher malignancy of tumor and poorer prognosis.

Expression of miR-449a in serum reflects that in tumor tissue and is correlated with tumor malignancy. The expression of *miR-449a* in serum was compared with that of the corresponding tissue sample. This revealed a tendency for positive correlation, as shown in Figure 3A (R^2=0.64, p=0.12). Regarding clinicopathological factors, *miR-449a* expression in serum was lower in those with dedifferentiated gastric cancer compared to differentiated cancer. For other factors such as tumor size, lymphovascular invasion and depth of invasion, there was no correlation to the *miR-449a* expression in serum samples. These results suggest that *miR-449a* in serum might reflect the expression of *miR-449a* in tumor tissue and could also be used as a biomarker preoperatively and in a less invasive manner than biopsy.

Discussion

In this study, the expression pattern and the significance of *miR-449a* in gastric cancer were demonstrated. It was revealed that a low expression of *miR-449a* is associated with increased level, of serum CEA and CA19-9. Although the type of cancer is different, *miR-449a* expression was reported to be inversely correlated with the level of serum CEA in colon cancer (7, 13). The expression level of *miR-449a* is reported to be frequently reduced in malignant tumor tissues, such as gastric and bladder cancer (16, 17). In these cancer types, *miR-449a* is considered to inhibit cell growth or induce senescence and apoptosis by activating the p53 pathway.

This present study demonstrated the significance of *miR-449a* as a prognostic indicator in the patients with gastric cancer. Previous reports also revealed that low expression of *miR-449a* would be a useful biomarker of bladder and prostate cancer, and medulloblastoma (18-21). In our previous work using knockdown of *miR-449a* in mice, we showed that the lack of *miR-449a* contributes to the tumorigenesis of colorectal cancer and that *miR-449a* regulated multL homolog 1 (*MLH1*) and Kirsten rat sarcoma viral oncogene homolog (*KRAS*) (12). Other reports demonstrated that *miR-449a* down-regulated oncogenes such

Table II. *Univariate analysis of 5-year cancer-specific survival (CSS).*

Factor	Subgroup	5-Year CSS (%)	*p*-Value
Age	<70/≥70 Years	80.0/59.7	0.16
Gender	Male/Female	58.9/69.8	0.43
Differentiation	tub1, tub2/poor, sig	67.1/54.7	0.58
LN metastasis	No/Yes	90.0/51.1	<0.05
Lymphatic invasion	No/Yes	93.3/55.5	<0.05
Vascular invasion	No/Yes	83.8/51.5	<0.05
CEA	<5/≥5 ng/ml	69.2/30.8	<0.05
CA19-9	</≥38 ng/ml	72.5/41.1	<0.05
miR-449a	Low/High	58.5/75.6	<0.05

tub1: Well-differentiated tubular adenocarcinoma; tub2: moderately differentiated tubular adenocarcinoma; poor: poorly differentiated adenocarcinoma; sig: signet-ring cell carcinoma; LN: lymph node; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9.

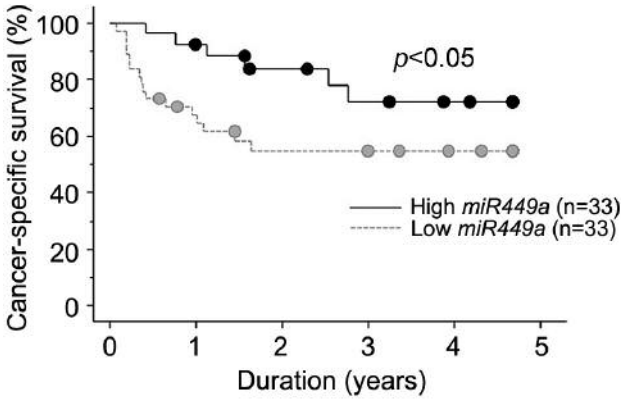

Figure 2. *Cancer-specific survival according to expression level of miR-449a in gastric tumor tissue. Patients with low expression of miR-449a had worse cancer-specific survival than those with high expression.*

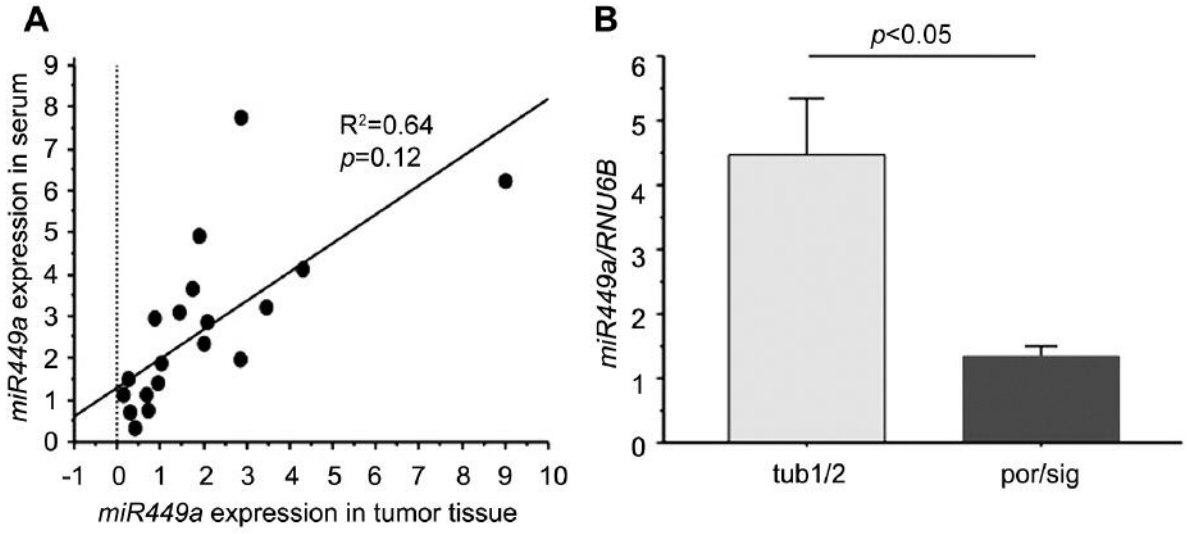

Figure 3. *The expression of miR-449a in serum samples reflected the expression in tumor tissue and correlated with tumor malignancy. A: Positive correlation between miR-449a expression in tumor tissue and corresponding serum. B: miR-449a expression in serum was lower in two dedifferentiated gastric cancer compared to differentiated cancer.*

as HDAC1, transforming growth factor-β (*TGFB*), special AT-rich sequence-binding protein 2 (*SATB2*), a disintegrin and metalloproteinase domain-containing protein 10 (*ADAM10*), *MYC* and mitogen-activated protein kinase 1 (*MAPK1*) (1-4, 17, 20).

Compared to diagnostics by tissue sampling, body fluid samples are easier to obtain and causes less harm to patients. Since Mitchell *et al*. confirmed that *miR-141* in plasma can be used as a diagnostic marker for prostate cancer (22), new circulating miRNAs have emerged as markers for tumor diagnosis. Serum expression levels of *miR-18a*-5p, *miR-21*-5p, *miR-29a*-5p, *miR-92a*-5p, *miR-143*-5p and *miR-378*-5p were reported to be significantly less expressed in patients with colorectal cancer (23). We demonstrated a tendency for positive correlation between serum and tissue levels of *miR-449a*, and *miR-449a* expression in serum was lower in those with dedifferentiated gastric cancer compared to differentiated cancer. miRNAs are stable in plasma, and there was no change in plasma expression level of miRNAs even when it was boiled at 100˚C for 10 min, kept at room temperature, or repeatedly frozen and thawed at room temperature (24). There is a current hypothesis that miRNAs

in plasma are present in exosomes, complexes, or microvesicles secreted by tumor cells (25). This makes miRNAs suitable biomarkers of disease.

The limitation of this study is the shortage of samples and the lack of detailed *in vitro* or *in vivo* analysis to determine the role of *miR-449a*. Further detailed examination is needed to confirm the descriptive data presented in this study.

In conclusion, *miR-449a* in serum appears to reflect the expression of *miR-449a* in tumor tissue and the results of this study suggest that a low expression of *miR-449a* might be a useful prognostic biomarker for the patients with gastric cancer.

Conflicts of Interest

None of the Authors has any potential financial conflicts of interest related to this study.

Authors' Contributions

Ishikawa D designed and carried out the experiment and wrote the initial draft of the article. Yoshikawa K and Takasu C contributed to analysis and interpretation of data. Tokunaga T and Higashijima J contributed to data collection and interpretation. Kashihara H and Nishi M assisted in the preparation of the article. Shimada M supervised the project. All Authors have critically reviewed the article, approved the final version of the article.

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