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The Significance of MicroRNA Let-7b, miR-30c, and miR-200c Expression in Breast Cancers

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Background: MicroRNA (miRNA) is a class of noncoding protein RNA as a promising biomarker for various diseases. In this study, the expression of let-7b, miR-30c, and miR-200c was studied in breast cancer tissues to evaluate the potential relationship with known clinicopathological parameters. **Methods:** Quantitative real-time polymerase chain reaction was performed to determine the expression level of three miRNAs in 37 pairs of noncancerous normal and cancer tissues and an additional 38 cancer tissues from patients with invasive ductal carcinoma. **Results:** miR-200c expression was higher in cancer tissues compared to noncancerous normal tissues, and its ratio was correlated with patient age at surgery, type of surgery, and Ki-67 expression. The expression level of let-7b in cancer tissues was inversely correlated with lymph node metastasis, histological grade, and Ki-67 expression but positively correlated with estrogen and progesterone receptor expression. miR-200c expression level was positively correlated with Her-2 expression. The miR-30c expression level in breast cancer was not correlated with any parameters. **Conclusions:** miR-200c and let-7b could be used as biomarkers in patients with breast cancer, but its pathological mechanism should be determined.

Key Words: Breast neoplasms; MicroRNAs; miR-200c; Let-7b; miR-30c

MicroRNA (miRNA) are small non-coding RNA molecules (19-25 nucleotides in length) that are evolutionary conserved and bind to the 3´-untranslated region of target mRNAs with sequence homology, resulting in mRNA degradation or suppression of their translation.[1] Lin-4 was the first miRNA identified from the nematode *Caenorhabditis elegans* in 1993 as a regulator of developmental processes.[2] According to miRBase release 16 (September 2010), more than 1,000 human miRNAs have been identified. It is estimated that 1-4% of genes in the human genome encode miRNAs.[3] Because of the mechanism of partial sequence homology between each miRNA and the target mRNA, one miRNA can regulate multiple targets and vice versa.[4] Using computational analysis, it is expected that over one-third of all human protein-coding gene expression is regulated by miRNAs,[5] and miRNAs are involved in the regulation of key cellular process including development, differentiation, cell proliferation, apoptosis, fat metabolism, angiogenesis, and inflammation.[6] Altered miRNA expression is likely to contribute to human disease, including cancer. In fact, certain groups of miRNAs expressed in solid cancers are very different from those of normal cells, and the predicted targets for differentially expressed miRNAs may be tumor suppressors or oncogenes.[7] Additionally, cancer metastasis may be promoted by overexpression of prometastatic miRNAs or downregulation of antimetastatic miRNAs.[8]

In recent years, miRNA profiling studies have been conducted on breast cancers, and some miRNAs acting as tumor suppressors or oncogenes have been identified.[9] Several miRNAs, including miR-206, miR-17-5p, miR-200, miR-34a, miR-31, miR-125a, b and let-7, miR-21, miR-155, miR-10b, and miR-373/520c play specific roles in breast cancers.[10] Among these, the role of miR-200 is controversial. One study reported that a decrease in the miR-200 family was associated with highly aggressive, metaplastic breast cancer,[11] and another study reported that miR-200 expression in mouse breast cancer cell lines enhances metastasis.[12] Another study showed that the region on chromosome 1 encoding the miR-200 cluster is amplified in breast cancer.[13] Although the breast cancer mechanism is not well understood, the miR-200 family seems to be important for regulating tumor progression and metastasis of breast cancer. The miRNA let-7 is poorly expressed or deleted in many human cancers. Enhanced let-7 expression in breast cancer cells

leads to decreased HMGA2 expression, a chromatin-remodeling protein that activates proinvasive and prometastatic genes.[14] miR-30c is the first biliary-specific hepatic miRNA.[15] Its high expression is associated with the benefits of tamoxifen treatment and with longer progression-free-survival,[16] suggesting an independent predictor in patients with breast cancer.

In the present study, we investigated the expression levels of let-7b, miR-30c, and miR-200c in breast cancer using quantitative real-time polymerase chain reaction (PCR) techniques and evaluated their expression with known biological predictors such as histological grade, tumor size, lymph node metastasis, clinical stage, patient age, survival, recurrence, and expression of the estrogen receptor (ER), progesterone receptor (PR), Her-2, and Ki-67.

MATERIALS AND METHODS

Materials

In total, 112 formalin-fixed paraffin embedded (FFPE) samples consisting of 37 pairs of cancer and noncancerous normal breast tissues from patients with breast cancer, and 38 breast cancer tissues were reviewed. All patients were operated on with a diagnosis of clinical stage I-III invasive ductal carcinoma between January 2002 and December 2004 at Korea University Anam Hospital. The patients were treated with either breast conserving surgery or a total mastectomy with or without an axillary lymph node dissection. Clinical stage was classified according to the American Joint Committee on Cancer classification system. Histological grade was evaluated using the modified Bloom and Richardson criteria. The mean age of the patients with breast cancer at surgery was 49.4 years (range, 32 to 75 years), and age 50 was used as a cut-off criterion for categorizing patients with breast cancer. The cut-off value for tumor size was 2 cm, and lymph node status was defined as positive if there was metastasis. ER and PR status was assessed using immunohistochemical staining and considered positive if the nuclear staining value for each hormone receptor was >10%. For Her-2, 0 and 1 were considered negative and 3 as positive. Breast cancers were divided into four subtypes according to ER, PR, and Her-2 status as follows: luminal A (ER+ and/or PR+, HER-2-), luminal B (ER+ and/or PR+, Her-2+), Her-2+ (ER-, PR-, Her-2+), and triple negative (ER-, PR-, Her-2-). The cut-off value for Ki-67 expression was 20%. Pathological analyses, including the identification of tumor and normal tissues, were performed by one pathologist. This study was approved by the Institutional Review Board of Korea University Hospital.

Reagents

All chemicals for de-paraffinization, such as xylene and alcohol, were supplied by Sigma (St. Louis, MO, USA). The Tri-reagent solution to purify total RNA containing miRNAs was purchased from Invitrogen (Carlsbad, CA, USA). The Taqman® microRNA reverse transcription kit, Taqman Universal M. Mix II, and the Taqman miRNA assay kit for reverse transcription and quantitative real-time PCR were obtained from Applied Biosystems (Foster City, CA, USA).

RNA purification from FFPE

Five sections of 10 μm thickness and approximately 100 mm^2 surface area from the FFPE samples were used for total RNA extraction as follows. Deparaffinization was performed by incubating the sections in xylene three times for 10 minutes, and then the sections were placed in absolute ethanol for three washes of 10 minutes each. After air drying for 5 minutes, the tissue sections were completely lysed with 0.8 mL Tri-reagent, followed by a 10 minutes incubation at room temperature. Chloroform (0.2 mL) was added, and the samples were mixed by vortexing followed by a 15 minute centrifugation at 14,000 rpm. The aqueous phase was transferred to a new 1.5 mL tube, followed by ethanol precipitation. After suspending the pellet in 20 μL of RNase-free deionized water, the quality and quantity of isolated total RNA were measured using a NanoDrop ND-1000 (Thermo Fisher Scientific, Rockford, IL, USA) spectrophotometer. Purified total RNA samples were stored at -80℃ before use.

Reverse transcription (RT) reaction

Total RNA (100 ng) was used for cDNA synthesis in 20 μL of total reaction volume using the Taqman® microRNA reverse transcription kit with specifically designed stem-loop RT primers for each mature miRNA in a multiplexing fashion. Briefly, a reaction mixture containing 2 μL 10× buffer, 0.4 μL dNTP mix (each 100 mM), 2 μL MultiScribe RTase, 0.25 μL RNase inhibitor, and the RNA sample were incubated for 30 minutes at 16℃, 30 minutes at 37℃, and 5 minutes at 85℃ using a mix of RT primers specific for let-7b, miR-30c, miR-200c, and U6 small nuclear RNA (snU6) in each 12.5 nM final concen-

tration and then stored at 4°C. Each reverse transcription product was diluted with an additional 80 μL of distilled water.

Quantitative real-time PCR

Real-time PCR was performed in a duplicated format with the Taqman Universal Mix II and Taqman miRNA assay using the StepOne™ Real-Time PCR System (Applied Biosystems) to quantify each miRNA (let-7b, miR-30c, miR-200c) and snU6 RNA. The PCR reaction was performed in 10 μL aliquots with 5 μL Taqman Universal Mix II, 0.5 μL Taqman miRNA for each 2 μL of miRNA. A 10 minute incubation at 95°C, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C was conducted. snU6 was used as the endogenous control to normalize expression levels. The relative expression level of each miRNA was measured using the ΔCt method and the following formula: $\Delta Ct = Ct_{(miRNA)} - Ct_{(snU6)}$, where Ct represents the threshold cycle number. The fold-change in the miRNA expression levels between normal and the tumor tissue pair or non small cell lung cancer cells was compared using the ΔΔCt method, $2^{\wedge\Delta([Ct(miRNA)-Ct(U6)]Tumor-[Ct(miRNA)-Ct(U6)]Normal)}$.

Statistical analyses

All statistical analyses were performed using SPSS ver. 14.0 (SPSS Inc., Chicago, IL, USA). Differences between the variables examined were analyzed using a t-test and one way analysis of variance. Pearson's correlation coefficient was used to evaluate the correlations between two variables. A p-value < 0.05 was considered statistically significant.

RESULTS

Expression of the miRNAs in noncancerous and cancerous tissues

The average standard deviations of each Ct value between the duplication data for snU6, let-7b, miR-30c, and miR-200c were 0.29 (0.04-0.51), 0.34 (0.09-0.54), 0.31 (0.04-0.49), and 0.22 (0.03-0.41), respectively. The expression levels of let-7b, miR-30c, and miR-200c were measured using quantitative real-time RT-PCR in 37 pairs of noncancerous and cancerous tissues. The miR-200c expression level was higher in cancerous tissues (1.07 ± 0.98) than that in noncancerous normal tissues (0.56 ± 0.80) (p = 0.019) (Fig. 1). However, no differences in let-7 and miR-30c expression were observed between cancerous and noncancerous tissues.

Expression ratio of the miRNAs in cancerous and noncancerous tissues (T/N ratio)

The expression level of each miRNA in cancer tissues was compared to paired normal tissue (T/N ratio), and classified into three groups: over-expression (≥ 2), intermediate (0.6-2), and under-expression (≤ 0.6). Let-7b expression in cancer tissues was under-expressed in 45.9%, whereas miR-200c was over-expressed in 48.6% compared to that in noncancerous normal tissue (Table 1). The T/N ratio of miR-200c expression was correlated with patient age at surgery (> 50 years), type of surgery (breast conserving surgery), and Ki-67 expression (> 20%) with Pearson's correlation coefficients of 0.348 (p = 0.035), -0.325 (p = 0.050), and 0.354 (p = 0.032), respectively. However, no

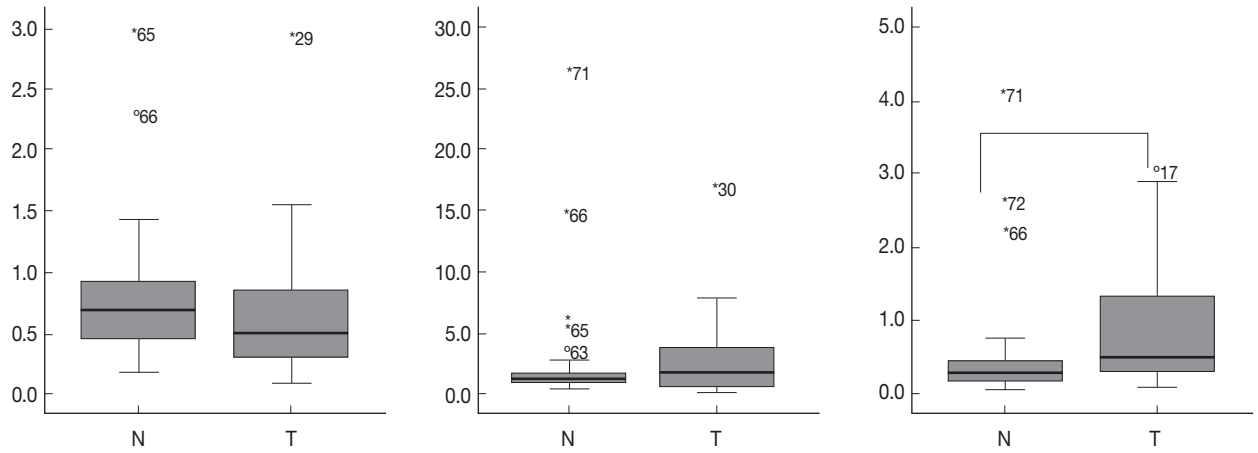

Fig. 1. Comparison of microRNAs expression between noncancerous normal tissue (N) and cancer (T) tissue of breast cancers.

Table 1. The T/N ratio of let-7b, miR-30c, and miR-200c expression in cancer and noncancerous normal tissues

T/N ratio	Let-7b	miR-30c	miR-200c
Over-expression (≥2)	5 (13.5)	4 (10.8)	18 (48.6)
Intermediate (0.6-2)	15 (40.5)	26 (70.3)	17 (45.9)
Under-expression (≤0.6)	17 (45.9)	7 (18.9)	2 (5.4)

Values are presented as number (%).

correlation in the T/N ratio of let-7b and miR-30c was observed with any clinicopathological parameter (Table 2).

Correlation of miRNA expression in cancer tissue with clinicopathological parameters

The expression levels of miR-200c, let-7b, and miR-30c were normalized to the snU6 RNA expression level, and the correlation with patient age; type of surgery; tumor size; histological grade; clinical stage; lymph node metastasis; expression of ER, PR, and Her-2; recurrence; and patient survival was conducted. Let-7b expression was inversely correlated with lymph node metastasis ($p=0.043$), histological grade ($p=0.002$), and Ki-67 expression ($p=0.014$), whereas it was positively correlated with ER ($p=0.038$) and PR ($p=0.005$) expression. The miR-200c expression level was positively correlated with Her-2 expression ($p=0.029$). No correlations were observed between miR-30c expression and the clinicopathological parameters (Table 3).

DISCUSSION

Abnormal expression of several miRNA plays an important role in breast carcinogenesis by regulating the expression of their target genes, including tumor suppressor genes and other tumor related genes, such as those for the cell cycle, development, differentiation, and apoptosis. Altered miRNA expression levels have been associated not only with tumorigenesis but also the occurrence of metastasis and a poor prognosis.[17] The let-7 family is one of the first identified mammalian miRNAs and known to be highly expressed during differentiation and in mature tissue but significantly under-expressed during the embryonic stage.[18] A recent study showed that the Raf kinase inhibitory protein inhibits the invasion of metastatic breast cancer cells and represses tumor cell intravasation and bone metastasis in a mouse model through mitogen-activated protein kinase (MAPK), G-protein coupled receptor kinase-2, and nuclear factor-κB signaling cascades.[14] Inhibiting MAPK decreases LIN28 transcription, an inhibitor of let-7 biogenesis, by reg-

Table 2. Correlation between T/N ratio of miRNAs and clinicopathologic parameters in breast cancer patients

Parameters	T/N ratio			
	No. of patients	Let-7b	miR-30c	miR-200c
Age (yr)				
≤50	28	0.952	1.414	**2.377**
>50	9	1.067	2.073	**4.944**
p-value		0.682	0.288	**0.035**
Surgery type				
Total mastectomy	21	0.879	1.287	**3.900**
BCS	26	1.113	1.952	**1.823**
p-value		0.333	0.216	0.050
Tumor size (cm)				
≤2	25	1.024	1.703	2.305
2< and ≤5	10	0.913	1.255	4.917
>5	2	0.780	1.560	2.160
p-value		0.572	0.577	0.171
Lymph node metastasis				
0	22	1.063	1.421	3.436
1-3	11	0.850	1.940	2.524
4-9	3	0.697	1.460	2.270
≥10	1	1.450	1.260	0.940
p-value		0.609	0.750	0.289
Stage				
I	16	1.062	1.528	2.592
II	16	0.954	1.665	3.794
III	5	0.820	2.097	4.213
p-value		0.479	0.998	0.972
Histologoic grade				
1	9	1.238	1.287	2.642
2	16	0.973	1.998	2.640
3	12	0.798	1.224	3.758
p-value		0.174	0.829	0.408
ER expression				
No expression	17	0.845	1.668	3.422
Expression	20	1.096	1.495	2.647
p-value		0.299	0.748	0.472
PR expression				
No expression	18	0.929	1.683	3.374
Expression	19	1.029	1.471	2.651
p-value		0.677	0.694	0.501
Her-2 expression				
0-1+	22	0.949	1.323	2.381
3+	15	1.027	1.942	3.915
p-value		0.749	0.254	0.156
Breast cancer type				
Luminal A & B	20	1.096	1.495	2.647
Her-2 & TNBC	17	0.845	1.668	3.422
p-value		0.299	0.748	0.472
Ki-67 expression (%)				
<20	15	1.177	1.751	**1.647**
≥20	22	0.846	1.453	**3.928**
p-value		0.174	0.585	**0.032**
Recurrence				
No	33	0.997	1.589	3.061
Yes	4	0.848	1.453	2.525
p-value		0.702	0.875	0.757
Death				
No	35	0.992	1.575	3.051
Yes	2	0.780	1.560	2.160
p-value		0.692	0.990	0.708

miRNA, micro RNA; BCS, breast conserving surgery; ER, estrogen receptor; PR, progesterone receptor; TNBC, triple-negative breast cancer.

Table 3. Correlation between expression of miRNAs and clinicopathologic parameters in breast cancer patients

Parameters	Expression level normalized to snU6			
	No. of patients	Let-7b	miR-30c	miR-200c
Age (yr)				
≤50	49	0.549	2.176	0.836
>50	26	0.527	1.849	0.791
p-value		0.805	0.509	0.787
Surgery type				
Total mastectomy	41	0.470	1.982	0.934
BCS	34	0.628	2.160	0.683
p-value		0.070	0.707	0.106
Tumor size (cm)				
≤2	49	0.539	2.053	0.748
2< and ≤5	22	0.569	2.143	0.940
>5	4	0.428	1.735	1.050
p-value		0.872	0.936	0.192
Lymph node metastasis				
No	54	**0.586**	2.124	0.891
Yes	21	**0.426**	1.904	0.637
p-value		**0.043**	0.406	0.163
Stage				
I	34	0.579	2.074	0.814
II	31	0.569	2.188	0.859
III	10	0.331	1.632	0.719
p-value		0.140	0.702	0.843
Histologic grade				
1	19	**0.674**	1.913	0.920
2	26	**0.646**	2.477	0.782
3	30	**0.367**	1.798	0.790
p-value		**0.002**	0.718	0.545
ER expression				
No expression	31	**0.435**	2.037	0.864
Expression	44	**0.617**	2.080	0.790
p-value		**0.038**	0.929	0.641
PR expression				
No expression	35	**0.413**	1.893	0.844
Expression	40	**0.654**	2.211	0.800
p-value		**0.005**	0.694	0.501
Her-2 expression				
0-1+	47	0.778	1.782	**0.691**
3+	28	0.526	2.534	**1.038**
p-value		0.792	0.120	**0.029**
Breast cancer type				
Luminal A & B	44	**0.617**	2.080	0.790
Her-2 & TNBC	31	**0.435**	2.037	0.864
p-value		**0.038**	0.929	0.641
Ki-67 expression (%)				
<20	20	**0.717**	2.196	0.943
≥20	55	**0.478**	2.014	0.775
p-value		**0.014**	0.733	0.341
Recurrence				
No	65	0.539	2.111	0.838
Yes	10	0.557	1.749	0.706
p-value		0.559	0.837	0.463
Death				
No	71	0.549	2.102	0.839
Yes	4	0.400	1.355	0.490
p-value		0.444	0.476	0.314

miRNA, micro RNA; BCS, breast conserving surgery; ER, estrogen receptor; PR, progesterone receptor; TNBC, triple-negative breast cancer.

ulating Myc expression. Enhanced let-7 expression in breast cancer cells decreases HMGA2 expression, a chromatin-remodeling protein that activates proinvasive and prometastatic genes, including Snail. Let-7 is considered a tumor suppressor gene that reduces cancer cell growth[19] and it is under-expressed in solid tumors such as ovarian and colorectal cancer.[20] Our study is the first report showing that let-7b expression in breast cancer tissue was inversely correlated with lymph node metastasis, histological grade, and the Ki-67 proliferation index, whereas it was positively correlated with ER and PR expression. Let-7b is one of the growth-inhibiting miRNAs in breast cancer. However, no correlation was found for the let-7b T/N ratio and any of the clinicopathological parameters. Because the noncancerous tissue was obtained from tissue adjacent to cancer, it may not represent true normal tissue and some genetic changes may have occurred. According to Johnson *et al.*,[19] the deregulated expression of let-7 in human cancer cells as an initial mechanism of tumor development is found by identifying its main target, Ras. The miR-200 family is comprised of five members (miR-200a, b, c, miR-141, and miR-429), and is strongly associated with epithelial phenotype. No general consensus has been reached as to whether miR-200 is upregulated or downregulated in advanced or metastasizing cancers.[21] Hyun *et al.*[22] showed that the human miR-200 family promotes cell growth when it is transfected into several cancer cell lines and increases apoptosis in the brain and smaller body size in the miR-8 (homolog of human miR-200 family in Drosophila)-null fly compared with wild types. A recent study showed that expression of the miR-200 family decreases in the presence of Akt2, suggesting that breast cancer metastasis may be under the control of the Akt-miR-200-E-cadherin pathway.[23] Upregulation of the miR-200 family has been reported in human ovarian cancers and correlates with decreased patient survival.[24] Another study showed that five members of the miR-200 family are all upregulated in endometrial adenocarcinoma compared to normal endometrial tissues.[25] In contrast, *in vivo* and *in vitro* studies have shown that miR-200a downregulation in brain tumors promotes tumor growth by increasing cyclin D1 and beta-catenin levels.[26] It has also been shown that overexpression of miR-200a inhibits nasopharyngeal carcinoma cell growth, migration and invasion by targeting beta-catenin, and Smad interacting protein 1.[27] The role of the miR-200 family also remains unknown in breast cancer. Our present study on 37 pairs of cancerous and noncancerous tissues from patients with breast cancer showed that miR-200c was upregulated in cancer tissues compared to that in normal tissues, and expression was correlated with high Ki-67 ex-

pression. Based on our results and those of previous reports, the miR-200c of miR-200 family plays an important role in breast cancer progression.

miR-30c is a member of the miR-30 family, which consists of miR-30a, 30b, 30c, 30d, and 30e and is located on chromosomes 1, 6, and 8. Previous studies have shown that the miR-30 family is associated with the regulation of connective tissue growth factor,[28] kidney development by targeting transcription factor Xlim1/Lhx1,[29] and control of the epithelial-to-mesenchymal transition in primary cultures of human pancreatic epithelial cells by inhibiting vimentin.[30] The role of miR-30c in breast cancer has not been well studied. A recent report showed that miR-30c overexpression is associated with a clinical benefit from tamoxifen treatment and with longer progression-free-survival and has been suggested to be a good prognostic indicator.[16] However, our study showed that miR-30c expression in cancer tissues was not correlated with any clinicopathological parameters.

Our results showed that miR-200c was frequently upregulated in breast cancer tissues, and that let-7b expression was associated with good prognostic parameters, whereas miR-200c expression was associated with Her-2 expression, suggesting that let-7b and miR-200c play a specific role in the progression of breast cancer.

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