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ORIGINAL ARTICLE

Prognostic Implications of MicroRNA-21 Overexpression in Invasive Ductal Carcinomas of the Breast

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Purpose: Among more than 500 microRNAs, microRNA-21 (miR-21) is known to act as an oncogene. The aim of this study was to investigate the significance of miR-21 expression level in relation with clinicopathological factors and prognosis in breast cancer. **Methods:** MicroRNA was extracted from cancer and normal breast tissue of 109 breast cancer patients who underwent surgery from 2002 to 2004 using the Taqman® MicroRNA Assay. The correlation between miR-21 expression and clinicopathologic features was analyzed and the significance of miR-21 as a prognostic factor and its relationship with survival was determined. **Results:** MiR-21 expression was higher in cancer tissues than in normal tissues ($p < 0.0001$). High miR-21 expression was associated with mastectomy, larger tumor size, higher stage, higher grade, estrogen receptor (ER) negative, human epidermal growth factor receptor 2 (HER2) positive, HER2 positive breast cancer subtype, high Ki-67 expression, and death. On multivariate analysis, prognostic factors for overall survival were ER and miR-21. High miR-21 expression was significantly related to lower overall survival ($p = 0.031$). **Conclusion:** This study supports the role of miR-21 as an oncogene and a biomarker for breast cancer with its high expression in cancer tissues and its relationship with other prognostic factors and survival.

Key Words: *Breast neoplasms, Human MicroRNA-21, Oncogene, Prognosis, Survival*

INTRODUCTION

Breast cancer is one of the most prevalent cancers and its incidence is further increasing, causing a major concern for women's health. Various modalities have been tried and the mortality rate is decreasing with medical advances. However, outcomes in breast cancer patients are variable even in the patients in same stage. To individualize treatment and to predict outcomes, novel biomarkers that will lead to molecular diagnostic tests are required.

MicroRNAs (miRNAs), small non-coding endogenous RNA gene products consisting of 18 to 25 nucleotides, were first discovered in 1993 [1]. After a multistage process commencing in the nucleus, pri-mRNAs are trimmed into pre-mRNAs and are exported to the cytoplasm. Following cleavage by the endoribonuclease dicer, pre-miRNAs are converted into mature miRNAs that are incorporated into the RNA-induced silencing complex (miRISC) and target messenger RNA (mRNA), resulting in cleavage or translational repression. By targeting mRNAs, miRNAs play critical roles in cell proliferation, differentiation, and apoptosis and, moreover, can act as tumor suppressors or oncogenes [2-6].

Iorio et al. [7] first reported 29 miRNAs associated with breast cancer and many more have since been discovered. Among these miRNAs, miR-21 is known to be overexpressed in breast cancer [7-10]. Studies have demonstrated that miR-21 functions as an oncogene by targeting tumor suppressor genes including tropomyosin 1 (TPM1), programmed cell death 4 (PDCD4), and phosphatase and tensin homolog (PTEN), leading to cell proliferation and inhibition of apoptosis and regulating cancer invasion and metastasis in breast cancer [11-13].

There has been a recent increase in studies involving miR-21, some of which propose that miR-21 is related to breast cancer patients' prognosis while others give opposing results [9-13]. To use miR-21 as a novel biomarker in breast cancer, its clinical relevance should be verified. This study was performed to understand the biological role of miR-21 expression in breast

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cancer by investigating the relationship between the expression of miR-21 and clinicopathological characteristics and survival in breast cancer patients.

METHODS

Materials

We collected clinical and pathologic reports of patients with invasive ductal breast cancer in stage I to III who underwent surgery between January 2002 and December 2004 in Korea University Anam Hospital. Among 252 patients, two patients had synchronous cancer in other organs, seven received neoadjuvant therapies, 15 had no follow up data, 13 had insufficient tumor or normal tissue, 16 had incomplete pathologic report, 81 lacked slides, and nine had $\Delta C_T > 30$; these patients were excluded from this study. Age was divided into two groups, under and over 50 years old. Patients were treated with either breast conserving surgery or total mastectomy. As for axillary dissection, patients who were not clinically suspected to have metastasis went through sentinel lymph node biopsy and if proven positive, axillary dissection was done. For those who were confirmed to have axillary metastasis from biopsy, axillary dissection was performed. The cut-off value for tumor size was 2 cm and lymph node status was defined as positive if there was metastasis. Clinical stage was classified according to the 7th American Joint Committee on Cancer (AJCC) classification [14].

Grade was evaluated using the Bloom and Richardson criteria suggested by the Nottingham City Hospital Pathologists. Assessment of estrogen receptor (ER) and progesterone receptor (PR) status was performed by standard immunohistochemical methods and considered positive if the value was > 10% of nuclear staining. For human epidermal growth factor receptor 2 (HER2) status, 0 and 1+ were considered as negative and 3+ as positive. HER2 status 2+, which was one case, was not included in statistical analysis. Subtypes were divided according to ER, PR, and HER2 status as follows: luminal A (ER+ and/or PR+, HER2-), luminal B (ER+ and/or PR+ HER2+), HER2+ (ER-, PR-, HER2+), and triple negative breast cancer (TNBC; ER-, PR-, HER2-). The p53 cut-off value was 10% and Ki-67 expression of < 20% was considered low expression. Pathological analyses, including identification of tumor and normal tissues, were performed by one pathologist. If there were questionable or missing data, the analysis was repeated. This study was approved by the Institutional Review Board of Korea University Anam Hospital (IRB No AN10050-002).

Methods

Using a microtome, 10 μm slices of formalin-fixed, paraffin-embedded tissue were obtained and placed in 1.5 mL microcentrifuge tubes. To isolate RNA, 0.75 mL TRI Reagent® (Applied Biosystems, Foster City, USA) was added, followed by incubation at room temperature for 5 minutes. After addition of 0.2 mL chloroform and thorough mixing, the sample was centrifuged for 10 minutes at 14,000 rpm and the clear supernatant was transferred into new tubes. After adding 0.5 mL isopropanol and mixing well, the samples were stored for 2 hours at -20°C and centrifuged for 15 minutes (14,000 rpm) at 4°C. The supernatant was removed without disturbing the pellet, and 1 mL 70% ice-cold ethanol was added to the pellet and re-centrifuged, again discarding the supernatant. The final pellet was dissolved in 50 μL DEPC-DW and RNA yield was determined using a UV spectrophotometer. Amounts of 0.5-1 μg were used in a reverse transcriptase reaction. After reverse transcription, a TaqMan® MicroRNA Assay (Applied Biosystems) was performed according to the manufacturer's protocol to detect miR-21 expression using Applied Biosystems using miR-21 as a primer (Assay ID: 000397) and small nRNA, RNU6 (Assay ID: 001093) as a control.

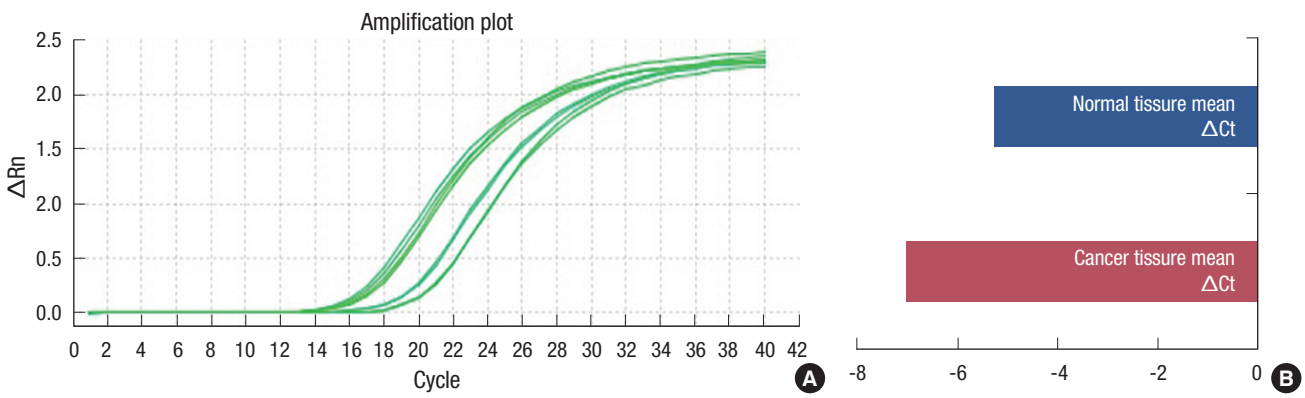

Figure 1. (A) Amplification plots with ΔCt values of miR-21 in breast cancer and normal tissues, (B) comparison of mean ΔCt of miR-21 between breast cancer tissue (-7.044 ± 1.26) and normal tissue (-5.92 ± 7.19, $p < 0.0001$).

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Expression of miR-21 was analyzed by relative quantity (RQ) using the equation $RQ = 2^{-}\Delta\Delta^{CT}$ (CT = threshold cycle to detect fluorescence). According to the definition of CT, lower value is consistent with faster detection of fluorescent which is due to higher expression of miR-21as shown in Figure 1A. $\Delta\Delta C_T$ is defined as the difference in miR-21 expression between tumor and normal tissue [$\Delta\Delta C_T = (C_{TmiRNA\text{-}21}\text{-}C_{TU6\ RNA})_{tumor}\text{-}(C_{TmiRNA\text{-}21}\text{-}C_{TU6\ RNA})_{normal}$]. Each analysis was performed in duplicate by StepOne™ Software v2.1 (Applied Biosystems, Foster City, USA) and samples with $\Delta C_T > 30$ were excluded for accuracy.

Statistical analysis

The relationship between miR-21 expression level and clinicopathologic features of the patients was analyzed using the chi-square test. Prognostic factors for breast cancer were examined by Cox proportional hazard regression model and survival curves were estimated by the Kaplan-Meier method. Statistical analysis was performed using SPSS version 13.0 software (SPSS Inc., Chicago, USA) and p-value < 0.05 were considered significant.

RESULTS

Patient characteristics

A total of 109 patients were analyzed in this study. The mean age was 48 years and 72 patients (66.1%) were under 50 years old. Sixty-eight patients (62.4%) had tumor size smaller than 2 cm, and 62 (56.9%) had no lymph node metastasis. When classified into breast cancer subtypes, 60 patients (55.5%) were luminal A, 14 (13%) were luminal B, 27 (25%) were HER2+, and seven (6.5%) were TNBC. The mean RQ was 5.92 ± 7.19, and 78 (71.6%) patients were classified as low miR-21 (≤ 5.92) and 31 (28.4%) as high miR-21 (> 5.92). Local recurrence o ccurred in 2 patients, regional recurrence in 1 patient, distant metastasis in 17 patients and six patients died. Patients' characteristics are outlined in Table 1. The mean overall and disease-free survival times were 54 and 36 months, respectively.

Table 1. Patient characteristics

Characteristics	No. of patients (%)
All patients	109 (100.0)
Age (yr)	
≤ 50	72 (66.1)
> 50	37 (33.9)
Operation method	
Total mastectomy	61 (56.0)
Breast conserving surgery	48 (44.0)
Tumor size (cm)	
≤ 2	68 (62.4)
> 2	41 (37.6)
Lymph node status	
Negative	62 (56.9)
Positive	47 (43.1)
Stage (TNM)	
I	45 (41.3)
II	42 (38.5)
III	22 (20.2)
Histologic grade	
1	25 (23.0)
2	42 (38.5)
3	42 (38.5)
Estrogen receptor status	
Negative	36 (33.0)
Positive	73 (67.0)
Progesterone receptor status	
Negative	44 (40.4)
Positive	65 (59.6)
HER2/neu expression	
Negative	67 (62.0)
Positive	41 (38.0)
Breast cancer subtype	
Luminal A	60 (55.5)
Luminal B	14 (13.0)
HER2+	27 (25.0)
TNBC	7 (6.5)
Ki-67 expression	
< 20%	37 (33.9)
≥ 20%	72 (66.1)
p53	
Negative	69 (63.3)
Positive	40 (36.7)
miR-21 RQ	
Low (≤ 5.92)	78 (71.6)
High (> 5.92)	31 (28.4)
Recurrence	
No	89 (81.7)
Yes	20 (18.3)
Death	
No	103 (94.5)
Yes	6 (5.5)

HER2 = human epidermal growth factor receptor 2; TNBC = triple negative breast cancer; RQ = relative quantity.

MiR-21 expression in breast cancer

We analyzed the miR-21 expression level in tumor and normal tissues acquired from the same breast cancer patients. The mean miR-21 expression level (ΔC_T) was -7.044 ± 1.26 for tumor tissue and -5.32 ± 1.34 for normal breast tissue; this difference was statistically significant with a p-value of < 0.0001 (Figure 1B).

MiR-21 expression and clinicopathologic features of breast cancer patients

Table 2 shows the correlation between clinicopathologic features and miR-21 expression. High miR-21 expression was related to mastectomy ($p = 0.005$) and tumor size > 2 cm ($p =$

Table 2. Correlation between miR-21expression and clinicopathologic factors

Characteristics	miR-21 RQ low (≤5.92) No. (%)	miR-21 RQ high (>5.92) No. (%)	*p*-value
Age (yr)			0.831
≤50	52 (66.7)	20 (64.5)	
>50	26 (33.3)	11 (35.5)	
Operation method			0.005
Total mastectomy	37 (47.4)	24 (77.4)	
Breast conserving surgery	41 (52.6)	7 (22.6)	
Tumor size (cm)			0.047
≤2	53 (67.9)	15 (48.4)	
>2	25 (32.1)	16 (51.6)	
Lymph node status			0.121
Negative	48 (61.5)	14 (45.2)	
Positive	30 (38.5)	17 (54.8)	
Stage			0.008
I	39 (50.0)	6 (19.4)	
II	26 (33.3)	16 (51.6)	
III	13 (16.7)	9 (29.0)	
Histologic grade			0.005
1	22 (28.2)	3 (9.6)	
2	32 (41.0)	10 (32.3)	
3	24 (30.8)	18 (58.1)	
Estrogen receptor status			0.002
Negative	19 (24.4)	17 (54.8)	
Positive	59 (75.6)	14 (45.2)	
Progesterone receptor status			0.053
Negative	27 (34.6)	17 (54.8)	
Positive	51 (65.4)	14 (45.2)	
HER2/neu expression			0.002
Negative	55 (71.4)	12 (38.7)	
Positive	22 (28.6)	19 (61.3)	
Breast cancer subtype			0.005
Luminal A	50 (64.9)	10 (32.3)	
Luminal B	9 (11.7)	5 (16.1)	
HER2+	13 (16.9)	14 (45.2)	
TNBC	5 (6.5)	2 (6.4)	
Ki-67 expression			0.044
<20%	31 (39.7)	6 (19.4)	
≥20%	47 (60.3)	25 (80.6)	
p53			0.784
Negative	50 (64.1)	19 (61.3)	
Positive	28 (35.9)	12 (38.7)	
Recurrence			0.531
No	64 (82.1)	25 (80.6)	
Yes	14 (17.9)	6 (19.4)	
Death			0.034
No	76 (97.4)	27 (87.1)	
Yes	2 (2.6)	4 (12.9)	

RQ=relative quantity; HER2=human epidermal growth factor receptor 2; TNBC=triple negative breast cancer.

0.047). Moreover, higher stage, higher grade, ER negativity, HER2 positivity, and high Ki-67 expression were significantly associated with high miR-21 expression. As hormone receptor (HR) status and HER2 expression is related to miR-21 expression level there was a substantially larger proportion of the HER2+ subtype (45.2%) in the high miR-21 expression group. However, no relationship was found between miRNA-21 expression and lymph node metastasis status and recurrence. High miR-21 expression level A was associated with a high death rate ($p=0.034$).

Table 3. Univariate analysis of prognostic factors associated with overall survival

Features	Univariate		
	HR	95% CI of HR	*p*-value
Age (yr)			
≤50 vs. >50	0.980	0.179-5.349	0.981
Size (cm)			
≤2 vs. >2	8.653	1.011-74.070	0.049
Lymph node status			
Negative vs. positive	1.312	0.265-6.503	0.738
Stage			
I and II vs. III	4.091	0.826-20.272	0.084
Histologic grade			
1 vs. 2 and 3	3.482	0.010-9.969	0.175
Estrogen receptor status			
Negative vs. positive	0.092	0.011-0.789	0.03
Progesterone receptor status			
Negative vs. positive	0.323	0.059-1.765	0.192
HER2/neu expression			
Negative vs. positive	1.824	0.151-4.497	0.822
Breast cancer subtype			
Luminal A vs. Luminal B	2.233	0.202-24.624	0.512
Luminal A vs. HER2	1.131	0.103-12.471	0.920
Luminal A vs. TNBC	10.298	1.445-73.393	0.02
Ki-67 expression			
Negative vs. positive	2.573	0.301-22.021	0.388
p53			
Negative vs. positive	1.718	0.347-8.512	0.507
miR-21 RQ			
Low vs. high	5.316	0.973-29.026	0.054

CI=confidence interval; HR=hormone receptor; HER2=human epidermal growth factor receptor 2; TNBC=triple negative breast cancer; RQ=relative quantity.

Table 4. Multivariate analysis of prognostic factors associated with overall survival

Features	Multivariate		
	HR	95% CI of HR	*p*-value
Size (cm)			
≤2 vs. >2			0.37
Estrogen receptor status			
Negative vs. positive	0.004	0.000-0.084	<0.001
miR-21 RQ			
Low vs. high	14.214	1.338-15.096	0.028

CI=confidence interval; HR=hormone receptor; RQ=relative quantity.

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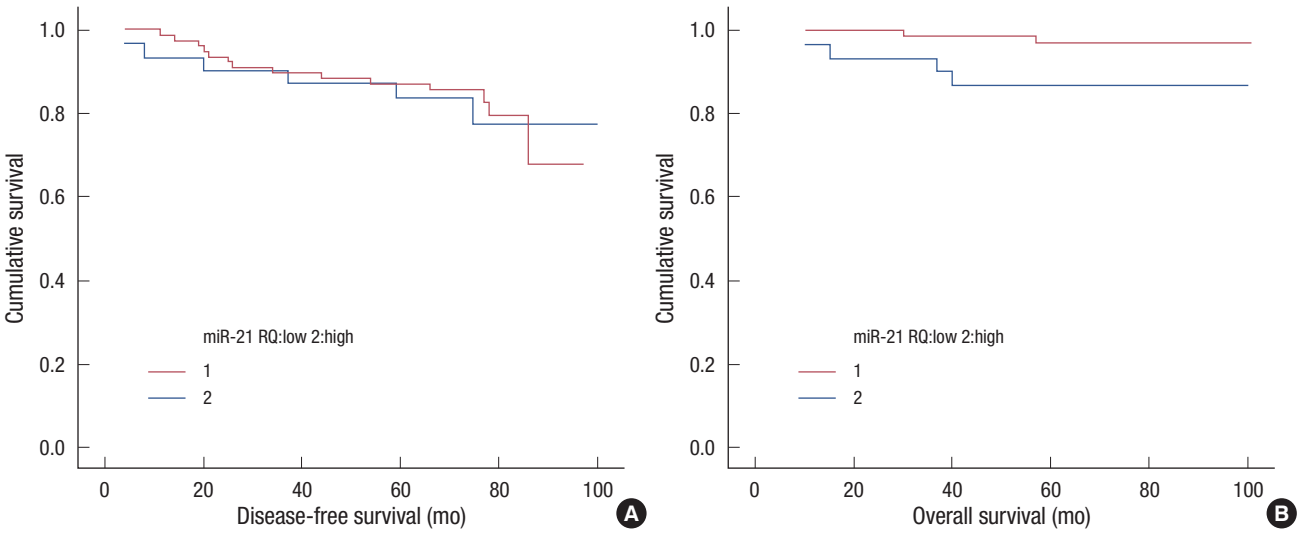

Figure 2. Disease-free survival ($p=0.862$) (A) and overall survival ($p=0.031$) (B) according to miR-21 relative quantity (RQ).

MiR-21 expression and prognosis and survival of breast cancer

On univariate analysis, factors associated with prognosis were tumor size ($p=0.049$) and ER status ($p=0.03$) (Table 3). For multivariate analysis, significant factors from univariate analysis and miR-21 expression were included. The prognostic significance of miR-21 expression could not be ignored since the *p*-value for high expression level was only slightly over 0.05 and the hazard ratio was 5.316. Multivariate analysis showed that high miR-21 expression and negative ER status were significantly related to poor prognosis (Table 4). Lymph node metastasis status, HER2 status, and proliferation rate (Ki-67 expression) were not associated with prognosis. On survival analysis, there were no significant differences in disease-free survival between low and high miR-21 expression level; however, overall survival was lower in the latter (Figure 2).

DISCUSSION

The potential of miRNAs as novel biomarkers is growing as more studies report the relationship between miRNAs and cancers [5-7,15-17]: however, interest in miRNA begun only a few years ago and there are not many reports on miR-21 in breast cancers. Our study shows that miR-21 expression is significantly increased in breast cancer tissue and that higher expression is related to aggressive tumor characteristics. Moreover, breast cancer patients with high miR-21 expression seem to have a poor prognosis.

Consistent with our results, previous researchers have found that miR-21 is frequently overexpressed in breast cancer [8-11]. This is explained by its function as an oncogene, inhibiting tumor suppressor genes. By array expression analysis of MCF-cells depleted of miR-21, Frankel et al. [12] reported a link between miR-21 and p53 tumor suppressor protein. Furthermore, their study showed that PDCD4 is directly regulated by miR-21. PDCD4 is a tumor suppressor gene that is downregulated in numerous human cancers and is known to function in the regulation of apoptosis [18]. Zhu et al. [13] searched for targets of miR-21 and found that TPM1 contains a putative miR-21 binding site and that regulation occurs at the translational level. They also demonstrated that overexpression of TPM1 suppresses cell growth *in vitro* [13]. Although the exact role of PTEN in breast cancer has not been elucidated, it has been found to be associated with tumor progression, acting as a tumor suppressor gene. Moreover, Huang et al. [11] proposed PTEN as a target gene of miRNA-21, suggesting that its expression is reduced by miR-21 in breast cancer.

We confirmed the clinical importance of miR-21 expression by showing its association with unfavorable clinicopathologic features. Higher expression of miR-21 leads to increased inhibition of tumor suppressor genes, resulting in increased tumor cell proliferation and inhibition of apoptosis; this explains the results of our study including its correlation with the proliferation marker Ki-67. However, axillary lymph node metastasis, which is one of the most important prognostic factors for breast cancer and recurrence, was not related to high miR-21 expression. This might be explained by the small population of this study. Even though there was no statistical significance, the proportion of patients with high miR-21 expression was 54.8% in those who had axillary lymph node metastasis which was higher than those who did not have metastasis (38.5%). If more patients were included, significant results might have been obtained. Furthermore, since invasion and metastasis including

axillary metastasis could not be explained by one mechanism but by regulation of interconnected molecular pathways or other undiscovered factors, this is just the beginning of the search for the role of miR-21. In a similar study by Yan et al. [10] with 113 patients, only stage and lymph node status were related to high levels of miR-21. They explained this result by suggesting that up-regulation of miR-21 developed during tumor progression and acquisition of metastatic potential. On the other hand, in a larger study of 344 patients, Qian et al. [9] analyzed miR-21 expression in fresh frozen tissues and did not find a relationship with clinicopathologic features other than grade, histologic type, and ER status.

HR and HER2 status plays an important role in the management and current classification of breast cancers [19-21]. There are numerous studies on molecular breast cancer subtypes, which are clinically divided into luminal A, luminal B, HER2+, and TNBC. HER2+ and TNBC types are reported to have poor prognosis due to the lack of targeting therapy and the biology of the tumor itself [22,23]. In this study it is likely that poor prognosis was related to HR negativity, which is linked to high miR-21 expression. Moreover, the high miR-21 group contained more patients with positive HER2 expression, which is known to have aggressive biology even though there is a targeting treatment, suggesting that patients with high miR-21 expression will have a poorer outcome. However, there are no consistent reports and no known mechanisms for these relationships and one can only propose that miR-21 might affect related pathways regulating ER and HER2 status or a common mRNA target. To clarify these mechanisms, further research seems to be necessary. Because of the importance of molecular profiling and identification of intrinsic subtypes, Lowery et al. [24] performed a study using artificial neural networks and concluded that miRNA could not only predict ER, PR, and HER2 status, but showed a superior accuracy over mRNA signatures in classifying subtypes.

Currently, mRNA molecular profiling of breast cancer is used commercially but faces problems regarding the accuracy of classical computational analysis, interference from inherent noise, the need for fresh frozen tissues, and overseas diagnosis, and for these reasons researchers are trying to find a universal, reproducible, and effective test to predict outcome and therapeutic response [17,24]. The ability of miR-21 expression to predict prognosis and its relationship to survival shown in our study and many other articles suggest its potential as a novel biomarker [3,4,16]. Although there were no significant difference between two groups regarding disease-free survival, patients with low miR-21 expression had better overall survival. This could reflect that disease progression after recurrence is worse in patients with high miR-21 expression, which may lead to other studies with new hypothesis. Numerous studies have demonstrated that the stability of miRNAs in formalin-fixed paraffin embedded tissues is superior to that of mRNAs and data regarding the feasibility and reliability of miRNAs as markers in other body fluids are emerging [16,25-29]. In addition to this, Si et al. [8] showed *in vitro* inhibition of breast tumor cell growth by anti-miR-21 and inhibition of tumor growth in a xenograft carcinoma mouse model, suggesting the possibility of therapeutic use. Furthermore, Calin and Croce [6] proposed a theory that if miRNAs are inherited in the germline, abnormal levels of expression of tumor-suppressor genes or oncogenes will have an effect on cancer development.

This study has several limitations. Although patient selection was consecutive from 2002 to 2004, lack of materials resulted in a small population for analysis and selection bias could have occurred. Because of the small population and short follow-up period, the number of deaths was small, which could have influenced the analysis. In addition, there is currently no international standardized definition of methods of analysis and expression levels for miRNAs and some studies use mean value of RQ to define high and low expression while other studies have different definitions for grouping the expression level. This discordant analysis can lead to disagreement among studies.

In conclusion, our study shows that miR-21 is overexpressed in breast cancer and high expression shows association with poor prognostic factors and poor survival. These findings suggest miR-21 to have profound potential as a marker to predict outcome, treatment response and as a method to treat breast cancer. To clarify the role of miR-21 and for its use as a biomarker and in targeting therapy, large worldwide population-based studies with a standardized definition of miR-21 expression level are necessary. Moreover, researches relating miR-21 with stem cells and heredity will bring advances of understanding, treating and preventing breast cancers.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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