Identification of recurrence-related microRNAs in the bone marrow of breast cancer patients

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Abstract. Recently, bone marrow has been considered as playing a critical role in the generation of both metastasis and recurrent disease. The accumulation of a single microRNA in the bone marrow has the potential to regulate the translation of multiple genes in cancer metastasis and may therefore serve as a prognostic marker for cancer recurrence. MicroRNA microarray analysis was performed to compare microRNA levels in bone marrow from 4 breast cancer patients with recurrent disease and 4 patients without recurrence. Accumulation of two of these microRNAs, miR-21 and miR-181a, in the recurrent breast cancer cases was validated by RT-PCR in bone marrow from 291 additional breast cancer cases. Expression of a common target gene, *PDCD4*, was also determined in bone marrow from 291 breast cancer cases. Increased miR-21 and miR-181a levels were significantly associated with shortened disease-free survival (DFS; p=0.0003, 0.0007) and overall survival (OS; p=0.0351, 0.0443), respectively. While low *PDCD4* expression was also significantly associated with poorer DFS (p=0.036). Multivariate analysis identified bone marrow miR-21 and mR-181a levels as valuable independent prognostic factors, with correlation coefficients that were significantly higher than that of the transcript of their common target gene. Accumulation of miR-21 and miR-181a in bone marrow appears to be associated with prognosis in breast cancer patients. The much higher significant correlation with microRNA levels and prognosis suggests epistatic effects on multiple target genes in the bone marrow of breast cancer patients.

Introduction

The morbidity rate of breast cancer has gradually increased in recent decades (1-3). The most serious problem in the diagnosis and treatment of breast cancer is metastasis and disease recurrence. As Rosen *et al* reported that 20% of early breast cancer patients eventually develop metastatic disease (4), identification of precise, early indicators of metastasis and recurrence is highly desirable. Much effort has been made to identify isolated tumor cells (ITCs) in the circulatory system and to determine how the presence of such ITCs impacts metastasis and recurrence (1,5-15). Because circulating ITCs have been identified in many breast cancer patients who appear to be relapse-free, we presume that metastasis and disease recurrence result from additional factors that enable cancer cells to acquire metastatic potential at distant sites.

Our recent work has suggested that both cancer cells as well as surrounding factors, including those located in the osteoblast and vascular niches of the bone marrow, are believed to be considerably important in the metastatic process (16). Indeed, breast cancer patients frequently develop bone metastases. However, our research has specifically focused on the presence of metastasis- or cancer recurrence-determining factors in the bone marrow.

Recently, alterations in microRNA levels have been shown to be involved in the initiation and progression of human cancers by inhibiting the translation of various target genes (17). Few studies have reported the presence of microRNAs in the circulatory system that regulates genes involved in cancer progression. Therefore, we elected to focus on microRNAs that are present in bone marrow and/or in peripheral blood of breast cancer patients with metastatic or recurrent disease. miR21 is known to inhibit expression of the tumor suppressor gene *programmed cell death protein 4 (PDCD4)* (18-21), and Target Scan showed that miR181a target PDCD4. Previous *in vitro* and *in vivo* studies have revealed that in cancer cells, miR-21 inhibits the translation of PDCD4 protein, resulting in an increased propensity of

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malignant cells to invade distant sites (22,23). Furthermore, loss of *PDCD4* expression has been observed in advanced primary breast cancer cases with a higher malignant potential, such as those that are HER2-positive, hormone-receptor-negative, and that have an invasive carcinoma histology (24,25). This study is the first to reveal the role of bone marrow microRNAs as prognostic indicators of disease-free survival (DFS) and overall survival (OS) in breast cancer patients. The findings presented herein, which were validated by quantitative RT-PCR analysis, may enable identification of bona fide markers for prediction of breast cancer metastasis or recurrence that can potentially serve as molecular therapeutic targets.

Patients and methods

Patients. A total of 291 breast cancer patients who underwent surgical treatment at the National Kyushu Cancer Center Hospital, Japan from 2000 to 2006 were enrolled in this study. All patients provided documented informed consent to participate in this study. The quantitative microRNAs analysis and *PDCD4* gene expression was evaluated in 291 cases. Among them, 84 cases were excluded because of the inadequate amount or quality of samples for quantitative RT-PCR, 207 cases were enrolled to examine both factors, *PDCD4* mRNA and microRNAs.

Patients who had distant metastases, had undergone neo-adjuvant chemotherapy, or who had been diagnosed with ductal carcinoma *in situ* were excluded. Twelve patients with no history of cancer who had undergone abdominal surgery (e.g., cholelithiasis) in our hospital from 2001 to 2004 were recruited as normal controls. The mean observation period ranged from 2 to 90 months (median, 61 months). Post-operative adjuvant therapy was performed according to the St. Gallen Consensus Conference guidelines (26,27).

Collection of clinical samples. Bone marrow aspiration was conducted immediately prior to surgery under general anesthesia. Bone marrow aspirates were obtained from the sternum using a bone marrow aspiration needle (MDTECH, Gainesville, FL, USA). The first 1 ml of bone marrow was discarded to avoid contamination by epithelial cells. Each 1-ml sample of bone marrow was immediately mixed vigorously with 4 ml Isogene (Nippon Gene, Toyama, Japan) and stored at -80˚C until RNA extraction. All clinical samples obtained at the National Kyushu Cancer Center were sent to our institute without knowledge of the histopathological or clinical results.

Total RNA extraction and first-strand cDNA synthesis. Total RNA was extracted according to the Isogene-LS manufacturer's protocols. All clinical samples obtained from the National Kyushu Cancer Center Hospital were sent to our institute. Reverse transcriptase (RT) reactions were performed as previously described (28,29). First-strand cDNA was synthesized from 2.7 μg total RNA in a 30-μl reaction mixture containing 5 μl 5X RT reaction buffer (Gibco BRL, Gaithersburg, MD), 100 μM hexadeoxynucleotide primer mixture, 50 U RNasin (Promega, Madison, WI, USA), 2 μl 0.1 M dithiothreitol, and 100 U Moloney murine leukemia virus RT (Gibco). The mixture was incubated at 37˚C for 60 min, 95˚C for 10 min, and then chilled on ice.

MicroRNA microarray analysis. We performed a microRNA microarray comparison between microRNA isolated from the bone marrow of 4 breast cancer cases that experienced cancer recurrence and 4 cancer cases without recurrence (Table I). The concentration and purity of total RNAs were assessed by a spectrophotometer, and RNA integrity was verified using an Agilent 2100 bioanalyzer (Agilent Technologies, Tokyo, Japan). Total RNA (100 ng) was directly labeled with cyanine 3-CTP (Cy3), without fractionation or amplification, using an Agilent protocol that produces precise measurements spanning a linear dynamic range from 0.2 amol to 2 fmol of input microRNA. We isolated 100 ng of total RNAs in bone marrow from 4 recurrent breast cancer patients and four non-relapsed breast cancer patients. We mixed up total RNA from those 8 cases to apply them as a control RNA in each hybridization reaction of the microRNA array (Agilent Microarray Design ID = 014947, Early Access version) containing 455 fragment of microRNAs, according to the manufacturer's protocol (16). A list of microRNAs contained in the array is available from version 8.2 of the Sanger microRNA database (http://microrna.sanger.ac.uk).

Quantitative RT-PCR. For *PDCD4*, the following primers were used: sense, 5'-CCCTCCAATGCTAAGGATACTG-3'; antisense, 5'-GTATGATGTGGAGGAGGTGGAT-3'. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control, with the following primers: sense, 5'-TGAA CGGGAAGCTCACTGG-3'; antisense, 5'-TCCACCACCCT GTTGCTGTA-3'. Real-time monitoring of PCR reactions was performed using the Light-Cycler™ system (Roche Applied Science, Indianapolis, IN, USA) and SYBR green I dye. Monitoring was performed according to the manufacturer's instructions, as described previously (30). In brief, a master mixture was prepared on ice, containing 500 ng cDNA of each gene, 2 μl LC DNA Master SYBR green I mix, 50 ng primers, and 2.4 μl 25 mM $MgCl_2$. The final volume was then adjusted to 20 μl with water. After the reaction mixture was loaded into glass capillary tubes, PCR was performed under the following cycling conditions: for amplification of *GAPDH*, an initial denaturation at 95˚C for 10 min was followed by 15 sec at 95˚C, 15 sec at 60˚C, and 13 sec at 72˚C by 40 cycles. For *PDCD4* amplification, an initial denaturation was followed by 15 sec at 95˚C, 15 sec at 56˚C, and 11 sec at 72˚C by 40 cycles.

Quantitative real-time PCR for microRNA. We examined quantitative analysis of miR-21 and miR-181a. We synthesized miR-21-, miR-181a-, and RNU6B (internal control)-specific cDNAs from total RNA using gene-specific primers according to the TaqMan MicroRNA Assays Protocol (Roche Applied Science). Reverse transcriptase reactions contained 10 ng total RNAs, 50 nmol/l stem-loop RT primer, 1X RT buffer, 0.25 mmol/l each deoxynucleotide triphosphate (dNTP), 3.33 U/μl MultiScribe reverse transcriptase, and 0.25 U/μl RNAase inhibitor. Reactions were incubated in a Bio-Rad i-Cycler (Bio-Rad Laboratories, Tokyo, Japan) in 96-well plates for 30 min at 16˚C, 30 min at 42˚C, 5 min at 85˚C, and

Table I. Clinicopathologic information of the microarraty analysis applied breast cancer cases.

Case no.	1	2	3	4	5	6	7	8
Age	36	65	73	64	54	54	56	61
Size of tumor (T factor)	1	1	1	1	2	3	1	1
Lymph node metastasis[a]	0	0	0	0	0	20	10	14
Stage of disease	1	1	1	1	2	3	2	2
Recurrencing site	-	-	-	-	Liver	Skin	Liver	Lymph node
Clinical outcome	Alive	Alive	Alive	Alive	Alive	Alive	Cancer death	Cancer death
Disease-free interval (months)	NA	NA	NA	NA	15	1	12	15
Follow-up period (months)	90	49	52	48	41	27	29	26

[a]T factor, T1 <2.0 cm, 2 cm ≤T2 5 cm, T3 >5 cm. NA, not applicable.

then maintained at 4˚C. Real-time PCR was done using an Applied Biosystems 7500 real-time PCR system. The 10-μl PCR mixture included 0.67 μl RT products, 1X TaqMan Universal PCR master mix, and 1 μl primers and TaqMan miRNA Assays probe mix. Reactions were incubated in 96-well optical plates at 95˚C for 10 min. Relative quantification of miRNA expression was calculated as follows: raw data were presented as the relative quantity of target miRNA normalized with respect to RNU6B and relative to a calibrated sample.

Data analysis. The intensity of each hybridization signal was evaluated using Feature Extraction Software (Agilent Technologies, Tokyo). Feature Extraction analysis examines multiple probes and multiple features per probe, and evaluates the measurements and errors for each miRNA. Observed values were imported into GeneSpring GX version 7.3 (Agilent Technologies).

PDCD4 and *GAPDH* mRNA expression levels were determined by comparison with cDNAs from the Human Universal Reference Total RNAs collection (Clontech, Palo Alto, CA, USA). After proportional baseline adjustment, the fit point method was employed to determine the cycle in which the log-linear signal was first distinguishable from baseline, and that cycle number was subsequently used as a crossing-point value. A standard curve was produced by measuring the crossing point of each standard value and plotting it against the logarithmic value of the concentrations. mRNA concentrations were calculated by plotting their crossing points against the standard curve, and were adjusted relative to *GAPDH* content. Taking into consideration the clinical application of the current study, the 95% confidence interval value was used as the upper limit of a normal case cutoff value (0.58). miR-21 and miR-181a levels were adjusted with respect to RNU6B levels, while *PDCD4* expression was adjusted with respect to *GAPDH* expression. We added as follows: the cutoff value was defined as the highest value of 95% confidence interval of miR-21 and miR-181a expression in normal control cases (miR-21: 5.84, miR-181a: 1.35). On the other hand, we set the cutoff value of *PDCD4* as the lowest value of 95% confidence interval in normal cases (0.58).

Statistical analysis. Differences between clinicopathological factors were analyzed by χ^2 tests for categorical variables. OS and DFS time were measured from the date of bone marrow aspiration until the date of death (for OS) or last follow-up (for DFS). Survival curves were determined using the Kaplan-Meier method. Statistical significance between groups was assessed using the log-rank test. Multivariate analysis was performed to assess the relative influence of prognostic factors on DFS or OS, using the Cox proportional hazards model in a forward stepwise procedure. Statistical analysis was performed by JMP 5.0.1a for Windows software (SAS Institute).

Results

MicroRNA microarray results. To achieve this goal, we used microRNA microarray analysis to compare microRNA expression in bone marrow from breast cancer patients with and without disease recurrence. As shown in Table II, we identified 10 representative microRNAs that were expressed at a much higher level in bone marrow isolated from 4 breast cancer patients with cancer recurrence than from 4 relapse-free breast cancer patients. Among a subgroup of microRNAs that appear to accumulate in the bone marrow of breast cancer patients, both miR-21 and miR-181a were identified as being expressed at a higher level in patients with recurrence than in those without recurrence (Table II). Among these, we focused on accumulation of miR-21 and miR-181a, which were both present at a significantly higher level in the recurrent cancer cases than in the relapse-free cases. We evaluated both of these microRNAs because the clinical significance of their common target gene, *PDCD4*, has been previously validated (19-21,24); these studies revealed that the loss of *PDCD4* plays a critical role in the progression of solid tumors.

Clinicopathologic significance of miR-21 and miR-181a in bone marrow of breast cancer patients. We searched for the target of miR21 and miR181a by Target Scan and found PDCD4 as a common target gene of these microRNAs. The correlation between bone marrow miR-21 levels and clinicopathological factors is summarized in Table III. A

Table II. Microarray analysis of microRNA level in bone marrow from 4 breast cancer cases with recurrence and 4 relapse-free cases.

		Rec[a] Normalized	Non-rec Normalized	Rec/non-rec	Target proteins, and note
1	hsa-miR-198	1.78	0.14	12.60	Cyclin T1,
2	hsa-miR-936	1.56	0.14	11.20	No data
3	hsa-miR-526b	1.35	0.14	9.97	No data
4	hsa-miR-21	0.85	0.10	8.58	RECK, **PDCD4**, TIMP3
5	hsa-miR-187[a]	2.42	0.29	8.29	NEUROG2, ARRDC3, BCL6, GRIA3
6	hsa-miR-518c[a]	1.43	0.18	8.06	Accumulated in retinoblastoma
7	hsa-miR-659	1.78	0.29	6.23	GRN
8	hsa-miR-92a-2[a]	1.08	0.18	5.84	miR17-92 clusters up-regulate c-Myc protein
9	hsa-miR-376c	2.05	0.36	5.71	No data
10	hsa-miR-181a	1.93	0.35	5.43	TIMP3, **PDCD4**, LIN28

[a]Rec, cases with recurrence.

Table III. Clinicopathological significance of miR-21 and miR181a in bone marrow from breast cancer cases.

	miR-21						miR-181a					
		Low		High				Low		High		
	n	244	%	47	%	p-value	n	220	%	71	%	p-value
Tumor						0.2188						0.6851
T1, T2	267	226	93	41	87		267	203	92	64	90	
T3, T4	24	18	7	6	13		24	17	8	7	10	
Histological grade						0.0011[a]						0.0058[a]
Grade 3	74	53	22	21	45		74	47	21	27	38	
Others	215	189	78	26	55		215	171	78	44	62	
Estrogen receptor						0.0132[a]						0.0158[a]
Positive	194	170	70	24	51		194	155	70	39	55	
Negative	97	74	30	23	49		97	65	30	32	45	
Progesterone receptor						0.0016[a]						0.0004[a]
Positive	166	149	61	17	36		166	138	63	28	39	
Negative	120	91	37	29	62		120	78	35	42	59	
HER2/neu status						<0.0001[a]						<0.0001[a]
Positive	33	17	7	15	32		33	15	7	18	25	
Negative	138	116	48	22	47		138	110	50	28	39	
Lymph node metastasis						0.0474[a]						0.0724
Positive	117	92	38	25	53		118	82	37	35	49	
Negative	174	152	62	22	47		174	138	63	36	51	
Recurrence						0.0014[a]						0.0022[a]
Positive	61	43	18	18	38		61	37	17	24	34	
Negative	230	201	82	29	62		230	183	83	47	66	
Overlapped with PDCD4	207						207					

Statistically significant at $p<0.05$. [a]p-value.

significantly higher proportion of cases with high miR-21 levels were both receptor-negative, HER2-positive, had lymph node metastasis, and experienced cancer recurrence compared to cases with low miR-21 levels (p=0.0132 and 0.0016, <0.0001, 0.0474 and 0.0014, respectively).

The clinicopathologic significance of miR-181a accumulation in bone marrow is also summarized in Table III. A significantly higher number of cases with high levels of miR-181a were nuclear histological grade 3, both hormone receptor-negative, HER2-positive, and experienced cancer recurrence compared to cases with low miR-181 levels (p=0.0058, 0.0158 and 0.0004, <0.0001 and 0.0022, respectively).

Clinical significance of PDCD4 mRNA in bone marrow of breast cancer patients. Patients were classified into two groups according to *PDCD4* expression: the low *PDCD4* expression group and the high *PDCD4* expression group. The correlation between *PDCD4* mRNA expression and clinicopathological factors was evaluated, and a significantly higher number of patients with low *PDCD4* expression were estrogen receptor (ER)-positive (p=0.0461) and progesterone receptor (PR)-positive (p=0.0036) compared to patients in the high *PDCD4* expression group. Similarly, a significantly higher number of cases with low *PDCD4* mRNA expression had lymph node metastasis (p=0.0364) and cancer recurrence (p=0.0090) compared to cases in the high *PDCD4* expression group. Notably, patients in the low *PDCD4* expression group experienced a significantly higher incidence of post-operative cancer recurrence than patients in the high *PDCD4* expression group (p<0.01).

Clinical importance of miR-21 and miR-181a as independent prognostic factors for DFS in breast cancer patients. As previously mentioned, we defined which is the dominant as an independent predictor of DFS of breast cancer patients between microRNAs and target gene. During the follow-up period of patients in which microRNAs were analyzed, breast cancer recurred in 60 patients (27.4%), 27 patients (12.3%) were died from breast cancer and 3 patients (1.4%) from other causes. As for *PDCD4* mRNA, breast cancer recurred in 65 patients (20.4%), 29 patients (9.1%) died from breast cancer and 4 patients (1.3%) from other causes. As shown in Fig. 1a and b, patients with high miR-21 expression experienced significantly shorter DFS (p=0.0003) and OS (p=0.0351) than patients with low miR-21 expression. Similarly, patients in the high miR-181a expression group had significantly shorter DFS (p=0.0007) and OS (p=0.0433) than patients in the low miR-181a expression group (Fig. 1c and d). Furthermore, patients in the low *PDCD4* expression group had significantly poorer DFS than patients in the high *PDCD4* expression group (p=0.0342; Fig. 2a). However, no significant difference in OS was observed between *PDCD4* expression groups (Fig. 2b).

In addition, we performed univariate analysis of miR-21, miR-181a and *PDCD4* in bone marrow from breast cancer cases individually for determination of the independent disease-free survival (Table IVA). As a result, both microRNAs and the gene were significant prognostic markers for disease-free survival in breast cancer cases.

Table IV. A, Univariate analysis for disease-free survival.

	Univariate analysis		
	RR	95% CI	p-value
Lymph node metastasis	0.42	0.32-0.55	<0.0001
Tumor (T1, T2/T3, T4)	0.48	0.37-0.65	<0.0001
Estrogen receptor	1.64	1.30-2.06	<0.0001
Progesterone receptor	1.51	0.18-1.92	0.0006
HER/neu2 status	0.67	0.51-0.89	0.0078
miR-181a accumulation	1.54	1.18-1.98	0.0017
miR-21 accumulation	1.63	1.23-2.13	0.0012
PDCD4 mRNA expression	0.77	0.60-0.98	0.0374

B, Multivariate analysis of miR-21 and miR-181a in bone marrow from breast cancer cases for disease-free survival.

	Multivariate analysis		
	RR	95% CI	p-value
Lymph node metastasis	0.37	0.25-0.53	<0.0001
Tumor (T1, T2/T3, T4)	0.68	0.49-0.97	0.0337
Estrogen receptor	0.91	0.60-1.40	0.6442
Progesterone receptor	1.23	0.80-1.85	0.3338
HER2/neu status	0.87	0.64-1.23	0.4208
miR-181a accumulation	1.69	1.17-2.39	0.0059
miR-21 accumulation	1.04	0.71-1.48	0.8531

Furthermore, we determined which factor is dominant among three markers simultaneously. Table IVB summarizes the results of a multivariate analysis performed to determine independent prognostic factors for DFS among clinicopathologic factors, high miR21 expression and high miR-181a expression.

Discussion

In vitro studies have revealed that diminished expression of PDCD4 protein in breast cancer cells appears to be regulated by the presence of microRNAs (19,20) rather than by hypermethylation of the *PDCD4* promoter region (25). Lu *et al* reported that PDCD4 protein levels are reduced by the presence of miR-21 in human breast cancer cells, whereas quantitative real-time PCR revealed little difference in *PDCD4* mRNA expression levels, suggesting translational regulation. In the current study, a correlation between *PDCD4* gene expression and accumulation of either miR-21 or miR-181a was observed in bone marrow samples from over 250 breast cancer patients, however, statistical significance was not observed between them. The results of the current study may indirectly support the results of previous studies uncovering that PDCD4 is regulated by miR-21 at the translational level.

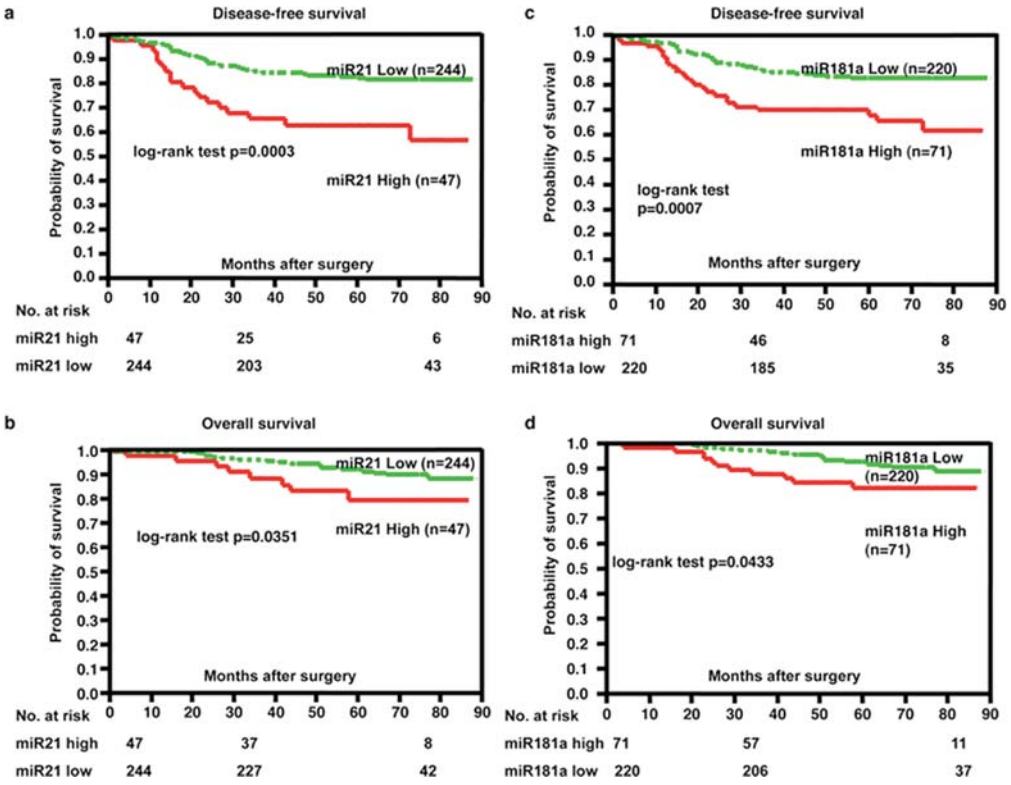

Figure 1. (a) Disease-free survival (DFS) and (b) overall survival (OS) in 47 breast cancer cases with high bone marrow levels of miR-21 and 244 cases with low miR-21 levels (p=0.0003 and 0.021, respectively). (c) DFS and (d) OS in 71 breast cancer cases with high miR-181a levels and 220 cases with low miR-181a levels (p=0.0007 and 0.0165, respectively).

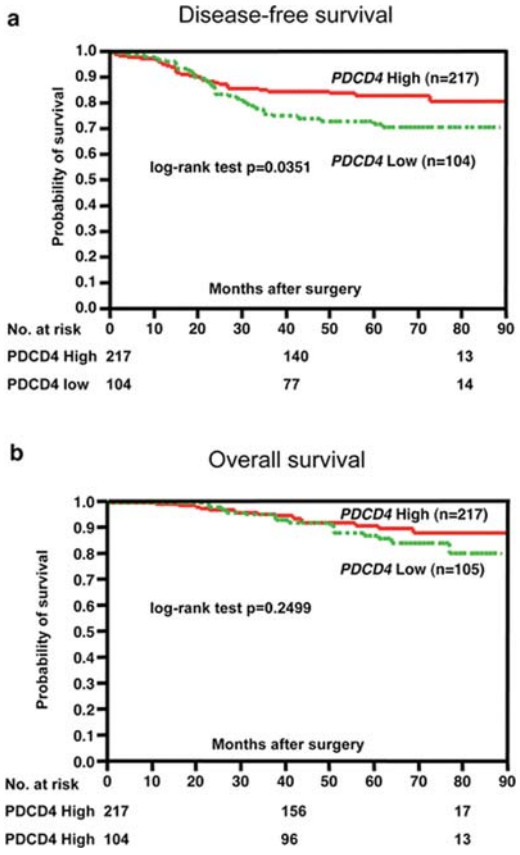

Figure 2. (a) DFS and (b) OS in 105 breast cancer cases with high *PDCD4* expression and 217 cases with low *PDCD4* expression. The difference in DFS was significant (p=0.0363).

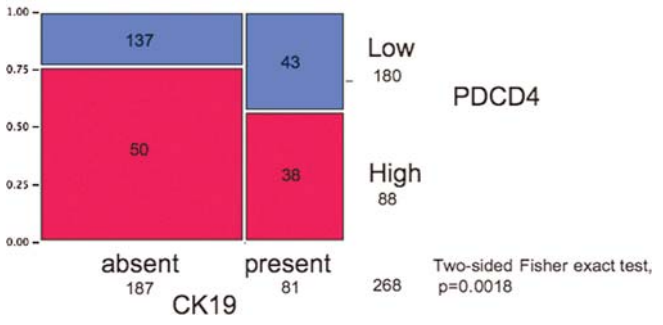

Figure 3. Among 268 cases of breast cancer, CK positive cases and PDCD4 high cases were 81 and 88, respectively. CK19 expression and PDCD4 expression showed inverse association with statistical significance (two-sided Fisher's exact test, p=0.0018).

Unfortunately, we were not able to determine whether diminished PDCD4 protein expression in bone marrow was due to inhibition at the translational level and/or mRNA degradation by miR-21 and miR-181a.

However, the clinical significance of *PDCD4* gene expression was observed with respect to its association with lymph node metastasis, ER-positivity, PR-positivity, and incidence of cancer recurrence (p=0.0379, 0.0492, 0.004, and 0.008, respectively). As for the positive relationship between hormone receptors and PDCD4, there was no direct structural and functional relationship between PDCD4 and hormone receptors with each other. Besides, ER/PR were not determined by the status of PDCD4 by multivariate analysis (data not shown). Therefore, we can predict the malignant potential and isolate

the cases to be cured by the status of hormone receptors as recognized at the St. Gallen Consensus meeting. As these factors were similar to those associated with bone marrow miR-21 and miR-181a levels, we postulated that *PDCD4* transcription may be regulated by miR-21 and/or miR-181a to some degree at the transcriptional level.

To identify new diagnostic markers and potential therapeutic targets in breast cancer patients, we compared the clinical significance of microRNA levels and expression of *PDCD4* in predicting DFS and OS. As shown in Table IVA and B, high miR-181a expression was identified as one of the best prognostic markers for DFS in breast cancer, followed by high miR-21 expression. As for miR181a, there were no definitive finding concerning the association between DFS and OS in breast cancer cases and the status of miR-181a. However, miR-181a was hypothesized to be relevant to prognosis and resistance to chemotherapy drugs, because miR-181 was reported to be up-regulated in the tamoxifen resistant MCF-7 cells (31). Therefore, in the near future, we must identify cancer cells with miR181a overexpression in bone marrow determining the resistance to chemotherapy leading to recurrence and poorer prognosis in breast cancer cases. In addition, miR-181a suppresses p27, which is necessary to arrest cell cycle progression and reach terminal differentiation, that enhances the malignant potential in HL60 cells (32,33). We need to clarify the miR-181a pathway leading to the cancer progression in breast cancer cases.

While *PDCD4* expression showed a significant association, albeit of a lower magnitude than that of the microRNAs, with DFS, *PDCD4* expression was not significantly associated with OS. Therefore, we speculate that changes in miR-21 and miR-181a levels may influence metastatic/recurrence potential by inhibiting the translation of multiple genes in addition to *PDCD4*, including *BCL2, SMAD7, SOX5, TIMP3*, and *TRIM2*. Further study is required to identify the full complement of proteins regulated by microRNAs at the translational level in bone marrow and to determine which factors are significantly associated with DFS and OS.

With respect to the origin of the microRNAs present in the bone marrow of breast cancer patients, we cannot presently define which cell types are responsible for microRNA processing. However, two potential scenarios may be considered: microRNA processing may occur in the cancer cells themselves, or in cells within the bone marrow microenvironment, such as hematopoietic progenitor cells (34,35), endothelial progenitor cells (36), and macrophages. As has been observed in previous studies reporting on the clinical significance of miR-21 accumulation in primary breast cancer tissue (19-21,37,38), the results of the current study unveiled a significant correlation between the incidence of lymph node metastasis and shortened survival of patients with increased bone marrow miR-21 accumulation. Together, these findings suggest that bone marrow miR-21 accumulation results from cancer cells. To confirm this, we must further clarify the full complement of proteins in primary cancer cells that are regulated by miR-21 and compare them to those present in bone marrow. Furthermore, we examined CK19 (39) and PDCD4 expression in bone marrow from breast cancer cases (Fig. 3), and found that the higher CK19 expressing cases showed significantly lower expression of PDCD4 (two-sided Fisher's exact test, p=0.0018). As CK19 was originally from tumor cells in circulating systems, the current study suggested that the diminished PDCD4 expression was probably inhibited by both microRNAs derived from circulating cancer cells as well.

Recently, it was proved clinically that the addition of zoledronic acid to adjuvant endocrine therapy improves disease-free survival in premenopausal patients with estrogen-responsive early breast cancer (40), although in molecular level, the role of zoledronic acid in breast cancer was not clarified yet. In multiple myeloma, bisphosphonate interferes with bone microenvironment inhibiting the survival of stromal cells and hampering the contact between plasma and stromal cells (41). It is suggested that zoledronic acid for breast cancer patients might affect the high expression of microRNAs, however, further studies will be required to clarify the precise mechanism of up-regulated microRNAs by zoledronic acid.

In conclusion, by multivariate analysis, high levels of miR-181a in the bone marrow of breast cancer patients appear to be significantly associated with inferior DFS and OS. However, the role of these microRNAs does not appear to be limited to direct regulation of transcription of their common target gene, *PDCD4*, as *PDCD4* expression levels are not significantly associated with survival. These results suggest that the accumulation of microRNAs that regulate multiple target genes in cancer cells in the bone marrow may determine the risk of breast cancer recurrence.

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