miR-206 Expression Is Down-regulated in Estrogen Receptor α–Positive Human Breast Cancer

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Abstract
Expression levels of estrogen receptor (ER) α govern estrogen-dependent growth, response to endocrine therapy, and prognosis in ERα-positive breast cancer. Multiple mechanisms involved in altering ERα gene expression in breast cancer have been identified, including ERα gene amplification as well as transcriptional silencing by DNA methylation of CpG islands within the ERα promoter and mutations within the open reading frame of ERα. However, expression levels of ERα in breast cancer tissues differ widely among patients, and frequently change during disease progression and in response to systemic therapies. Recent evidence has shown that microRNA mutations or misexpression correlate with various human cancers, and miR-206 is reported to decrease endogenous ERα mRNA and protein levels in human MCF-7 breast cancer cells via two specific target sites within the 3′-untranslated region of the human ERα transcript. In this study, we show for the first time that miR-206 expression is markedly decreased in ERα-positive human breast cancer tissues assayed by quantitative reverse transcription-PCR analysis. Moreover, we observe that miR-206 expression is inversely correlated with ERα but not ERβ mRNA expression in breast cancer tissues. Transfection experiments revealed that introduction of miR-206 into estrogen-dependent MCF-7 breast cancer cells inhibits cell growth in a dose- and time-dependent manner. Our results suggest that miR-206 could be a novel candidate for endocrine therapy that targets only ERα in breast cancer. [Cancer Res 2008;68(13):5004–8]

Introduction
Estrogen receptor (ER) α is essential for estrogen-dependent growth, and its level of expression is a crucial determinant of response to endocrine therapy and prognosis in ERα-positive breast cancer. There is no doubt that the more ERα present in the tumor cells, the greater the likelihood of a favorable response to endocrine therapy (1). However, little is known about how the expression of ERα in human breast cancer is regulated.

ERα acts as a ligand-activated oncogene product in breast tissues. Expression of the human ERα gene (ESR1) is controlled at the transcriptional level by seven different promoters used in a cell-specific manner (2). This complex transcriptional unit can undergo alternative splicing, which has been shown to generate shorter ERα isoforms (3). Multiple mechanisms involved in the silencing of ESR1 gene expression in breast cancer have been identified, including mutations within the open reading frame (3) and transcriptional silencing by DNA methylation of promoter-proximal CpG islands (4). It was recently reported that ERα gene amplification is frequent in breast cancer and results in ERα protein overexpression (5). However, breast cancer patients show a wide range of ERα expression levels, and the levels of expression in individual patients change during disease progression and in response to systemic therapies. Therefore, other mechanisms may also regulate ERα expression in breast cancer.

Recently, Adams and colleagues (6) reported that the microRNA miR-206 decreases endogenous ERα mRNA and protein levels in human MCF-7 breast cancer cells by acting through two specific miR-206 target sites within the 3′-untranslated region (UTR) of the human ERα transcript. MicroRNAs (miRNA) are a class naturally occurring small noncoding RNAs that control gene expression by targeting mRNAs for translational repression or cleavage (7). Primary miRNA transcripts are cleaved into 70- to 80-nucleotide precursor miRNA (pre-miRNA) hairpins by RNA Dicer into 19- to 25-nucleotide miRNA duplexes. One strand of each duplex is degraded, and the other strands become mature miRNAs, which, incorporated into the RNA-induced silencing complex, recognize sites in the 3′-UTR of the target mRNAs and cause translational repression or mRNA cleavage. miRNAs are a new player among gene regulation mechanisms, and their functions have not been fully explored but are known to include the regulation of cellular differentiation, proliferation, and apoptosis. Recent evidence has shown that miRNA mutations or misexpression are associated with various human cancers and indicates that miRNAs can function as tumor suppressors and oncogenes (8). MicroRNA expression profiling also revealed that miRNAs are differently expressed among molecular subtypes in breast cancer (9–11). Furthermore, recent studies have also shown that loss or gain of function of specific miRNAs, such as let-7 (12), miR-10b (13), miR-21 (14), and mir-17-5p (15), contributes to breast epithelial cellular transformation and tumorigenesis.

The present study was undertaken to assess the role of miR-206 in human breast cancer. We examined expression of miR-206 in ERα-positive and ERα-negative human breast cancer tissues, and found for the first time that miR-206 expression was markedly decreased in ERα-positive breast cancer. Growth inhibition of estrogen-dependent breast cancer cells transfected with miR-206 was also analyzed.

Materials and Methods
Breast cancer tissues and immunohistochemical analysis. Breast tumor specimens from female patients with invasive breast carcinoma, who were treated at Nagoya City University Hospital between 1996 and 2001, were included in this study. The study protocol was approved by the institutional review board and conformed to the guidelines of the 1975 Declaration of Helsinki. All patients had undergone surgical treatment for
primary breast cancer (either mastectomy or lumpectomy). The samples were chosen from the continuous series of invasive carcinoma tissues. ERα protein expression status was confirmed by immunohistochemistry (IHC) as follows. One 4-μm section of each submitted paraffin block was stained first with H&E to verify that an adequate number of invasive carcinoma cells were present and that the fixation quality was adequate for IHC analysis. Serial sections (4 μm) were prepared from selected blocks and float mounted on adhesive-coated glass slides, for staining with monoclonal mouse anti-human ERα antibody (1D5; DAKO) at 1:100 dilution as described previously (16). The DAKO Envision system (DAKO EnVision–labeled polymer, peroxidase) was used for detection. After the entire slide was evaluated by light microscopy, expression of ERα was scored by proportion and intensity, according to Allred’s procedure (17). In brief, the proportion score represented the estimated proportion of tumor cells staining positive was as follows: 0 (none), 1 (<1/100), 2 (1/100 to 1/10), 3 (1/10 to 1/3), 4 (1/3 to 2/3), and 5 (>2/3). Any brown nuclear staining in invasive breast epithelium counted toward the proportion score. The intensity score represented the average intensity of the positive cells was as follows: 0 (none), 1 (weak), 2 (intermediate), and 3 (strong). The proportion and intensity scores were then added to obtain a total score, which could range from 0 to 8. Among 383 cases from which paired frozen tissue samples were available, 203 cases with HER2-negative tumors were used in this study from 246 cases which HER2 status was known. Forty-five tumors with scores of 0 to 2 made up the ERα-negative group in this study, and 49 tumors with score 8 composed the ERα-positive group. HER2-positive tumors were excluded in this study (Table 1).

**Table 1. Clinicopathologic characteristics of patients and breast tumors**

<table>
<thead>
<tr>
<th></th>
<th>ERα-positive (%)</th>
<th>ERα-negative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of patients</td>
<td>49</td>
<td>45</td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>6 (12)</td>
<td>18 (40)</td>
</tr>
<tr>
<td>≥50</td>
<td>43 (88)</td>
<td>27 (60)</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤2</td>
<td>16 (33)</td>
<td>9 (20)</td>
</tr>
<tr>
<td>&gt;2</td>
<td>33 (67)</td>
<td>36 (80)</td>
</tr>
<tr>
<td>No. of positive lymph nodes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>29 (59)</td>
<td>30 (67)</td>
</tr>
<tr>
<td>1–3</td>
<td>13 (27)</td>
<td>11 (24)</td>
</tr>
<tr>
<td>&gt;3</td>
<td>7 (14)</td>
<td>4 (19)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive ductal carcinoma</td>
<td>41 (84)</td>
<td>38 (84)</td>
</tr>
<tr>
<td>Special types</td>
<td>8 (16)</td>
<td>7 (16)</td>
</tr>
<tr>
<td>HER2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>49 (100)</td>
<td>45 (100)</td>
</tr>
<tr>
<td>Positive</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
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</table>

**Figure 1.** miR-206 expression is down-regulated in ERα-positive human breast cancer. A, quantitative RT-PCR detection analysis shows that expression levels of miR-206 are much higher in the ERα-negative tumors than in the ERα-positive tumors ($P = 0.0001$). B, quantitative RT-PCR detection analysis shows that expression levels of miR-30c are not different between ERα-negative and ERα-positive tumors. C, the relationship between ERα protein expression scores and miR-206 expression in breast cancer tissues. miR-206 expression is gradually decreased as ERα protein scores increase.
mRNA expression were calculated from the relevant signals by normalization with the signal for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. The assay numbers for ERα, ERβ, progesterone receptor (PR), cyclin D1, p52, and GAPDH were as follows: Hs00174860_m1 for ERα, Hs00230957_m1 for ERβ, Hs00172183_m1 for PR, Hs00277039_m1 for cyclin D1, Hs00170216_m1 for p52, and Hs99999905_m1 for GAPDH (Applied Biosystems).

**Cell culture and transfections.** MCF-7 cells (American Type Culture Collection) were grown in RPMI 1640 containing 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine and penicillin-streptomycin (50 IU/mL and 50 mg/mL, respectively), at 37°C with 5% CO2. Transfections of pre-miR-206 precursor (hsa-miR-206; Ambion) were performed with Cell Line Nucleofector kits (Amaxa) using a Nucleofector device (Amaxa) according to the manufacturer’s instructions. A nonspecific control miRNA (Anti-miR miRNA Inhibitors-Negative Control #1; Ambion) was used as a negative control.

**Cell proliferation assay.** After transfection, MCF-7 cells (4,000 cells per well) were plated in 96-well plates and incubated in RPMI 1640 containing 10% FBS or in RPMI 1640 without phenol red containing 10% charcoal/dextran-treated FBS (HyClone Laboratories), in the presence or absence of 17β-estradiol (E2, 10 nmol/L; Sigma). Cell growth was measured using Cell Proliferation Reagent WST-1 (Roche Applied Science) by incubating cells for 3 h with the reagent and recording absorbance at 450 nm with a 96-well plate reader.

**Statistical analysis.** The unpaired Student’s t test was used to compare expression levels of miRNAs and mRNAs in ERα-positive and ERα-negative tumors. The differences between miR-206 expression levels corresponding to various ERα protein scores were compared by one-way ANOVA followed by the Tukey-Kramer test. Correlation between miR-206 expression and ERα and ERβ mRNA expression was assessed by regression analysis. Differences between treatments on cell growth were compared by one-way ANOVA followed by the Dunnett’s test.

**Results**

**miR-206 expression is down-regulated in ERα-positive human breast cancer.** We first examined expression levels of miR-206 in ERα-positive and ERα-negative human breast cancer tissues. Quantitative RT-PCR detection analysis showed that the expression levels of miR-206 were much higher in the ERα-negative tumors (0.67 ± 0.52; range, 0.15–2.25) than in the ERα-positive tumors (0.31 ± 0.34; range, 0.06–1.66; P = 0.0001; Fig. 1A). We then tested expression of another miRNA, miR-30c, in the same tissues because it was reported that miR-30c expression, as assayed by microRNA microarray analysis, was increased in ERα-positive compared with ERα-negative breast cancer (9). Our quantitative RT-PCR results showed no differences in miR-30c expression between ERα-negative and ERα-positive tumors (30.8 ± 25.4 versus 24.7 ± 20.8, P = 0.21; Fig. 1B). We then extended the analysis of miR-206 expression in breast cancer tissues to include all scores for ERα protein expression, assessed semiquantitatively by IHC (Fig. 1C). miR-206 expression levels were gradually decreased as ERα protein scores increased. We conclude that miR-206 expression is markedly decreased in ERα-positive human breast cancer tissues.

**miR-206 expression is inversely correlated with ERα mRNA expression in human breast cancer.** We next examined ERα mRNA expression in human breast cancer tissues. As expected, the expression levels of ERα mRNA were much higher in the ERα-positive tumors (16.1 ± 12.1; range, 0.08–50.00) than in the ERα-negative tumors (0.38 ± 0.82; range, 0.005–3.20; P < 0.0001; Fig. 2A). To analyze the association between miR-206 expression and ERα mRNA expression, expression levels of miR-206 and ERα mRNA were plotted. The scatterplots showed that miR-206 expression is inversely correlated with expression of ERα but not ERβ in human breast cancer tissues. A, quantitative RT-PCR detection analysis shows that expression levels of ERα mRNA are much higher in the ERα-positive tumors than in the ERα-negative tumors (P < 0.0001). B, scatterplots show inverse correlation between miR-206 and ERα mRNA expression in breast cancer tissues (P = 0.002). C, scatterplots show ERβ mRNA expression is not correlated with miR-206 expression in breast cancer tissues.

**Figure 2.** miR-206 expression is inversely correlated with expression of ERα but not ERβ in human breast cancer tissues. A, quantitative RT-PCR detection analysis shows that expression levels of ERα mRNA are much higher in the ERα-positive tumors than in the ERα-negative tumors (P < 0.0001). B, scatterplots show inverse correlation between miR-206 and ERα mRNA expression in breast cancer tissues (P = 0.002). C, scatterplots show ERβ mRNA expression is not correlated with miR-206 expression in breast cancer tissues.

instructions (Applied Biosystems). The relative levels of miRNA expression were calculated from the relevant signals by normalization with the signal for U6B miRNA expression. The assay names for miR-206, miR-30c, and U6B were as follows: hsa-mir-206 for miR-206, hsa-mir-30c for miR-30c, and RNU6B for U6B (Applied Biosystems).

**Quantitative RT-PCR detection of miRNA.** Total RNA (1 μg) was subjected to reverse transcription with random primers in a 20 μL reaction volume using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). mRNA expression was measured by quantitative reverse-transcription PCR (RT-PCR) with the Taq Man Universal PCR Master Mix using a 7500 ABI PRISM Sequence Detector System according to the manufacturer’s instructions (Applied Biosystems). The relative levels of mRNA expression were much higher in the ERα-negative tumors (0.67 ± 0.52; range, 0.15–2.25) than in the ERα-positive tumors (0.31 ± 0.34; range, 0.06–1.66; P = 0.0001; Fig. 1A).
expression was inversely correlated with ERα mRNA expression in human breast cancer \( (P = 0.002; \text{Fig. 2B}) \). In contrast, no association was found between miR-206 expression and ERβ mRNA expression \( (P = 0.43; \text{Fig. 2C}) \).

**miR-206 suppresses ERα expression and inhibits growth of MCF-7 cells.** To assess the role of miR-206 in the growth of ERα-positive breast cancer, pre–miR-206 precursor was introduced into proliferating MCF-7 cells. Cells were transfected with either control miRNA (200 nmol/L) or pre–miR-206 precursor at 20 or 200 nmol/L and incubated for up to 72 hours in a medium containing 10% FBS. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based cell proliferation assay revealed that miR-206 strongly inhibited cell growth in a dose- and time-dependent manner, whereas MCF-7 cells transfected with control miRNA continued to grow during the period (Fig. 3A). Introduction of miR-1, which is expressed in muscle cells and promotes their differentiation as well as miR-206 \( (19) \), also did not inhibit cell growth (data not shown). When MCF-7 cells were transfected with pre–miR-206 precursor at various concentrations and incubated for 72 hours, miR-206 induced a dose-dependent inhibition of cell growth (Fig. 3B). Introduction of pre–miR-206 precursor at 200 nmol/L into proliferating MCF-7 cells produced a significant 55% decrease in cell number compared with cells transfected with negative control \( (P < 0.0001; \text{Fig. 3B, top left}) \).

Expression levels of ERα mRNA and miR-206 were quantitatively measured by quantitative RT-PCR. In C, MCF-7 cells were transfected with either control miRNA (200 nmol/L) or pre–miR-206 precursor (200 nmol/L) and incubated for up to 96 h in phenol red–free RPMI 1640 containing 10% charcoal/dextran-treated FBS with or without 17β-estradiol (E2, 10 nmol/L). Cell growth was measured by an MTT-based cell proliferation assay.

**Figure 3.** Transfection of miR-206 into estrogen-dependent MCF-7 breast cancer cells suppresses ERα expression and inhibits cell growth. Representative experiments are shown in triplicate along with SD. A, MCF-7 cells were transfected with either control miRNA (200 nmol/L) or pre–miR-206 precursor at 20 or 200 nmol/L and incubated for up to 72 h in a medium containing 10% FBS. Cell growth was measured by an MTT-based cell proliferation assay. B, MCF-7 cells were transfected with either control miRNA (200 nmol/L) or pre–miR-206 precursor at 20 to 200 nmol/L and incubated for 72 h in a medium containing 10% FBS. ERα mRNA levels, miR-206 levels, and mRNA levels of PR, cyclin D1, and pS2 were measured by quantitative RT-PCR. C, MCF-7 cells were transfected with either control miRNA (200 nmol/L) or pre–miR-206 precursor (200 nmol/L) and incubated for up to 96 h in phenol red–free RPMI 1640 containing 10% charcoal/dextran-treated FBS with or without 17β-estradiol (E2, 10 nmol/L). Cell growth was measured by an MTT-based cell proliferation assay.
measured using parallel samples. Transfection with pre-miR-206 induced a dose-dependent repression of ERα mRNA levels, whereas it produced a dose-dependent increase of miR-206 levels (Fig. 3B, top right and bottom left). To further validate the effect of miR-206 on growth of MCF-7 cells, mRNA expression of ERα-target genes, such as PR, cyclin D1, and pS2, was quantitatively measured using parallel samples (Fig. 3B, bottom right). Introduction of pre-miR-206 produced a dose-dependent decrease of mRNA expression of these genes. We then extended our analysis to determine whether miR-206 would affect estrogen-induced growth of MCF-7 cells. Similar inhibition of growth by introduction of pre-miR-206 precursor at 200 nmol/L was observed when cells were cultured in a medium containing charcoal/dextran-trasnf BSA in the presence of E2 (control +E2 versus pre–miR-206 +E2; Fig. 3C). In the absence of E2, cell growth was also inhibited when cells were transfected with pre–miR-206 (control -E2 versus pre–miR-206 -E2; Fig. 3C). The cells may have been affected by low levels of estrogen present in serum because MCF-7 cells transfected with negative control grew slightly in the absence of added E2. From these analyses, we conclude that introduction of miR-206 into estrogen-dependent breast cancer cells leads to suppression of ERα mRNA expression and inhibition of growth.

Discussion

In the present study, we have extended the basic, mechanistic insight by Adams and colleagues (6) to actual breast cancer by using clinically obtained tissue samples, and found that miR-206 expression is markedly decreased in ERα-positive human breast cancer tissues. We also showed that introduction of miR-206 into estrogen-dependent breast cancer cells leads to suppression of ERα expression and growth inhibition.

In experimental models, a single mirRNA can regulate a number of genes (20). Previous studies have shown that miR-206 is expressed in skeletal and cardiac muscle and promotes muscle differentiation by down-regulating the p180 subunit of DNA polymerase α and myogenic transcription factors, Is1-3 and MyoR (19). Adams and colleagues (6) recently identified two miR-206 binding sites within the 3′-UTR of human ERα and showed that miR-206 down-regulates ERα mRNA and protein expression in breast cancer cells. Our results show that the expression levels of miR-206 are much higher in ERα-negative than in ERα-positive breast cancer tumors, and that the more ERα-positive cells present in the tumor, the less miR-206 expression seen, suggesting that miR-206 is a key factor for the regulation of ERα expression in development and progression of human breast cancer.

Endocrine therapy has become the most important treatment option for women with ERα-positive breast cancer, and ~70% of primary breast cancers express ERα. Although endocrine therapy inhibits estrogen-dependent growth via ERα, the currently available treatments, such as selective ER modulators and estrogen deprivation therapy, affect the functions of both ERα and ERβ. In this study, we show that miR-206 expression is inversely correlated with expression of ERα but not ERβ in human breast cancer tissues, and that introduction of miR-206 into estrogen-dependent breast cancer cells suppresses ERα expression and inhibits cell growth. Our results suggest that miR-206 would be a novel tool for endocrine therapy that targets only ERα.

To analyze the role of miR-206 in development of breast cancer could also be important for identifying the different molecular mechanisms between ERα-positive and ERα-negative breast cancers. In addition, miR-206 may serve as a key factor that regulates ERα expression during the development of normal breast epithelium because the expression of miR-206 is under hormonal regulation (6), although the role of miR-206 in normal breast tissues has not been studied.

In conclusion, the present data indicate for the first time that miR-206 expression is markedly decreased in ERα-positive human breast cancer tissues and that introduction of miR-206 into estrogen-dependent breast cancer cells inhibits cell growth. miR-206 could be a novel candidate for ERα-specific endocrine therapy in breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

3. Adams BD, Furneaux H, White BA. The micro-RNA (miRNA) miR-206 targets the human estrogen receptor-α gene and represses allosteric regulation (6), although the role of miR-206 in normal breast tissues has not been studied.
4. Gascoyne RB, Lees CR, Gong R, et al. The micro-RNA (miRNA) miR-206 targets the human estrogen receptor-α gene and represses allosteric regulation (6), although the role of miR-206 in normal breast tissues has not been studied.