Int J Clin Exp Pathol 2016;9(11):11708-11717
www.ijcep.com /ISSN:1936-2625/IJCEP0039013

Original Article

Comparison of microRNA expression levels between primary and recurrent breast cancer: microRNA-133a, microRNA-191, and microRNA-204 can predict local recurrence of breast cancer

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Received August 29, 2016; Accepted September 21, 2016; Epub November 1, 2016; Published November 15, 2016

Abstract: Background: Identification of patients with the potential of local recurrence after resection of breast cancer can help achieve an optimal management. We evaluated microRNA (miRNA) expression as a predictable biomarker in primary breast cancers and corresponding breast cancers. Methods: MiRNA expression array was used to search candidate miRNAs between primary and recurrent breast cancer from 5 patients. These miRNAs were further verified by real-time RT-PCR in a cohort of 25 patients. Results: 8 miRNAs including miRNA-26a, miRNA-133a, miRNA-133b, miRNA-186, miRNA-191, miRNA-194, miRNA-204, miRNA-296-5p were selected as candidates in a miRNA expression array. 2 miRNAs (miRNA-133a and miRNA-191) showed significantly different expression level between primary and recurrent tumor in the validation cohort, with down-regulation of miRNA-133a and up-regulation of miRNA-191 were identified in recurrent tumor. The initial expression level of miRNAs was another potential biomarker. 2 miRNAs (miRNA-191 and miRNA-204) were significantly correlated with disease-free survival, with higher expression of miRNA-191 and lower expression of miRNA-204 revealed worse prognosis. Conclusions: We demonstrate that miRNA-133a, miRNA-191, and miRNA-204 can be useful biomarkers for predicting breast cancer recurrence. MiRNA-191 is supposed to be a potential oncogenic miRNA, and miRNA-133a and miRNA-204 are supposed to play as tumor suppressors in the process of breast cancer recurrence.

Keywords: Breast neoplasms, recurrence, microRNA-133a, microRNA-191, microRNA-204

Introduction

Breast cancer is one of the most common cancers and the leading cause of cancer mortality in women. Over the past few decades, active screening examinations through mammography and ultrasonography have led to early detection of breast cancer patients. Development of treatment options including appropriate surgical techniques, neo-adjuvant and/or adjuvant systemic chemotherapy and radiotherapy has also improved the outcomes of breast cancers [1, 2]. Despite the advances in the management of breast cancer, a subset of patients will experience local recurrence after a curative resection with or without an adjuvant chemo/radiotherapy that follows. Local recurrence brings about negative influences to the patients, and there is no single standard treatment to be considered exclusively. Identification of patients with the potential of local recurrence can help achieve an optimal management. Therefore, the need for discovery and definition of biomarkers for predicting recurrence has always been strongly suggested.

MicroRNAs (miRNAs) are small noncoding single-stranded RNAs that regulate the expression of specific target genes, usually by translational regression and silencing. MiRNAs play important roles in various physiologic processes, such as embryonic development, cell differentiation, cell proliferation, and apoptosis, and they are involved in pathologic conditions

including carcinogenesis as well [3-5]. Dysregulation of miRNA expression associated with degree of aggressiveness, local recurrence, or metastasis has been described in various types of cancer including breast [6-10], lung [11], thyroid [12], brain [13], and colorectal cancer [14]. But we still know little about the miRNA signatures related to local recurrence breast cancer. Moreover, there has not yet been any study investigating the sequential change of miRNA expression levels in breast cancer or the comparison of miRNA expression levels between primary diagnosed tumor tissue and recurrent tumor tissue of the same patients.

In this study, we aimed to identify miRNAs differentially expressed in primary breast cancers and corresponding recurrent breast cancers. We also evaluated the prognostic impact of miRNA expression levels in resected breast cancers for predicting recurrence.

Materials and methods

Patient population and sample collection

For this study, breast cancer patients who underwent resection of primary and recurrent tumor in Kyungpook National University Hospital (KNUH) between 2001 and 2010 were identified. A total of 25 patients who were available of both primary and recurrent tumor tissue as formalin-fixed, paraffin-embedded (FFPE) tissue blocks were enrolled in the current study. In addition, 10 normal breast tissue samples and 10 breast tissue samples of benign condition were used as controls. The electronic medical records and pathologic reports were reviewed for identifying the clinical data, including age, type of cancer, type of surgery, tumor size, presence of metastatic regional lymph nodes, tumor grade, tumor estrogen receptor (ER)/progesterone receptor (PR)/human epidermal growth factor receptor 2 (HER2) status, and time to recurrence. All FFPE blocks were cut and stained with hematoxylin and eosin. Each slides was reviewed and marked for representative tumor areas by two pathologists (JYJ and JYP). All personal identifiable information of the enrolled patients was removed, and a newly given study identification number was used. This study was approved by the Institutional Review Board at KNUH.

RNA extraction

Total RNA was extracted using Qiagen miRNeasy FFPE kit (Qiagen, Hilden, Germany). Each samples was sectioned by 10 μm, and the isolation procedure was done according the manufacturer's manual. The quality of isolated RNA was confirmed by Agilent's 2100 Bioanalyzer system (Agilent, Santa Clara, CA, USA).

MiRNA expression array

As the screening method for identifying miRNAs differentially expressed in 5 primary breast cancers and 5 corresponding recurrent breast cancers, we evaluated the expression of 158 miRNAs using PANArray miRNA expression profiling system (Panagene, Korea) according to the manufacturer's instructions. In detail, a total of 400 ng RNA denaturation mixture from each sample mixed with RNase-free water was prepared. Hybridization and labeling by pCp-Cy3 were performed. After washing the microarray slides, the slides were scanned using Genepix 4000B scanner (Molecular devices, Sunnyvale, CA, USA). Normalization of data was done by normalization using RNA6B as internal control and control samples. The signal intensity was analyzed using the PANAGENE software (Panagene, Korea), and the final normalized ratio was calculated. MiRNAs which showed more than twofold the expression ratio comparing primary and recurrent breast cancer sample in more than 3 out of 5 patients are considered as candidate miRNAs for validation test by real time RT-PCR.

MiRNA real time RT-PCR

The measurement of the expression levels of individual miRNAs was performed using miRNA specific primers (Applied Biosystems, Foster City, CA, USA). Candidate miRNAs were mainly selected on the basis of the result of the preperformed miRNA expression array. 8 miRNAs including miR-26, miR-133a, miR-133b, miF-186, miR-191, miR-194, miR-204, and miR-296-5p were evaluated in a validation set including 25 patients.

In detail, 10 ng of total RNA was reverse transcribed using High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using ABI 7500

Table 1. Clinicopathological characteristics of primary breast cancers and recurrent breast cancers

Patient number	Age at first surgery	Tumor size (mm)	Positive lymph nodes[a]	Tumor grade[b]	ER status of primary breast cancer[c]	PR status of primary breast cancer[c]	HER2 status of primary breast cancer[d]	Time to recurrence (weeks)
1	57	45	1	3	0	1	0	15
2	52	28	0	2	1	0	1	27
3	42	17	0	3	1	0	0	11
4	33	35	1	3	1	0	1	26
5	38	31	1	2	1	1	0	87
6	48	40	1	2	0	0	0	37
7	48	41	1	2	0	0	0	14
8	56	23	0	2	1	1	1	54
9	69	15	1	3	0	0	1	15
10	32	22	0	2	1	1	1	21
11	33	41	1	2	1	1	1	35
12	36	15	0	3	0	0	1	22
13	46	40	1	2	N/A	N/A	N/A	60
14	66	30	1	2	0	0	1	25
15	51	15	0	2	1	1	0	51
16	39	35	1	3	1	0	0	42
17	69	20	0	2	0	0	0	43
18	50	40	1	3	1	0	1	25
19	55	34	1	3	0	0	1	40
20	49	23	0	3	0	0	0	4
21	42	30	1	2	0	0	0	6
22	23	18	N/A	3	1	1	0	26
23	48	45	1	3	0	0	1	7
24	42	55	1	3	1	1	0	17
25	71	28	1	2	0	0	1	7
Total (Range)	48 (23-71)	30 (15-55)	16/24	2	12/24	8/24	12/24	25 (4-87)

ER, Estrogen receptor; PR, Progesterone receptor; HER2, Human epidermal growth factor receptor 2; [a]0, no metastatic lymph node; 1 positive metastatic lymph node; [b]Modified Bloom-Richardson grading system; [c]0, negative in immunohistochemical stain (Allred score 0-2); 1, positive in immunohistochemical stain (Allred score 3-8); [d]0, normal expression; 1, overexpression and/or gene expression (3+ in immunohistochemical stain and/or amplification in SISH).

Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), and all RT-PCRs were performed in duplicate. RNU6 was used for normalization of amplified values. The expression level of each miRNA was evaluated according to the $2^{-\Delta\Delta CT}$ method.

The value calculated by the $2^{-\Delta\Delta CT}$ method was used directly for comparing miRNA expression levels between primary and recurrent breast cancer. The grade of the expression level was used for survival analysis. Grade 1 of the expression level was defined as down-regulation that showed expression level below 1, grade 2 represented low up-regulation that showed expression level from 1 to 10, and grade 3 was high up-regulation that showed expression level above 10.

Statistical analysis

The statistical analyses were performed using SPSS for Windows (version 23.0, SPSS Inc., Chicago, IL, USA). Differences in miRNA expression levels between primary breast cancer and corresponding recurrent breast cancer were evaluated with paired *t* test. The disease-free survival (DFS) was defined as the time from the first diagnosed surgery to the second surgery with recurrence. The DFS rates were estimated using the Kaplan-Meier method and analyzed by a log-rank test. The multivariate analysis for evaluating the association between DFS and individual miRNA was performed using a Cox proportional hazards regression model. *P* value ≤ 0.05 was considered as statistically significant.

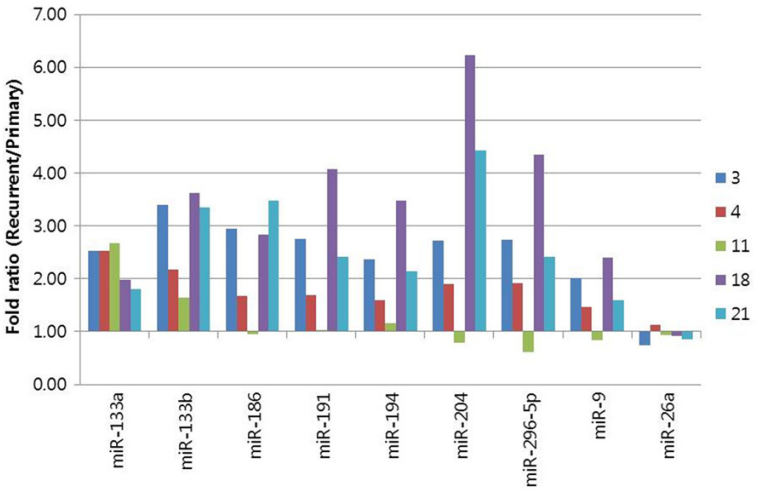

Figure 1. Expression level ratio of candidate miRNAs in primary breast cancers and recurrent breast cancers. MiRNA-133a, miRNA-133b, miRNA-186, miRNA-191, miRNA-194, miRNA-204, and miRNA-296-5p show more than twofold the expression level in recurrent breast cancers as compared with corresponding primary breast cancers in more than 3 out of 5 patients (3, 4, 11, 18, and 21 shown in the right side indicate patient number). MiRNA-26a reveals down-regulated expression in 4 patients. MiRNA-9 is up-regulated more than double only in 2 patients.

Results

Patient characteristics

The patients' clinic pathological characteristics are summarized in **Table 1**. The median age of the patient population was 48 years (ranging from 23 to 71 years). The median tumor size was 30 mm (ranging from 15 to 55 mm). Regional lymph node metastasis was identified in 16 (66.7%) out of 24 available patients. 13 patients (56%) showed grade 2, and 12 patients (44%) showed grade 3 evaluated by modified Bloom-Richardson grading system. ER, PR, and HER2 were positive in 12 (50%), 8 (33.3%), and 12 (50%) patients respectively. DFS was 25 weeks in median ranging from 4 to 87 weeks. Although not shown in the **Table 1**, all enrolled patients were diagnosed as "invasive ductal carcinoma (invasive carcinoma of no special type)", and all patient underwent curative resection with negative surgical resection margin by total mastectomy or modified radical mastectomy.

MicroRNA expression array by using PANArray

Among 158 miRNAs which could be detected in PANArray microRNA expression profiling system, we found considerable miRNAs differently expressed between primary and recurrent breast cancers. We selected miRNAs that showed more than twofold the expression ratio comparing primary and corresponding recurrent breast cancer. 1 miRNA (miRNA-133b) showed more than twofold the expression ratio in the recurrent breast cancer comparing with the primary breast cancer in 4 out of 5 patients. 6 miRNAs including miRNA-133a, miRNA-186, miRNA-191, miRNA-194, miRNA-204, and miRNA-296-5p were highly expressed to be more than double in recurrent tumors than primary tumors in 3 out of 5 patients. Another 13 miRNAs including miRNA-1, miRNA-127, miRNA-181b, miRNA-181c, miRNA-181d, miRNA-192, miRNA-222, miRNA-25, miRNA-27a, miRNA-30b, miRNA-30c, miRNA-9, and miRNA-371-5p revealed more than double the expression ratio in 2 out of 5 patients. 2 miRNAs including miRNA-124a and miRNA-23b were more than double in only 1 patient. Interestingly, miRNA-26a was down-regulated in the recurrent tumor in 4 patients and showed similar expression level even in 1 remaining patient. We choose 7 miRNAs that revealed more than double the expression ratio in more than 3 out of 5 patients as candidate miRNAs for predicting recurrence. So, 8 miRNAs including 7 miRNAs with more than twofold the expression ratio and miRNA-26a were considered as our main candidates.

Expression level ratio of miRNA-9 (expression level in recurrent tumor/expression level in primary tumor) which was reported as potential useful marker for predicting recurrence by literature review [6] revealed 2.01, 1.47, 0.84, 2.40, and 1.60 in each of 5 patients. Expression ratios of the 8 candidate miRNAs and miRNA-9 in 5 patients of screening set are shown in **Figure 1**.

MicroRNA expression level by using real-time RT-PCR and prognostic implication

Expression levels of 8 candidate miRNAs of primary and corresponding recurrent breast cancers in 25 patients of validation set are listed in

Table 2. Expression levels of 9 microRNAs in tumor samples of primary (1st) and recurrent (2nd) resected breast cancers

Patient number	miRNA-26a		miRNA-133a		miRNA-133b		miRNA-186		miRNA-191		miRNA-194		miRNA-204		miRNA-296-5p	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
1	5.73	0.02	8.07	N/A	N/A	0.01	2.76	0.06	3.01	0.05	6.65	0.01	2.54	N/A	1.40	N/A
2	1.43	6.52	23.66	0.05	14.61	0.42	1.40	4.51	0.82	3.65	7.47	4.03	7.49	3.62	N/A	2.38
3	23.60	0.23	52.73	0.00	159.01	0.02	7.14	0.23	3.05	0.32	4.79	0.09	6.99	0.04	0.65	N/A
4	131.11	2.04	N/A	0.01	20.07	0.06	4.37	5.49	1.65	7.46	3.00	1.95	1.19	0.48	1.21	1.67
5	9.16	3.44	19.03	0.05	22.77	0.52	5.01	6.96	0.31	9.58	4.58	2.88	55.38	5.39	0.49	1.62
6	4.15	3.23	16.38	0.02	32.78	0.23	1.59	2.39	0.58	1.44	2.00	2.53	5.12	2.57	N/A	4.12
7	15.62	2.84	75.41	106.62	203.62	655.91	9.46	1.25	7.16	0.75	26.06	0.72	39.56	3.33	3.49	N/A
8	0.95	0.77	25.75	0.02	7.05	0.11	1.65	0.84	0.22	0.43	7.16	0.93	1.34	0.74	1.25	1.31
9	8.63	2.42	N/A	0.05	38.86	N/A	2.60	1.30	1.00	2.55	0.48	0.32	11.85	N/A	0.24	1.35
10	5.04	0.16	66.03	1.54	33.73	19.66	8.07	0.20	2.13	0.04	2.91	0.06	21.99	0.08	N/A	N/A
11	8.02	6.13	21.45	0.05	110.37	0.32	6.26	2.14	3.72	8.13	2.38	3.05	107.47	9.03	N/A	1.95
12	0.50	0.22	0.01	0.03	0.01	0.24	0.42	0.34	0.31	0.15	1.87	0.50	5.64	0.34	N/A	0.78
13	0.81	0.04	1.17	0.01	1.09	0.06	0.44	0.04	0.32	0.03	0.33	0.22	5.20	0.22	0.71	0.43
14	2.59	0.00	0.46	0.00	0.24	N/A	1.41	N/A	1.38	N/A	N/A	N/A	7.83	0.02	0.80	0.00
15	0.42	4.63	0.01	0.09	0.01	0.79	0.34	2.87	0.21	1.86	3.07	1.13	1.26	2.97	192.15	2.27
16	0.16	4.04	0.00	0.04	0.00	0.32	0.49	10.66	0.31	2.65	0.46	5.44	0.30	10.97	0.44	1.16
17	1.26	4.49	8.48	0.02	3.57	0.12	0.80	2.98	0.61	2.42	0.34	1.24	1.02	6.31	0.57	1.59
18	0.52	1.25	6.58	0.02	4.96	0.13	0.76	1.52	0.42	2.08	0.53	4.01	2.02	0.75	1.07	1.68
19	0.24	1.87	3.85	0.08	4.02	0.48	0.28	3.64	0.33	3.32	0.55	2.25	1.02	11.83	0.71	3.23
20	0.14	2.95	2.67	0.11	1.16	0.63	0.36	2.65	0.25	5.04	1.09	1.86	0.09	1.99	0.46	1.01
21	0.17	10.73	3.52	0.09	2.97	0.49	0.20	2.80	0.09	14.61	0.27	2.10	0.80	3.91	0.30	2.83
22	0.51	0.06	7.47	0.01	9.81	0.02	0.18	0.03	0.67	0.01	1.07	0.03	3.56	0.17	0.22	N/A
23	0.12	13.87	5.37	0.06	2.85	0.38	0.30	4.62	0.49	18.21	0.26	6.39	0.32	17.36	0.41	3.38
24	0.51	2.03	0.07	0.06	0.05	0.46	0.70	4.21	0.25	1.85	0.02	3.75	0.59	10.82	0.62	2.24
25	0.27	3.65	7.23	0.01	2.29	0.74	0.21	5.34	0.42	1.46	0.62	3.75	0.02	5.41	0.12	0.72
Median	0.95	2.42	7.23	7.27	4.49	0.32	0.80	2.52	0.49	1.97	1.48	1.91	2.54	2.97	0.64	1.64
P value	0.291		0.016		0.929		0.589		0.030		0.345		0.149		0.347	

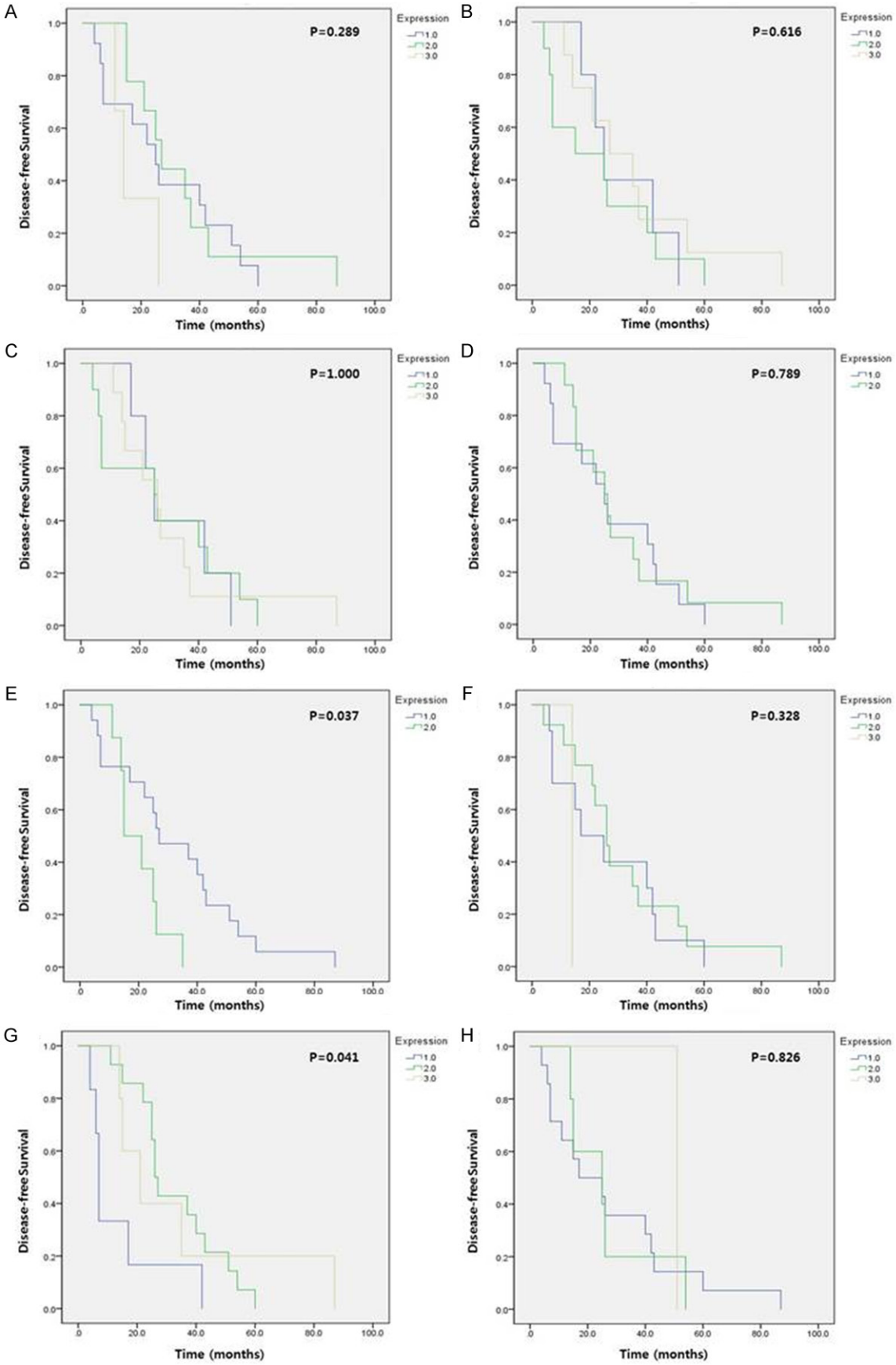
A
P=0.289
B
P=0.616
C
P=1.000
D
P=0.789
E
P=0.037
F
P=0.328
G
P=0.041
H
P=0.826
Disease-free Survival
Time (months)
Expression
1.0
2.0
3.0

Figure 2. Kaplan-Meier survival curve for disease-free survival (DFS). Survival curves for DFS according to initial expression levels of each candidate miRNAs are displayed. The expression levels are graded in three groups: 1 represents down-regulation (expression level 0-1, blue line), 2 represents low up-regulation (expression level 1-10, green line), and 3 represents high up-regulation (expression level >10, yellow line) compared to the control group. High expression level of miRNA-191 (E) and low expression level of miRNA-204 (G) are associated with poorer outcome. MiRNA-26a (A), miRNA-133a (B), miRNA-133b (C), miRNA-186 (D), miRNA-194 (F), and miRNA-296-5p (H) are not associated with DFS.

Table 3. Multivariate analysis of prognostic factor affecting disease-free survival

Prognostic factors	*P* value	Hazard ratio	95% confidence interval
Age >50 years vs ≤50 years	0.415	0.537	0.121-2.390
Tumor size	0.271		
>20 but ≤50 mm vs ≤20 mm	0.312	2.931	0.364-23.571
>50 mm vs ≤20 mm	0.109	34.264	0.455-2581.355
Positive lymph nodes vs negative lymph node	0.029	0.217	0.055-0.856
Tumor grade 3[a] vs grade 2	0.201	2.569	0.605-10.908
miRNA-191 expression level grade 2[b] vs expression level grade 1	0.001	20.053	3.529-113.938
miRNA-204 expression level	<0.001		
Expression level grade 2 vs expression level grade 1	<0.001	0.022	0.003-0.157
Expression level grade 3 vs expression level grade 1	<0.001	0.020	0.002-0.178

[a]Modified Bloom-Richardson grading system; [b]Expression level grades are defined as follows: 1, down-regulation (expression level 0-1 by the $2^{-\Delta\Delta CT}$ method); 2, low up-regulation (expression level 1-10); 3, high up-regulation (expression level >10).

Table 2. Comparison of the individual expression levels of 8 microRNAs between the first and second resected breast cancers using paired *t* test revealed miRNA-133a (P=0.016) and miRNA-191 (P=0.030) were significantly different. 18 patients showed down-regulation and 4 patients showed up-regulation in recurrent tumor for miRNA-133a, and 7 patients showed down-regulation and 17 patients showed up-regulation in recurrent tumor for miRNA-191.

In the univariate analysis, the patients with high expression level of miRNA-191 (P=0.037) and low expression level of miRNA-204 (P=0.041) at the time of initial diagnosis showed worse prognosis compared to the patients with low expression level of miRNA-191 and high expression level of miRNA-204 (**Figure 2E** and **2G**). Other candidate miRNAs including miRNA-26a, miRNA-133a, miRNA-133b, miRNA-186, miRNA-194, and miRNA-296-5p were not associated with DFS (**Figure 2A-D**, **2F**, and **2H**). In the multivariate analysis by Cox proportional hazards regression model (**Table 3**), expression levels of miRNA-191 and miRNA-204 were identified as independent prognostic factor for DFS. Presence of metastatic lymph nodes was associated with DFS independently also.

Discussion

Increased understanding of molecular biology of breast cancer improves the development of effective treatments and the survival of the patients. Despite remarkable advances in the therapies of breast cancer, a subset of patient still experiences local recurrence after initial curative surgery. Several factors associated with higher recurrence rate have been reported so far, which include positive surgical resection margins, multicentric tumors, younger age, family history, and other various clinicopathological characteristics [6, 15, 16]. But there is no clear and definite biomarker for predicting recurrence of breast cancer, so identification appropriate biomarkers which can distinguish high risk of recurrence is essential. To the best of our knowledge, a study about comparison of miRNA expression level in a paired tumor sample has not been reported so far.

Studying recurrent breast cancer tumor tissue in comparison to its corresponding primary tumor tissue might represent a possibility for new biomarkers associated with local recurrence. We have investigated the sequential change and compared miRNA expression levels in primary and corresponding recurrent breast cancer. We evaluated the prognostic impact of

each miRNAs for predicting recurrence as well. In the current study, we have identified initial expression level of miRNA-191 & miRNA-204 and sequential follow-up expression level of miRNA-133a & miRNA-191 can predict the local recurrence of breast cancer.

8 candidate miRNAs including miRNA-26a, miRNA-133a, miRNA-133b, miRNA-186, miRNA-191, miRNA-194, miRNA-204, and miRNA-296-5p were selected in a screening test by miRNA expression array analysis (PANArray). Zhou X *et al.* reported miRNA-9 is potential biomarker for breast cancer local recurrence [6]. Unlike in the current study, Zhou *et al.* investigated in two different groups of patients which included tumor samples with local recurrence and without recurrence, not in the same patients. In our study, miRNA-9 was not differently expressed in primary and corresponding recurrent tumor of the same patients that were verified both by miRNA expression array analysis and by real-time RT-PCR.

When comparing each expression level of 8 candidate miRNAs between primary and recurrent breast cancer in a validation test by real-time RT-PCR, miRNA-133a and miRNA-191 showed significantly different expression level. The median expression level of miRNA-133a was slightly higher in recurrent tumor (expression level 7.27) than primary tumor (expression level 7.23), but 18 patients showed down-regulation and only 4 patients showed up-regulation in recurrent tumor. Thus, down-regulation or declining tendency of miRNA-133a over time is considered as potential biomarker for breast cancer recurrence. The discordance of median expression level seemed to be due to one patient who showed exceptionally high expression level (patient number 7, expression level 106.62). The expression level of miRNA-191 was higher in 17 patients and lower in 7 patients in recurrent tumor than primary tumor, and the median expression level was also higher in recurrent tumor (expression level 1.97) than primary tumor (expression level 0.49). So up-regulation or increasing tendency of miRNA-191 over time is considered as potential biomarker for predicting recurrence as well.

Several studies investigated miRNA-133a in breast cancer. Wu ZS *et al.* reported that loss of miRNA-133a expression was associated with poor survival and restoration of miRNA-133a expression inhibited growth and invasion of breast cancer [17]. Cui W *et al.* identified that miRNA-133a regulated the cell cycle and proliferation by targeting epidermal growth factor receptor (EGFR) through the downstream signal molecule Akt [18]. These studies suggested that miRNA-133a might act as a tumor suppressor in breast cancer in common, and our results also corresponded with the previously reported data. Some studies about miRNA-191 in breast cancer have been also conducted recently. Mar-Aguilar F *et al.* reported that up-regulation of miRNA-191 was found in breast cancer, so it seemed to be associated with tumorigenesis of breast cancer [19]. Di Leva G. *et al.* demonstrated that miRNA-191 had fundamental impact on breast cancer initiation and progression by reducing the expression of an extensive network of genes [20]. Nagpal N *et al.* identified estrogen/ER/miRNA-191/SATB1 cascade and miRNA-191 seemed to be significantly associated with estrogen signaling pathway as oncogenic player [21]. All mentioned investigators suggested miRNA-191 as an oncogenic miRNA in breast cancer in the same way as our results.

The expression level of specific miRNAs at the time of primary tumor resection was associated with breast cancer recurrence in the current study. We found that initial expression level of miRNA-191 show positive correlation with tumor recurrence through survival analysis, which strengthens the oncogenic role of miRNA-191 in breast cancer. The initial expression level of miRNA-204 was negatively correlative with tumor recurrence in survival analysis. This result was different from the screening test by miRNA expression array analysis. In the miRNA expression array, miRNA-204 showed higher expression level in recurrent tumor than primary tumor in 4 out of 5 patients. But in the validation test by survival analysis, lower initial expression level of miRNA-204 was predictable biomarker for poor prognosis and recurrence. The difference might point towards to limited sample number of our study. Studies about miRNA-204 in breast cancer have been little reported and controversial so far. Pollari S *et al.* reported that miRNA-204 down-regulated the expression of several genes in transforming growth factor-beta (TGF-beta) signaling pathway and associated with bone metastatic process in breast cancer [22]. According to their

results, miRNA-204 is supposed to be oncogenic miRNA. On the other hand, Wang X *et al.* identified that miRNA-204 targets Janus kinase 2 (JAK2) and suppressed JAK2 in breast cancer, which further induces cell apoptosis by inhibiting anti-apoptotic STAT3/Bcl-2/survivin pathway [23]. Therefore, further study in larger number of tumor samples will be needed to identify the significance of miRNA-204.

To sum up, we report the discovery of 3 miRNAs, miRNA-133a, miRNA-191, and miRNA-204, which can predict local recurrence of breast cancer and can be useful new biomarkers to identify patients at high risk of breast cancer recurrence. In case of miRNA-191 and miRNA-204, higher initial expression level of miRNA-191 and lower initial expression level of miRNA-204 could predict the local recurrence. One of them (miRNA-191) showed significance as a sequential expression level as well as an initial expression level. The increasing tendency of miRNA-191 sequential level was associated with local recurrence. In addition, the declining tendency of miRNA-133a in a timed sequence was associated with local recurrence also.

Acknowledgements

This work was supported by Biomedical Research Institute grant, Kyungpook National University Hospital (2012).

Disclosure of conflict of interest

None.

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