Usefulness of miRNA profiles for predicting pathological responses to neoadjuvant chemotherapy in patients with human epidermal growth factor receptor 2-positive breast cancer

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Abstract. Pathological complete response (pCR) is considered to be a useful prognostic marker for neoadjuvant chemotherapy to improve the survival rate of patients with operable breast cancer. In the present study, we identified differentially expressed microRNAs (miRNAs) between pCR and non-pCR groups of patients with human epidermal growth factor receptor 2 (HER2)-positive breast cancer who received neoadjuvant chemotherapy with trastuzumab. Expression profiles were examined by miRNA microarrays using total RNA extracted from formalin-fixed, paraffin-embedded tissues from pretreatment biopsy specimens. Significant differences were observed in miRNAs associated with pCR between the luminal B-like (HER2-positive) and HER2-positive (nonluminal) subtypes, which were further classified according to their estrogen receptor (ER) status. Prediction models constructed with differentially expressed miRNAs performed well. In conclusion, the combination of miRNA profiles and ER status may improve the accuracy of pCR prediction in patients with HER2-positive breast cancer and enable the development of personalized treatment regimens.

Introduction

Human epidermal growth factor receptor 2 (HER2) is overexpressed in ~20% of primary invasive breast cancer cases,

while higher amplification has been correlated with more aggressive behavior and poorer clinical outcomes (1,2). Trastuzumab, a monoclonal antibody that binds to the extracellular domain of HER2, is used as a targeted molecular therapy in combination with chemotherapy and has been demonstrated to improve the survival rate of patients with HER2-positive metastatic breast cancer (3). Furthermore, the addition of trastuzumab to adjuvant chemotherapy has been demonstrated to improve disease-free survival (DFS) and overall survival (OS) of patients with HER2-positive operable breast cancer (4). Therefore, trastuzumab has become a significant therapeutic agent for patients with HER2-positive breast cancer.

The use of neoadjuvant chemotherapy is a recent treatment strategy for breast cancer that offers survival benefits equivalent to those of adjuvant chemotherapy and provides a means to predict prognosis (5,6). Pathological complete response (pCR), which is characterized by the disappearance of invasive tumors in surgical specimens, is considered a useful prognostic marker, since patients who achieve pCR exhibit significant improvement in DFS and OS. Therefore, the identification of biomarkers to predict pathological responses may help to maximize the benefit of treatment and minimize the risk of adverse effects. The addition of trastuzumab to neoadjuvant chemotherapy for patients with HER2-positive breast cancer significantly improves the rate of pCR for these patients (7,8); thus, it was recommended by the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer in 2013 (9).

HER2-positive breast cancer is divided into two subtypes according to estrogen receptor (ER) status using immunohistochemical analysis as a surrogate for the classification of intrinsic subtypes by molecular assays, while ER-positive tumors are classified as either 'HER2-positive (nonluminal)' or 'luminal B-like (HER2-positive)' subtypes, respectively (9). However, there exists a discrepancy in the pathological responses and survival benefits of neoadjuvant chemotherapy between these two HER2-positive subtypes (10). Therefore,

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new predictive markers in combination with conventional markers, including immunohistochemical ER and HER2 status, are required.

MicroRNAs (miRNAs) are short noncoding RNAs, ranging in length from 20 to 25 nucleotides, which regulate protein synthesis at the post-transcriptional level by binding to the 3' untranslated region of mRNAs (11). Numerous miRNAs are reported to be related to dysregulation and various cancers. For example, miR-21, which is a well-studied oncomiR, was reported to be associated with HER2-positive breast cancer. Higher expression of miR-21 is associated with positive HER2 status, and miR-21 inhibits apoptosis through targeting tumor suppressors including phosphatase and tensin homolog (12). miRNAs play essential roles in breast cancer pathogenesis through a complex gene regulation network, and several have been implicated in resistance and sensitivity to breast cancer therapeutic drugs (13). miRNAs are well-preserved in a range of specimen types, including plasma, urine and formalin-fixed paraffin-embedded (FFPE) tissues (13). Since miRNA expression profiles differ among breast cancer subtypes, as determined by gene expression profiles and immunohistochemical findings (14,15), we speculated that miRNAs may be efficient predictive markers of the response of neoadjuvant chemotherapy.

In the present study, we evaluated the use of differentially expressed miRNA profiles of biopsy specimens collected prior to neoadjuvant chemotherapy with trastuzumab for patients with HER2-positive breast cancer between pCR and non-pCR patient groups to predict the pathological response of neoadjuvant chemotherapy. To the best of our knowledge, few studies have investigated miRNA profiles associated with the pathological response of neoadjuvant chemotherapy for patients with HER2-positive breast cancer according to ER status. The aim of this study was to develop a more reliable prediction of neoadjuvant chemotherapy outcomes using differentially expressed miRNA profiles in combination with ER and HER2 status.

Materials and methods

Patient population. The study protocol was approved by the Bioethics Committee for Human Genome and Gene Analysis of Jichi Medical University, Tochigi, Japan (approval no. 13-10), and written informed consent was obtained from all participants. We retrospectively reviewed the medical records of 47 consecutive patients with HER2-positive breast cancer who received neoadjuvant chemotherapy with trastuzumab at the Department of Breast Surgery, Jichi Medical University Hospital, between January 2006 and December 2011. All patients had pathologically confirmed HER2-positive invasive breast cancer by ultrasound-guided core needle biopsies prior to treatment. FFPE blocks of pretreatment biopsy specimens were available for 40 cases (85.1%). The remaining seven cases were excluded from this study as the biopsies were performed in other institutions.

Treatment. Neoadjuvant chemotherapy consisted of anthracycline-based regimens, followed by taxane-based regimens. The following three anthracycline-based regimens were used: three or four cycles of FEC (fluorouracil 500 mg/m², epirubicin 100 mg/m², and cyclophosphamide 500 mg/m²

Table I. Summary of yield and quality of extracted RNA samples.

Variables	Median	Range
Concentration (ng/ μ l)	102.10	24.40 to 374.50
A260/280	1.96	1.70 to 2.06
A260/230	1.74	1.00 to 1.95
RNA integrity number	2.40	1.70 to 2.50

A260/280, ratio of absorbance at 260 and 280 nm. A260/230, ratio of absorbance at 260 and 230 nm.

every 3 weeks); four cycles of AC (adriamycin 60 mg/m² and cyclophosphamide 600 mg/m² every 3 weeks); and four cycles of EC (epirubicin 90 mg/m² and cyclophosphamide 600 mg/m² every 3 weeks). The three taxane-based regimens were as follows: three or four cycles of docetaxel (100 mg/m² every 3 weeks); four cycles of docetaxel (75 mg/m² every 3 weeks); and 12 cycles of paclitaxel (80 mg/m² every weeks). All patients received trastuzumab regimens (4 mg/kg as a loading dose and 2 mg/kg from the second dose onward every week) concomitantly with a taxane-based regimen. Surgery was performed 3-4 weeks after the final dose of the neoadjuvant chemotherapy regimen.

Pathological evaluation. Expression levels of ER (SP1; Roche Diagnostics Deutschland GmbH, Mannheim, Germany), progesterone receptor (PR; 1E2; Roche Diagnostics) and HER2 (4B5; Roche Diagnostics) in the biopsy specimens were routinely subjected to immunohistochemical analysis. The cut-off value for ER and PR positivity was $\geq 10\%$ positive cells. HER2 expression was scored on a scale of 0-3+, in accordance with the American Society of Clinical Oncology/College of American Pathologists guidelines (16). A score of 0-1+ was considered negative, while a score of 3+ was considered positive. For 2+ cases, HER2 amplification was verified by fluorescence *in situ* hybridization using a PathVysion HER-2 DNA Probe kit (Abbott/Vysis, Des Plaines, IL, USA). Tumors were categorized into two subtypes on the basis of ER and HER2 status of the biopsy specimens as surrogate markers: the luminal B-like (HER2-positive) subtype (ER- and/or PR-positive, HER2-positive) and the HER2-positive (nonluminal) subtype (ER- and PR-negative, HER2-positive). Following surgery, residual tumors in the resected specimens were pathologically evaluated. The pathological responses of neoadjuvant chemotherapy were determined according to the status of residual invasive tumors. A pCR was defined as no pathological evidence of residual invasive cancer in breast and axillary lymph nodes, irrespective of the remaining intraductal components.

Isolation of total RNA. Four serial 10- μ m-thick sections of FFPE tissue specimens were mounted onto Leica PEN-membrane slides (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) and stained with cresyl violet (Ambion Life Technologies, Austin, TX, USA). Neoplastic portions of each specimen were collected by laser capture microdissection using a laser microdissection (LMD) system (LMD7000;

Figure 1. Example of laser capture microdissection. (A) FFPE section (4 μ m) was stained with hematoxylin-eosin. (B) FFPE section (10 μ m) was stained with cresyl violet on a PEN-membrane slide. (C) Neoplastic portions of specimen were defined before cutting (red lines). (D) Specimen was cut along the definition. Dissectates were collected by gravity. FFPE, formalin-fixed paraffin-embedded.

Figure 2. Example of electrophoretic RNA measurement recorded with an Agilent 2100 bioanalyzer. The electropherogram represents the size of distribution in nt and FU. Peak of RNA fragments was located at $\sim$ 100 nt in length. RNA integrity number of this sample was 2.3. FU, fluorescence units.

Leica Mikrosysteme Vertrieb GmbH; Fig. 1). RNA extraction was performed using an miRNeasy FFPE kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA in the samples was quantified using a Qubit RNA HS Assay kit and Qubit 2.0 fluorometer (Life Technologies, Palo Alto, CA, USA), while RNA quality was assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA; Fig. 2). Total RNA was extracted from FFPE sections of pretreatment biopsy specimens from all 40 patients. The yield and quality of all samples were checked prior to subsequent analyses (Table I). The median concentration of extracted RNA was 102.1 ng/ μ l (range, 24.4-374.5 ng/ μ l). The median 260/280 and 260/230 nm ratios of absorbance were 1.96 (range, 1.70-2.06) and 1.74 (range, 1.00-1.95), respectively. The median RNA integrity number, which indicates the degree of RNA degradation, was 2.4 (range, 1.7-2.5).

miRNA microarray expression analysis. Total RNA (100 ng) was labeled with cyanine 3-pCp using the Agilent miRNA Complete Labeling and Hyb kit (Agilent Technologies, Inc.). Labeled RNA was hybridized to an Agilent SurePrint G3 Human miRNA microarray (8x60 K, release 19.0; Agilent

Technologies, Inc.) for 20 h at 55°C in a hybridization chamber. Following hybridization, the array was washed and scanned with an Agilent G2505C microarray scanner (Agilent Technologies, Inc.) at 3 μ m resolution. miRNA expression data were extracted from the scanned images with Feature Extraction software (version 10.7.3.1; Agilent Technologies, Inc.).

Data processing and statistical analysis. miRNA expression data were analyzed using GeneSpring software (version 13.0; Agilent Technologies, Inc.) and normalized using a 90th percentile normalization algorithm, followed by preprocessing baseline to median data of all samples. miRNAs were filtered based on signal intensity values with a lower percentile cut-off value of 20% in more than 50% of the samples in any one category. Unsupervised hierarchical cluster analysis was performed on filtered data. Expression differences between objective groups were determined using a moderated t-test. Differences of more than two-fold with a probability P-value <0.05 were considered statistically significant. Prediction models for pathological responses were developed using a partial least squares discrimination (PLSD) model with GeneSpring software, and a prediction model for non-pCR was based on expression values of differentially expressed miRNAs. Prediction results are presented as *t*-scores computed with Prism 5 statistical software

Table II. Patient characteristics.

Characteristics	All patients	Luminal B-like (HER2-positive)	HER2-positive (nonluminal)	P-value
Number of patients	40	17	23	
Median age (range)	55 (31-83)	49 (33-66)	58 (31-83)	0.01
Median tumor size (range, cm)	3.4 (1.2-6.8)	3.1 (1.3-6.8)	3.5 (1.2-6)	0.78
Clinical axillary node status				0.37
Metastasis	34 (85.0%)	13 (76.5%)	21 (91.3%)	
No metastasis	6 (15.0%)	4 (23.5%)	2 (8.7%)	
Clinical stage				0.31
2	26 (65.0%)	13 (76.5%)	13 (56.5%)	
3	14 (35.0%)	4 (23.5%)	10 (43.5%)	
Regimen of neoadjuvant chemotherapy				0.49
Anthracycline followed by taxane	38 (95.0%)	17 (100%)	21 (91.3%)	
Taxane-based (without anthracycline)	2 (5.0%)	0 (0%)	2 (8.7%)	
Pathological response				0.04
pCR	15 (37.5%)	3 (17.6%)	12 (52.2%)	
Non-pCR	25 (62.5%)	14 (82.3%)	11 (47.8%)	

HER2, human epidermal growth factor receptor 2; pCR, pathological complete response.

(version 5.0f; GraphPad Software, Inc., La Jolla, CA, USA). The *t*-scores were plotted and compared between the pCR and non-pCR groups using the Mann-Whitney U-test. Receiver operating characteristic (ROC) curves were drawn and the area under the ROC curve (AUC) was calculated. Patient characteristics were compared between the HER2-positive (nonluminal) and luminal B-like (HER2-positive) subtypes using Prism 5 software. Fisher's exact test was used to compare categorical data. The Mann-Whitney U-test was used to compare continuous data.

Results

Patient characteristics. Medical records from a total of 40 females with stage II or III breast cancer were included for analysis (Table II). The median age at diagnosis was 55 years (range, 31-83 years). A total of 17 and 23 patients, respectively, were diagnosed as the luminal B-like (HER2-positive) or HER2-positive (nonluminal) subtype. Patients with HER2-enriched tumors (ER-negative) were older than those with luminal-HER2 hybrid tumors (ER-positive) ($P=0.01$). There were no significant differences in median tumor size, clinical axillary node status, clinical stage or neoadjuvant chemotherapy regimen between subtypes ($P=0.78$, 0.37, 0.31 and 0.49, respectively). The pCR of the HER2-positive (nonluminal) subtype was significantly greater than that of the luminal B-like (HER2-positive) subtype ($P=0.04$).

miRNA expression profiles associated with pathological response of neoadjuvant chemotherapy. Unsupervised hierarchical clustering using the full dataset of 2,024 miRNA expression profiles of all 40 patients was insufficient to differentiate between the pCR and non-pCR groups (Fig. 3A). Unsupervised cluster analyses were also performed following

categorization into HER2-positive (nonluminal; Fig. 3B) and luminal B-like (HER2-positive) subtypes (Fig. 3C); however, these were also insufficient to predict the pCR group.

Twenty-one miRNAs were differentially expressed between the non-pCR and pCR groups in the analysis of all 40 patients with HER2-positive breast cancer (Table III). Nine of these 21 miRNAs are reported to be associated with breast cancer (17-25). The other 12 miRNAs have no reported correlation with breast cancer. Furthermore, differential miRNA expression was separately examined according to the luminal B-like (HER2-positive) and HER2-positive (nonluminal) subtypes. For the luminal B-like (HER2-positive) subtype, 17 miRNAs were differentially expressed (Table IV), and nine of these 17 miRNAs are reported to be correlated with breast cancer (20-22,24,26-30). For the HER2-positive (nonluminal) subtype, 14 miRNAs were differentially expressed (Table V), and five of these are reported to be correlated with breast cancer (17,23,25,31,32). The miRNA expression profiles associated with pathological response differed completely between the HER2-positive (nonluminal) and luminal B-like (HER2-positive) subtypes.

Generation of a prediction model for pathological response to neoadjuvant chemotherapy of HER2-positive breast cancer. After evaluating differential miRNA expression, a prediction model for pathological response was constructed using the 21 differentially expressed miRNAs identified by analysis of all 40 patients. The distribution of *t*-scores significantly differed between the pCR and non-pCR groups ($P<0.001$; Fig. 4A). Performance of the prediction model was evaluated using discriminant analysis with ROC curves. The AUC was 0.8827, indicating moderate accuracy of this model in predicting pathological responses. We further examined the results of this prediction model separately for the HER2-positive

Figure 3. (A) Unsupervised hierarchical cluster analysis using the full dataset of 2,024 miRNA expression profiles of all 40 patients. (B) Unsupervised hierarchical cluster analysis using the full dataset of 2,024 miRNA expression profiles of patients with HER2-positive (nonluminal) breast cancer. (C) Unsupervised hierarchical cluster analysis using the full dataset of 2,024 miRNA expression profiles of patients with luminal B-like (HER2-positive) breast cancer. The insets show details from the main heat map. The categorical variables were pCR and non-pCR, listed below the insets. HER2, human epidermal growth factor receptor 2; pCR, pathological complete response.

(nonluminal) and luminal B-like (HER2-positive) subtypes. There were significant differences in the distribution of t-scores ($P < 0.001$) between the pCR and non-pCR groups in the HER2-positive (nonluminal) subtype (Fig. 4B), whereas no significant difference was observed for the luminal B-like (HER2-positive) subtype ($P = 0.051$; Fig. 4C). In discriminant analyses with the ROC curve, the AUCs were 0.9242 for the HER2-positive (nonluminal) subtype and 0.8810 for the luminal B-like (HER2-positive) subtype, respectively (Fig. 4B and C). These results demonstrate that the predictive ability for the HER2-positive (nonluminal) subtype

Figure 4. (A) Distribution of t-scores and ROC curve to predict the pCR of all 40 patients. (B) Distribution of t-scores and ROC curve to predict the pCR of patients with HER2-positive (nonluminal) breast cancer. (C) Distribution of t-scores and ROC curve to predict the pCR of patients with luminal B-like (HER2-positive) breast cancer. The t-scores were computed using differentially expressed miRNAs between the pCR and non-pCR groups. ROC, receiver operating characteristics; pCR, pathological complete response; AUC, area under the ROC curve; HER2, human epidermal growth factor receptor 2.

is better than that for the luminal B-like (HER2-positive) subtype. A prediction model of pathological response was also constructed using the 14 miRNAs differentially expressed in the HER2-positive (nonluminal) subtype. The distribution of t-scores significantly differed ($P < 0.001$) between the pCR and non-pCR groups, and the AUC was 0.9621 (Fig. 5). These results indicate that the prediction model for the HER2-positive (nonluminal) subtype was more suitable than that of the prediction model using 21 differentially expressed miRNAs in the analysis of all 40 patients. A model for the luminal B-like (HER2-positive) subtype could not be constructed as the data were not suitable for classification using the PLSD algorithm.

Discussion

In the present study, we identified miRNA expression profiles of biopsy specimens from patients with HER2-positive

Table III. Differential miRNA expression between non-pCR and pCR in all 40 patients.

miRNA	P-value	Fold change	Regulation	Function correlated with breast cancer
miR-106b-3p	0.046	2.97	Down	Downregulated in bone metastasis patients with breast cancer (17)
miR-1180	0.035	4.64	Down	N/A
miR-1238-5p	0.046	3.00	Down	N/A
miR-142-5p	0.035	2.73	Down	Upregulated in human breast cancer stem cells (18)
miR-150-5p	0.030	2.05	Down	Overexpression promotes growth and reduces apoptosis in breast cancer cells (19)
miR-181c-5p	0.016	4.27	Down	Predictive miRNA corresponding with HER2 status in early-stage breast cancer (20)
miR-182-5p	0.021	3.18	Down	N/A
miR-200a-5p	0.028	4.85	Down	Higher level of circulating miR-200a in CTC-positive MBC patients (21)
miR-210	0.046	2.22	Up	Higher expression correlates with poor prognosis of patients with breast cancer (22)
miR-218-5p	0.013	5.99	Down	Downregulated in cisplatin-resistant breast cancer cell lines (23)
miR-31-3p	0.043	2.04	Up	Upregulated in chemoresistant breast cancer tissues (24)
miR-3609	0.026	3.84	Down	N/A
miR-362-5p	0.049	3.33	Down	N/A
miR-3620-3p	0.046	3.72	Down	N/A
miR-4418	0.030	4.40	Down	N/A
miR-449a	0.028	6.44	Up	N/A
miR-449b-5p	0.037	3.15	Up	N/A
miR-4506	0.015	4.95	Down	N/A
miR-4657	0.049	3.37	Down	N/A
miR-505-3p	0.026	3.44	Down	Tumor suppressive miRNA, which correlates inversely with drug sensitivity (25)
miR-505-5p	0.012	3.75	Down	

pCR, pathological complete response; N/A, not applicable; CTC, circulating tumor cell; MBC, metastatic breast cancer.

Figure 5. Distribution of t-scores and receiver operating characteristics curve to predict pCR using a prediction model constructed from the 14 miRNAs differentially expressed between the pCR and non-pCR groups of the human epidermal growth factor receptor 2-positive (nonluminal) subtype. pCR, pathological complete response; AUC, area under the ROC curve.

breast cancer prior to neoadjuvant chemotherapy with trastuzumab. The miRNAs associated with pathological response

significantly differed between the HER2-positive (nonluminal) and luminal B-like (HER2-positive) subtypes. The prediction

Table IV. Differential miRNA expression between non-pCR and pCR in luminal B-like (HER2-positive) subtype.

miRNA	P-value	Fold change	Regulation	Function correlated with breast cancer
miR-148b-3p	0.041	5.035	Up	Higher level of circulating miR-148b in breast cancer patients (26)
miR-151a-3p	0.043	4.635	Up	N/A
miR-152	0.016	4.490	Up	Upregulation indirectly interacts with MAPK signaling pathway (27)
miR-203a	0.027	18.271	Up	Higher level of circulating miR-203 in CTC-positive MBC patients (21)
miR-210	0.022	10.120	Up	Higher expression correlates with poor prognosis of patients with breast cancer (22)
miR-28-5p	0.039	4.214	Up	N/A
miR-301b	0.007	3.485	Down	Oncogenic miRNA, miR-301 attenuation decreases cell proliferation and invasion (28)
miR-34b-5p	0.037	4.754	Up	miR-34 and p53 regulate epithelial-mesenchymal transition of cancer cells (29)
miR-376c-3p	0.019	21.261	Up	Upregulated in plasma of patients with breast cancer (30)
miR-377-3p	0.039	11.814	Up	Predictive miRNA corresponding with PR status in early-stage breast cancer (20)
miR-3907	0.006	4.389	Up	N/A
miR-429	0.037	7.079	Up	Overexpressed in chemoresistant breast cancer cells (24)
miR-4291	0.006	5.189	Up	N/A
miR-4737	0.007	4.726	Down	N/A
miR-487b	0.040	9.634	Up	N/A
miR-5684	0.036	4.578	Up	N/A
miR-582-5p	0.007	4.721	Down	N/A

pCR, pathological complete response; HER2, human epidermal growth factor receptor 2; N/A, not applicable; MAPK, mitogen-activated protein kinase; CTC, circulating tumor cell; MBC, metastatic breast cancer; PR, progesterone receptor.

models of pathological responses constructed using miRNAs differentially expressed between the pCR and non-pCR groups demonstrated considerable reliability.

Breast cancer may be classified into subtypes according to gene expression profiles (33). Surrogate subtype classification obtained by immunohistochemical analyses of ER, PR, Ki-67 and HER2 is often used in clinical practice (9). In previous studies, miRNA expression profiles differed according to the breast cancer subtype (14,15). However, several previous studies of miRNA expression profiles of neoadjuvant chemotherapy for patients with HER2-positive breast cancer did not classify patients according to hormone receptor status (34,35). The pathological response and survival benefit of neoadjuvant chemotherapy with trastuzumab reportedly differs between two HER2-positive subtypes divided by hormone receptor status (10). Therefore, we classified HER2-positive breast cancer into subtypes based on ER status and subsequently analyzed miRNA expression. The results of our study revealed significant differences in the miRNA profiles associated with pathological responses between the HER2-positive (nonluminal) and luminal B-like (HER2-positive) subtypes. We further constructed models to predict pathological responses using differentially expressed miRNAs. The combination of breast cancer subtypes and miRNA expression profiles offers a means to accurately and efficiently predict pathological responses.

We identified a total of 52 differentially expressed miRNAs in the analysis of all 40 patients: 21 according to pCR status (Table III), 17 by analysis of the luminal B-like (HER2-positive) subtype (Table IV), and 14 by analysis of the HER2-positive (nonluminal) subtype (Table V), respectively. A total of 48 miRNAs were selected due to the overlap of four miRNAs (i.e., miR-210, miR-106b, miR-505 and miR-218). miR-210 was upregulated in the analyses of all 40 patients and the luminal B-like (HER2-positive) subtype. Correlations between increased miR-210 expression and poor prognosis in various cancers, particularly in breast cancer, as well as associations with the hypoxic pathway in a hypoxia-inducible factor-dependent manner have been reported elsewhere (22,36). miR-106b-3p, miR-218-5p and miR-505-5p were downregulated in the analyses of all 40 patients and the HER2-positive (nonluminal) subtype. Downregulation of miR-106b involves bone metastasis of breast cancer with negative regulation of matrix metalloproteinase 2 (17). miR-218 was downregulated in cisplatin-resistant breast cancer cell lines and regulated chemosensitivity by targeting BRCA1 (23). miR-505 has been reported as a tumor-suppressive miRNA, and is inversely correlated with Akt3, which modulates drug sensitivity (25). The remaining differentially expressed 15 miRNAs between the non-pCR and pCR groups are reportedly involved in the progression of breast cancer (Tables III-V). To the best of our

Table V. Differential miRNA expression between non-pCR and pCR in HER2-positive (nonluminal) subtype.

miRNA	P-value	Fold change	Regulation	Function correlated with breast cancer
let-7a-3p	0.048	2.387	Up	Tumor suppressive miRNA, which decreased breast cancer cell migration and invasion (31)
miR-106b-3p	0.014	4.186	Down	Downregulated in bone metastasis patients with breast cancer (17)
miR-1237-3p	0.048	2.876	Up	N/A
miR-136-5p	0.044	7.102	Down	N/A
miR-181a-3p	0.028	7.355	Down	N/A
miR-196a-5p	0.044	2.222	Down	N/A
miR-218-5p	0.019	7.687	Down	Downregulated in cisplatin-resistant breast cancer cell lines (23)
miR-342-5p	0.016	4.669	Down	Downregulation associates with early recurrence in patients with breast cancer (32)
miR-362-5p	0.026	3.357	Down	N/A
miR-376a-3p	0.027	3.941	Down	N/A
miR-376c-3p	0.018	5.352	Down	N/A
miR-505-5p	0.017	4.369	Down	Tumor suppressive miRNA, which correlates inversely with drug sensitivity (25)
miR-550a-5p	0.049	3.647	Up	N/A
miR-6515-3p	0.010	6.535	Up	N/A

pCR, pathological complete response; N/A, not applicable.

knowledge, the involvement of the other 28 miRNAs identified in this study with breast cancer has not been previously reported.

In a study using pretreatment biopsy specimens, Kolańska *et al* (37) reported correlations between miRNA expression profiles and the pathological response of neoadjuvant chemotherapy. This group investigated the expression profiles of 19 miRNAs in frozen biopsy specimens collected from patients with ER-negative, PR-negative and HER2-negative (triple-negative) breast cancer, and observed that miR-200b-3p, miR-190a and miR-512-5p were associated with a better pathological response. However, there were no differences in the expression levels of these three miRNAs between the non-pCR and pCR groups in our study. Most triple-negative breast cancers belong to different molecular subtypes of ER- and/or HER2-positive breast cancers (38), and miRNA expression of triple-negative breast cancers differed compared with other subtypes (14,15). Triple-negative breast cancer tends to behave more aggressively than other subtypes; there are currently no therapies targeting the endocrine system or HER2 for this subtype of breast cancer. Taken together, it appears that there are differences in miRNA profiles associated with pathological response according to subtype.

miRNAs associated with the pathological response of neoadjuvant chemotherapies in patients with HER2-positive breast cancer have been reported in several studies using circulating miRNA. Circulating miRNAs may be exploited as noninvasive biomarkers since miRNAs derived from tumors are stable and detectable in serum (39,40). For example, Jung *et al* (34) reported that the expression level of circulating miR-210 was associated with the sensitivity of

neoadjuvant chemotherapy in patients with HER2-positive breast cancer, while Müller *et al* (35) reported that serum levels of miR-21, miR-210 and miR-373 were higher in patients with HER2-positive breast cancer than in healthy females, although no associations between circulating miRNAs with pCR were noted. The present study revealed that miR-210 upregulation was associated with non-pCR (Tables III and IV). Therefore, upregulation of miR-210 may be useful to predict the pathological response of neoadjuvant chemotherapy with trastuzumab, although these results cannot be validated due to the differences in analyses and chemotherapy regimens.

In this study, we determined miRNA profiles in FFPE sections of pretreatment biopsy specimens that were obtained prior to neoadjuvant chemotherapy. miRNA is stable and intact in FFPE, while mRNA is fragmented in FFPE tissue compared with fresh tissue (41). Our results also revealed degradation of RNA, and RNA fragments extracted from FFPE tissue were as short as 100 nt (Fig. 2). miRNAs appear to be better preserved, possibly due to their intrinsically shorter lengths (42). Microarray analysis of miRNA expression profiles demonstrated good correlations between frozen and FFPE samples (43). FFPE tissues are often the only available tissue samples in several institutions, and have usually been archived for long periods. This stability and availability in the clinical setting are advantages of miRNA study using FFPE. As another advantage of this study, an LMD system was used during the process of RNA extraction. The LMD system enables access to specific regions within tissue samples to collect relevant information (44). RNA was extracted from tumor tissue sections collected from biopsy specimens. Since the validity of microarray analysis of microdissected tumor

tissues from FFPE samples has been reported (45), we consider that our approach was reasonable.

There are certain limitations to this study that should be addressed. First, the sample size was relatively small and this retrospective study was limited to a single institution. Second, there exists no consensus on a method to normalize miRNA microarray data (46). Data normalization is necessary to minimize the effects of systemic experimental bias and technical variations. Although our data were analyzed using 90th percentile normalization, other normalization methods may lead to different results. Third, our results have not yet been verified, and therefore we cannot discount the possibility that the results were random. The candidate miRNAs should be verified by quantitative polymerase chain reaction analysis, and the prediction models should ideally be verified against a validation cohort.

In conclusion, the results of our study demonstrated differential miRNA expression profiles between pCR and non-pCR groups, following neoadjuvant chemotherapy with trastuzumab in patients with HER2-positive breast cancer. The combination of miRNA profiles and ER status may improve the accuracy of prediction of pathological responses to enable the design of personalized treatment regimens. The results of this study may be verified in larger and multicenter studies as this study used FFPE samples, which are generally available in most institutions. The findings of this study may be helpful to identify new predictive biomarkers to monitor the response of neoadjuvant chemotherapies.

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