miR-29b is an indicator of prognosis in breast cancer patients

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Abstract. MicroRNA-29b (*miR-29b*) targets numerous important genes that mediate carcinogenesis and tumor development in breast cancer *in vitro* and *in vivo*. The aim of the present study was to determine the clinical significance of *miR-29b* expression in primary breast cancer patients. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of *miR-29b* and certain target genes of *miR-29b*, such as DNA methyltransferase 3A (*DNMT3A*), ten-eleven translocation 1 (*TET1*) and thymine DNA glycosylase (*TDG*), was performed in 94 primary breast cancer samples. Low expression of *miR-29b* in primary tumors was significantly associated with poorer disease-free survival (DFS) (P=0.0075) and overall survival (OS) (p=0.0012). Multivariate analysis indicated that *miR-29b* expression was an independent prognostic factor for OS [relative risk=15.6 (2.33-348), P=0.0026]. In addition, a significant inverse correlation was identified between the expression levels of *DNMT3A* and *miR-29b* in estrogen receptor-positive breast cancer patients (P=0.027). To the best of our knowledge, this is the first study to investigate the clinicopathological significance of *miR-29b* in breast cancer cases and *miR-29b* is shown to act as a tumor suppressive microRNA in breast cancer and as a potential marker for recurrence and metastasis in breast cancer patients.

Introduction

Breast cancer is a leading cause of cancer-related mortality among women in industrialized countries. Despite advances in the technologies used for its diagnosis and treatment, recurrence and metastasis of breast cancer remain serious clinical issues. Therefore, there is an urgent need to identify biomarkers or techniques to be used for the early detection of carcinogenesis or recurrence following curative surgery using minimally invasive tests.

MicroRNAs (miRs) are small non-coding RNAs consisting of 20-22 nucleotides. Changes in the levels of miRs are involved in the initiation and progression of human cancers due to the altered translation of various target genes (1). The recent increase in miR interest is attributed to the breakthrough discovery of their role in numerous pathological processes, including malignant transformation (2). In fact, miRs have been reported as potential biomarkers of various malignancies (3,4).

MicroRNA-29b (*miR-29b*) regulates a number of important genes that mediate carcinogenesis and tumor development in breast cancer (5-8). For example, *miR-29b* targets a network of pro-metastatic regulators involved in angiogenesis, collagen remodeling and proteolysis, thereby inhibiting metastasis (5). Furthermore, *miR-29b* directly targets DNA methyltransferase 3A (*DNMT3A*), *DNMT3B*, ten-eleven translocation 1 (*TET1*) and thymine DNA glycosylase (*TDG*), all of which play crucial roles in the progression and metastasis of various cancers by altering the DNA methylation status (9-16). Although almost all the studies investigating the numerous important roles of *miR-29b* in breast cancer have been experimental studies conducted *in vitro* and *in vivo* (5-8,16), the clinicopathological significance of *miR-29b* in breast cancer cases has not been determined clinically. The present study evaluated the importance of *miR-29b* in breast cancer cases and additionally showed the associations between *miR-29b* and several target genes of *miR-29b* indicated in the regulation of DNA methylation status in clinical samples.

Materials and methods

Patients. Breast cancer patients (n=94) who underwent surgical treatment at several hospitals [National Hospital Organization Kyushu Cancer Center (Fukuoka, Fukuoka) Kyushu University Beppu Hospital (Beppu, Oita), Oita Prefectural Hospital (Yufu, Oita) and Takada-Chuo Hospital (Yokohama, Kanagawa), all in Japan)] between 1990 and 1999 were enrolled in the study. Prior to sample acquisition, each patient provided written informed consent at the respective hospital. The study was approved by the ethics committees of Kyushu University. Patients were excluded who had been diagnosed

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with ductal carcinoma *in situ*. Three patients who had distant metastasis at first diagnosis received no neo-adjuvant chemotherapy. Post-operative adjuvant chemotherapy and endocrine therapy were performed according to the St. Gallen Consensus Conference guidelines (17). Among the 94 patients, 63 were estrogen receptor (ER)-positive. The expression levels of the HER2 protein could not be confirmed in the cases, as the measurements used for HER2 expression were not common when the surgeries were performed. The mean observation period ranged from 1 to 124 months (median, 54 months). Among the 94 patients, only 30, 81 and 57 patients were examined for *TET1*, *TDG* and *DNMT3A* expression, respectively, due to the deficiency of samples for quantification of each cDNA by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Total RNA extraction and first-strand cDNA synthesis. The resected tumor tissue specimens were frozen immediately in liquid nitrogen and stored at -80˚C until analysis. The total RNA extraction from the primary tumors was performed according to the ISOGEN-LS (Nippon Gene Co., Ltd., Tokyo, Japan) manufacturer's instructions. The reverse transcription reactions and first-strand cDNA synthesis were performed as described previously (18).

RT-qPCR for miR-29b, TET1, TDG and DNMT3A. Quantitative analysis was performed of *miR-29b*- and *RNU6B* (internal control)-specific cDNAs derived from total RNA extracted from resected tumors using gene-specific primers, according to the TaqMan MicroRNA Assay protocol (Assay IDs: 000413 for hsa-*miR-29b*-3p and 001093 for *RNU6B*; Applied Biosystems, Carslbad, California, USA). The procedures were as described previously (18). The raw miR expression levels were normalized to *RNU6B* expression for calculation of the relative miR expression values. To determine the relative expression levels of *TET1*, *TDG* and *DNMT3A*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the internal control. RT-qPCR was performed using the LightCycler® 480 system and the LightCycler® 480 Probes Master kit (Roche Applied Science, Penzberg, Germany). The sequences of the primers for *TET1*, *TDG* and *DNMT3A* were as follows: *TET1* sense, 5'-TCTGTTGTTGTGCCTCTGGA-3' and antisense, 5'-GCCTTTAAAACTTTGGGCTTC-3'; *TDG* sense, 5'-ATGCAGCAGTGAACCTTGTG-3' and antisense, 5'-GTCATCCACTGCCCATTAGG-3'; and *DNMT3A* sense, 5'-AAGGAGGAGCGCCAAGAG-3' and antisense, 5'-ATC ACCGCAGGGTCCTTT-3'. The expression of *DNMT3B* was not detected in the samples.

Statistical analysis. For *miR-29b* analysis, differences between clinicopathological factors were analyzed using χ^2 tests for categorical variables. Disease-free survival (DFS) and overall survival (OS) times were measured from the time of the first surgery until the date of mortality or last follow-up. Survival curves were determined by the Kaplan-Meier method and statistical significance between groups was assessed using the Wilcoxon test. Multivariate analysis was performed to assess the relative influence of prognostic factors on OS using the Cox proportional hazards model with a forward stepwise procedure. Statistical analysis was performed by JMP® Pro version 9.0.2 for Mac OS (SAS Institute Japan, Tokyo, Japan). P<0.05 was considered to indicate a statistically significant difference.

Table I. *miR-29b* expression and clinicopathological factors.

Factors	*miR-29b* expression		P-value
	Low (n=47), no. (%)	High (n=47), no. (%)	
Age, mean years ± SD	55±11	54±11	
ER			
Positive	31 (66)	32 (68)	0.59
Negative	15 (32)	12 (26)	
Progesterone receptor			
Positive	26 (55)	31 (66)	0.24
Negative	18 (38)	13 (28)	
T factors			
T1	13 (28)	25 (53)	0.01
T2-4	34 (72)	22 (47)	
Lymph node metastasis			
Absent	22 (47)	27 (57)	0.31
Present	25 (53)	20 (43)	
Lymphatic invasion			
Absent	18 (38)	16 (34)	0.73
Present	23 (49)	24 (51)	
Venous invasion			
Absent	33 (70)	34 (72)	0.60
Present	7 (15)	6 (13)	
Stage			
Stage I	7 (15)	17 (36)	0.02
Stages II-IV	40 (85)	30 (64)	

miR-29b, microRNA-29b; SD; standard deviation, ER, estrogen receptor.

Results

Low miR-29b expression in primary tumor tissues is a prognostic factor for breast cancer patients. *miR-29b* expression was assessed in primary tumor tissues from 94 breast cancer patients. Patients were divided into *miR-29b* high and low expression groups according to the median value of *miR-29b* expression. Clinicopathological factors were subsequently analyzed in association with *miR-29b* levels. The *miR-29b* low expression group exhibited a significantly larger tumor size and more advanced clinical stages compared to the *miR-29b* high expression group (Table I). In terms of DFS and OS, the *miR-29b* low expression group showed a significantly poorer prognosis than that of the *miR-29b* high expression group (Fig. 1). Among the ER-positive cases, the low *miR-29b* expression group had significantly poorer DFS and OS compared to the high *miR-29b* expression group (Fig. 2). Among the ER-negative cases, low *miR-29b* expression correlated with a poorer OS only (Fig. 3).

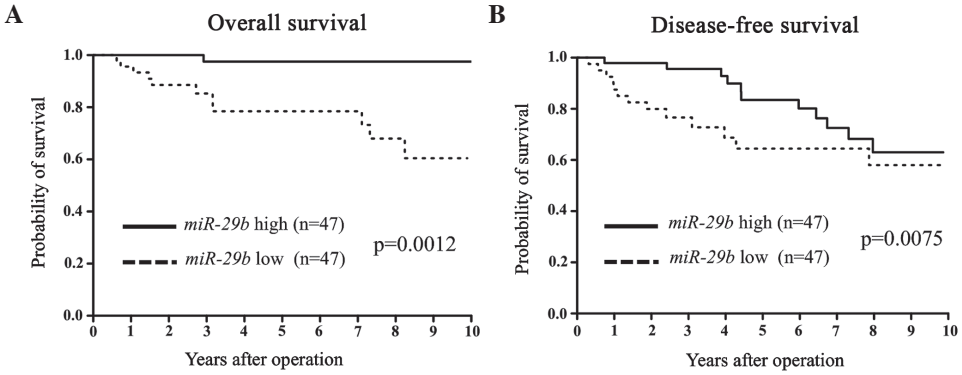

Figure 1. (A) Overall survival (OS) and (B) disease-free survival (DFS) curves for breast cancer patients according to the expression levels of microRNA-29b (*miR-29b*) in primary tumors. The differences in OS and DFS were significant (P=0.0012 and 0.0075, respectively).

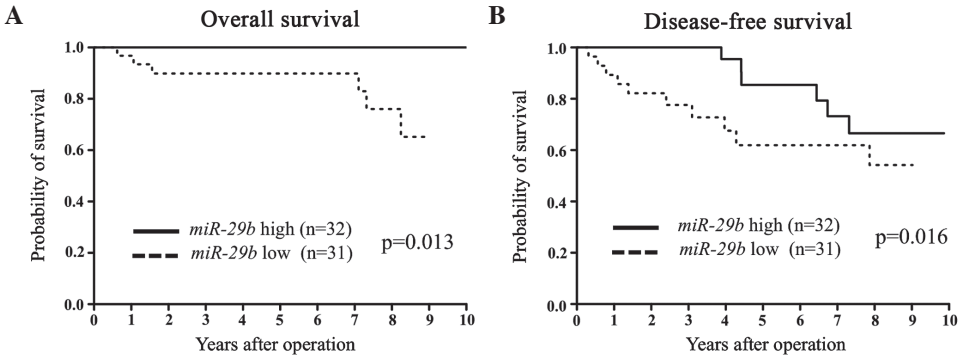

Figure 2. (A) Overall survival (OS) and (B) disease-free survival (DFS) curves for breast cancer patients according to the expression levels of microRNA-29b (*miR-29b*) in estrogen receptor (ER)-positive primary tumors. The differences in OS and DFS between the *miR-29b* low and high expression levels were significant (P=0.013 and 0.016, respectively).

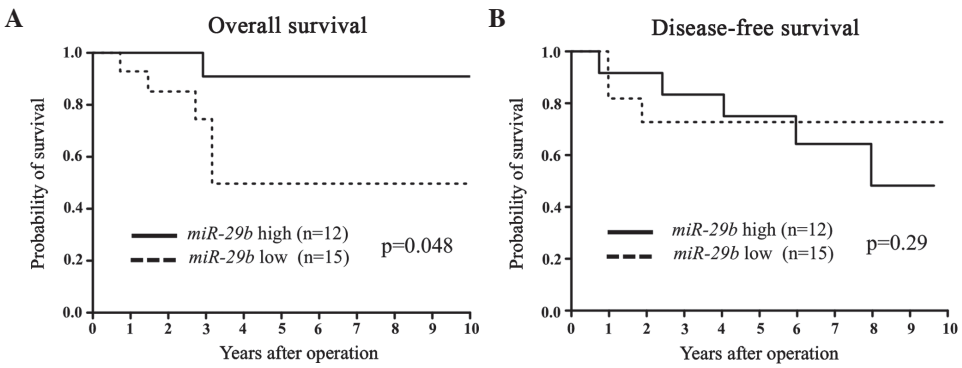

Figure 3. (A) Kaplan-Meier overall survival (OS) and (B) disease-free survival (DFS) curves for breast cancer patients according to the expression level of microRNA-29b (*miR-29b*) in estrogen receptor (ER)-negative primary tumors. The difference in OS between the *miR-29b* low and high expression levels was significant (P=0.048).

Multivariate analysis of OS showed that the low level of *miR-29b* expression was an independent prognostic predictor in all patients (Table II).

Evaluation of TET1, TDG and DNMT3A expression levels and their comparison with miR-29b levels in breast cancer patients. Additionally, the expression levels of *TET1*, *TDG* and *DNMT3A* were examined in breast cancer primary tumor tissues. These levels were subsequently compared between the *miR-29b* high and low expression groups. There were no significant differences in any of the patients between the *miR-29b* high and low expression groups (Fig. 4). However, in analyses of the ER-positive patients, *DNMT3A* showed significantly higher expression in the *miR-29b* low expression compared to the high expression group (P=0.027; Fig. 5).

Discussion

In the present study, low *miR-29b* expression in primary breast tumors correlated significantly with poor DFS and OS in breast cancer patients. This was consistent with previous *in vitro* and *in vivo* findings that *miR-29b* acts as a tumor suppressive

Table II. Results of multivariate analysis of clinicopathological factors for overall survival (Cox proportional hazards model).

	Multivariate analysis	
Factors	RR (95% CI)	P-value
T factor (T1/2-4)	3.14 (0.39-19.5)	0.250
Lymph node metastasis	1.15 (0.13-24.9)	0.910
Lymphatic invasion	11.4 (0.61-743)	0.120
Venous invasion	2.59 (0.56-12.7)	0.220
Stage (I/II-IV)	2.57 (0.04-211)	0.650
miR-29b expression	15.6 (2.33-348)	0.003

RR, relative risk; CI, confidence interval; *miR-29b*, microRNA-29b.

miR (5-7). For example, Chou *et al* (5) showed that *miR-29b* was induced by GATA3 and inhibited metastasis by targeting various genes (*ANGPTL4*, *LOX*, *MMP* and *VEGFA*) involved in modifying the tumor microenvironment.

With respect to the clinicopathological factors, larger tumor sizes and more advanced stages were detected in the *miR-29b* low expression compared to the high expression group. This finding suggested that the suppression of *miR-29b* is associated with tumor progression. To clarify how *miR-29b* contributed to breast cancer progression, the study focused on candidate target genes of *miR-29b* according to TarBase 6.0 (19). Among 103 genes, we were interested in those that regulate epigenetic status, such as *TET1*, *TDG* and *DNMT3A*. The direct interactions between *miR-29b* and *TET1*, *TDG* and *DNMT3A* were confirmed by luciferase assays and western blot analysis (16). Although there are numerous pathways that regulate the levels of *TET1* (13,20), *TDG* (21) and *DNMT3A* (22) in breast cancer, significant inverse correlations were identified between the expression levels of *miR-29b* and *DNMT3A* in ER-positive patients. The overexpression of *DNMT3A* correlates with a poor prognosis in numerous cancers, including breast cancer (10,23). Starlard-Davenport *et al* (24) demonstrated that transfection of pre-*miR-29b* into breast cancer cell lines inhibited cell proliferation, decreased *DNMT3A* and *DNMT3B* mRNA levels and decreased the promoter methylation status of several tumor suppressor genes.

With respect to breast cancer subtypes, the present results showed a significant correlation between low *miR-29b* expression and poor OS, independent of the ER status. According to the results of the multivariate analysis, *miR-29b* is a powerful biomarker for predicting patient outcomes in all the subtypes of breast cancer.

In conclusion, *miR-29b* expression in breast cancer primary tumors was an independent prognostic factor for OS. Low *miR-29b* expression in primary tumors may predict poor OS and DFS in breast cancer patients. Additionally, in ER-positive cases, a significant inverse correlation between the expression levels of *miR-29b* and *DNMT3A* was identified.

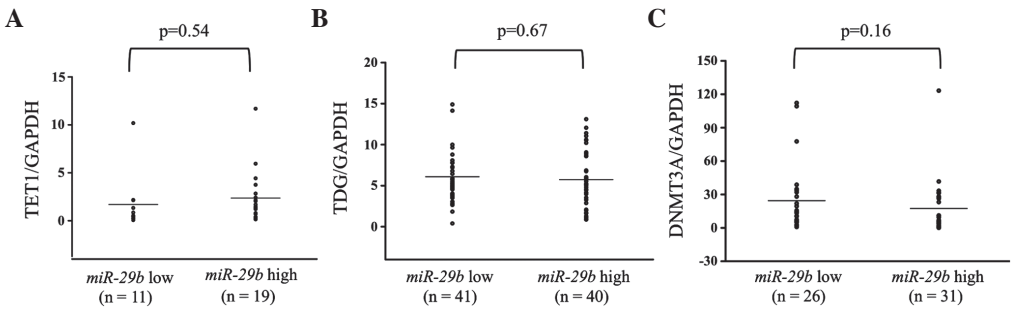

Figure 4. MicroRNA (miR) expression of (A) *TET1*, (B) *TDG* and (C) *DNMT3A* in *miR-29b* low- and high-expressing primary breast cancer tumors. The high *miR-29b* expression level was above and the low *miR-29b* expression level was below the mean expression value of all the samples (n=94). The horizontal line in the graph represents the mean of each group. There were no significant differences in any of the samples. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

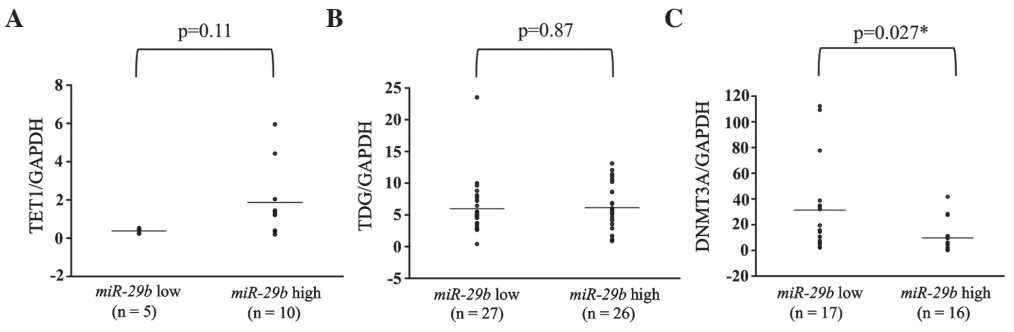

Figure 5. MicroRNA (miR) expression of (A) *TET1*, (B) *TDG* and (C) *DNMT3A* in *miR-29b* low- and high-expressing primary estrogen receptor (ER)-positive breast cancer tumors. The high *miR-29b* expression level was above and the low *miR-29b* expression level was below the mean expression value of all the samples. The horizontal line in the graph represents the mean of each group. The difference in *DNMT3A* expression was significant (*P=0.027). *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

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