Decreased miR-206 Expression in BRCA1 Wild-type Triple-negative Breast Cancer Cells After Concomitant Treatment with Gemcitabine and a Poly(ADP-ribose) Polymerase-1 Inhibitor

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Abstract. *No targeted-therapy has been established for triple-negative breast cancer accompanied by mutations in breast cancer susceptibility gene1 (BRCA1) mutation. In the present study, using BRCA1 wild-type cells (MDA-MB-231) and BRCA1-mutated cells (MDA-MB-436), we investigated miRNA expression and apoptosis on day 1 after addition of gemcitabine-alone and in combination with poly ADP-ribose polymerase-1 (PARP1) inhibitor. After drug treatment, there were significantly fewer apoptotic BRCA1 wild-type cells than BRCA1-mutated cells. Expression of miRNA-26a, -29b, -100, and -148a increased in BRCA1 wild-type cells exposed to gemcitabine-alone and in combination with the PARP1 inhibitor. The addition of PARP1 inhibitor reduced miR-206 expression in BRCA1 wild-type cells but increased it in BRCA1-mutated cells. It was suggested that miR-206 serves as a target molecule of PARP1 inhibitor combination therapy for BRCA1 wild-type triple-negative breast cancer cells.*

Breast cancer is the most frequent cancer in women. To prevent and treat this disease, the relationships between certain pathological factors and breast cancer therapy have been reported. Breast cancer is divided into different subtypes based on gene expression analysis. Triple-negative breast cancer (TNBC) is a subtype negative for the gene expression of estrogen receptor (ER), progesterone receptor (PgR), and human epithelial growth factor receptor (HER2) (1). TNBC accounts for 10%-15% of breast cancer cases, and the prognosis is poor, being accompanied by distant metastasis in 30%. Since there is no specific target molecule and hormone therapy and anti-HER2 treatment are ineffective, making cure difficult, the establishment of a treatment method is urgently needed (2). The DNA injury-type TNBC carrying mutant breast cancer susceptibility gene 1 (*BRCA1*), P53 mutation, or tumor-histologically poorly differentiated cells has been reported to be markedly sensitive to anti-cancer drugs, and poly (ADP-ribose) polymerase (PARP) inhibitor that induces cancer cell apoptosis is also used to treat TNBC (3-5).

The present study focused on microRNA (miRNA) to identify a method to efficiently induce the apoptosis of cancer cells with wild-type BRCA1. miRNA inhibits mRNA and protein expressions by complementarily binding to nucleotides of the 3'-untranslational region (UTR) of the specific target of mRNA. The objective of this study was to analyze miRNA specifically expressed in TNBC carrying wild-type *BRCA1* and identify the target miRNA to effectively induce tumor cell apoptosis on with concomitant treatment PARP1 inhibitor.

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Materials and Methods

Cell culture. MDA-MB-231 (*BRCA1* wild-type) and MDA-MB-436 (*BRCA1*-mutated) human TNBC cells (Japanese Cancer Resources Bank, Osaka, Japan), Dulbecco's modified Eagle's Medium culture medium (DMEM) added 10% inactivated fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin (GIBCO Penicillin-Streptomycin liquid; Invitrogen, Carlsbad, CA, USA). Cells were incubated at 37˚C under 5% CO_2 in 95% air.

Drug combination studies. MDA-MB-231 and MDA-MB-436 cells were seeded in six -well plates and treated with a final concentration of 1 mg/ml gemcitabine (Yakult, Tokyo, Japan) alone or combination of gemcitabine and PARP1 inhibitor (Wako, Tokyo, Japan) ($C_{15}H_{12}N_2O_2$: MW=252.27, final concentration, 1%). PARP1 inhibitor dilution series were prepared in 100% dimethylsulfoxide (DMSO) and diluted 1 ml in DMEM. 20 μl of PARP1 inhibitor solution was added to 1800 μl of DMEM in each well, with a final PARP-1 inhibitor concentration of 1%.

Cell viability. Cell growth (as evidenced by increases in the number of cells) was determined using a BIO RAD TC20™ Automated Cell

Counter (BIO RAD, Tokyo, Japan). To determine cell viability, MDA-MB-231 cells and MDA-MB-436 cells were incubated in 6-well plates for three days at 37˚C until confluence was reached, at which point the cell number was 5×10^4 cells/plate, after treatment with gemcitabine alone and the combination of gemcitabine and PARP1 inhibitor, for one day.

Apoptosis detection. Apoptosis was detected by staining cells for MUSETM Annexin V Dead cell kit (MUSE, Darmstadt, Germany). Cells were plated overnight in six-well plates followed by treatment with gemcitabine alone and the combination of gemcitabine and PARP1 inhibitor, for one day.

miRNA measurement. MiRNA was extracted from cells using a miRNeasy Mini kit (Qiagen, Tokyo, Japan). miRNA MiScript SYBR Green Human Breast Cancer miRNA PCR Kit were detected by an ABI PRISM 7000 Sequence System (Applied Biosystems Inc, Foster City, CA, USA) under the following conditions: 1 cycle at 95˚C for 10 minutes, 40 cycles of 94˚C for 15 seconds, annealing at 60˚C for 30 seconds, and extension at 72˚C for 30 seconds. Data were analyzed by the ΔΔ Ct method (original expression level=$2^{=-\Delta\Delta Ct}$). Data were expressed as the Ct level of the cells subjected to drug treatment as a ratio of that of non-drug-treated cells. Differential miRNA expression was defined as differences in miRNA expression between the drug-treated and untreated groups of more than ±100-fold.

Statistical analysis. The significance of differences between groups was determined using unpaired Student's *t*-test (two-tailed), with the level of significance set at $p<0.001$.

Results

Cell viability after treatment with gemcitabine-alone and in combination with PARP inhibitor. The mean counts of viable MDA-MB-231 and MDA-MB-436 cells after the addition of gemcitabine alone were no significantly different from that of the control cells ($p=0.067$). The mean MDA-MB-436 cell counts after the addition of concomitant gemcitabine and PARP1 inhibitor significantly decreased ($p<0.001$) (Figure 1).

Cell apoptosis after treatment with gemcitabine alone and in combination with PARP1 inhibitor. The apoptotic cell counts significantly increased in MDA-MB-436 cells after the addition of gemcitabine ($p<0.001$). The apoptotic cell counts after the addition of concomitant gemcitabine and PARP1 inhibitor were also significantly increased in MDA-MB-436 cells (n=3) ($p<0.001$) (Figure 2).

Analysis of miRNA expression in MDA-MB-231 cells. The addition of gemcitabine-alone increased the expression of miR-18a, -19a, -19b, -26a, -26b, -29b, -29c, -100, -148a, -195 and -424 by more than 100 times, respectively. In contrast, the ΔΔCT values of miR-125b-1, -205 and -206 were low (Figure 3). The addition of concomitant gemcitabine and PARP1 inhibitor increased the expression of miRNA-26a, -26b, -100 and -148a by more than 100 times, respectively. In contrast, the ΔΔCT values of miR-125b-1, -202, -205, and -206 were low (Figure 4).

Analysis of miRNA expression in MDA-MB-436 cells. The addition of gemcitabine-alone reduced the miR-202 expression level by more than 100 times, (Figure 3). However the combination of gemcitabine and PARP1 inhibitor increased it to 111.43. Similarly, that of miR-206 increased by more than 100-fold (Figure 4).

Discussion

The miR-206 expression level in *BRCA1* wild-type cells (MDA-MB-231 cells) decreased after the addition of concomitant gemcitabine and PARP1 inhibitor, but it increased in *BRCA1*-mutated cells (MDA-MB-436 cells). Expression of miR-206 was changed by more than 100-fold. The expression levels of four miRNAs, miR-26a, -29b, -100, and -148a were increased in *BRCA1* wild-type cells treated with gemcitabine-alone and its combination with PARP1 inhibitor compared to those in untreated control cells. These miRNAs were not previously reported in the expression profile after chemotherapy of TNBC cells.

BRCA1 mutation induces abnormalities in the DNA repair pathway and control of, proliferation, which effectively induces cell death through the inhibition of PARP1 (6-10). However, the inhibition of PARP1 does not kill cancer cells carrying wild-type *BRCA1* because homologous recombination through BRCA1, which is the double-stranded DNA repair system, is activated (11, 12). O'Shaughnessy performed biomarker analysis involving 123 patients the TNBC in a phase II clinical study, and observed that the concomitant administration of a PARP1 inhibitor (BSI-201), gemcitabine, and carboplatin delayed cancer progression and improved the overall survival rate (12). However, in a phase III clinical study involving 519 patients with TNBC, the overall and progression-free survival times were not significantly improved when the patients were divided into those treated with monotherapy and combination therapy with PARP1 inhibitor (13). This may have been due to a low rate (19.5%) of *BRCA1* mutant-type TNBC, all others having wild-type *BRCA1* in the phase III clinical study (14). In our study, cell growth significantly decreased on day 1 after the addition of concomitant gemcitabine and PARP1 inhibitor in *BRCA1*-mutated cells comparted with *BRCA1* wild-type, clarifying that the combination of gemcitabine and PARP1 inhibitor is effective for *BRCA1*-mutated cells (Figure 1). Upon measurement of apoptosis, significantly more *BRCA1*-mutated cells than *BRCA1* wild-type cells were apoptotic on day 1 after the addition of gemcitabine alone and the combination of gemcitabine and PARP1 inhibitor, clarifying that apoptosis was induced by both gemcitabine-alone and its combination with PARP1 inhibitor in *BRCA1*-mutated cells (Figure 2). These findings strongly suggest that the presence or absence of *BRCA1* mutation and the repair function of PARP1 are related to TNBC cell death.

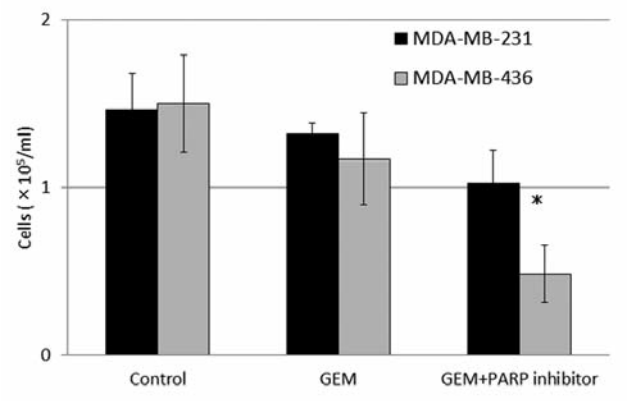

Figure 1. *Cell viability after treatment with gemcitabine alone and the combination of gemcitabine and poly(ADP-ribose) polymerase-1(PARP1) inhibitor. The mean counts of MDA-MB-231 and MDA-MB-436 cells after the addition of gemcitabine alone were not significantly different (p=0.067). The mean counts after the addition of concomitant gemcitabine and PARP1 inhibitor significantly decrease in MDA-MB-436 cells (*p<0.001). Data are the mean±SD (n=6).*

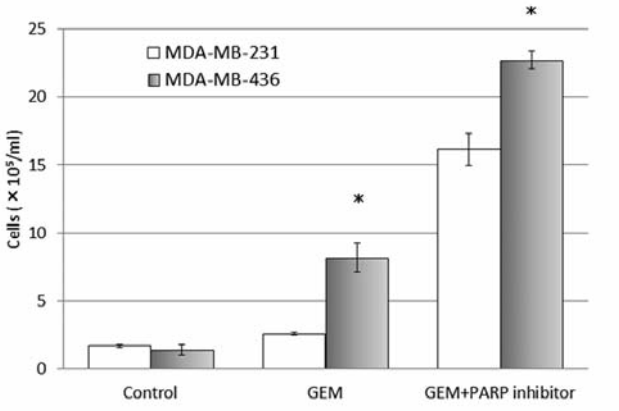

Figure 2. *Number of apoptotic cells after treatment with gemcitabine alone and the combination of gemcitabine and PARP1 inhibitor. The apoptotic cell counts after the addition of gemcitabine alone and concomitant gemcitabine and PARP1 inhibitor significantly decreased in MDA-MB-231 cells (*p<0.001). Data are the mean±SD (n=3).*

The expression levels of miR-26a, -29b, -100, and -148a were specifically increased by gemcitabine-alone and its combination with PARP1 inhibitor in *BRCA1* wild-type cells, but increases in their expression was not observed in *BRCA1*-mutated cells (Figures 3 and 4). The expression levels of miR-29b, -100, and -148a were lower after the addition of concomitant gemcitabine and PARP1 inhibitor than after the addition of gemcitabine alone, whereas that of miR-26a was higher after the addition of concomitant gemcitabine and PARP1 inhibitor. miR-26a induces tumor cell apoptosis in a P53-dependent manner, and it has been reported that its expression was correlated with a favorable vital prognosis in patients with breast and prostate cancer (15, 16). An increase in miR-26a expression was also observed in our study, suggesting that P53 was activated and inhibited cancer cell proliferation. The protein expression-inhibiting function of miRNA is considered to be a cause of stronger apoptosis induction by the combination of gemcitabine and PARP1 inhibitor than gemcitabine-alone. miR-29b has been reported to target phosphatase and tensin homolog deleted on chromosome ten (PTEN) in MDA-MB-231 cells. PTEN expression has been reported to promote tumor growth and cancer cell proliferation by activating AKT-mechanistic target of rapamysin (AKT-mTOR) and be associated with recurrence and metastasis. Increased miR-29b expression inhibits PTEN function, and the inactivation of downstream mediators promotes apoptosis (17). We also observed the enhancement of miR-29b expression and promotion of apoptosis by the addition of gemcitabine alone and combined with PARP1 inhibitor, suggesting that apoptosis was induced through a pathway other than the Insulin-like growth factor-mTOR (IGF-mTOR) signaling cascade, because gemcitabine injures nuclear DNA and inhibits double-stranded DNA repair (17). miR-100 targets IGF2, which is a metabolism- and cell growth-regulatory apparatus. miR-100 has been reported to inhibit the proliferation and promote the apoptosis of breast cancer cells by inhibiting sIGF-mTOR signaling (18). We also observed that gemcitabine-alone and in combination with PARP1 inhibitor enhanced miR-100 expression and promoted apoptosis, suggesting that gemcitabine injured nuclear DNA and induced apoptosis through a pathway other than the IGF-mTOR signaling cascade, similarly to miR-29b. The addition of gemcitabine-alone and with PARP1 inhibitor enhanced miR-148a expression. High expression of miR-148a acts on the transcription factor-binding site in colorectal cancer cells and inhibits the expression of the target, BCL2 (19). miR-148a induced inhibition of anti-apoptotic BCL2 expression promotes cytochrome *c* release from the mitochondrial membrane and increases apoptotic cells. Apoptotic cells were increased by the addition of concomitant PARP1 inhibitor, and this was not through cell death regulated by the target, BCL2, but through a reduced cellular repair function for double-stranded DNA cut by gemcitabine and bound by PARP1 inhibitor, with a high affinity for the DNA repair site. Opposite effects on miR-206 expression were noted after the addition of concomitant PARP1 inhibitor between *BRCA1* wild-type and *BRCA1*-mutated. The combination of gemcitabine and PARP1 inhibitor reduced the miR-206 expression level in *BRCA1* wild-type cells but increased it

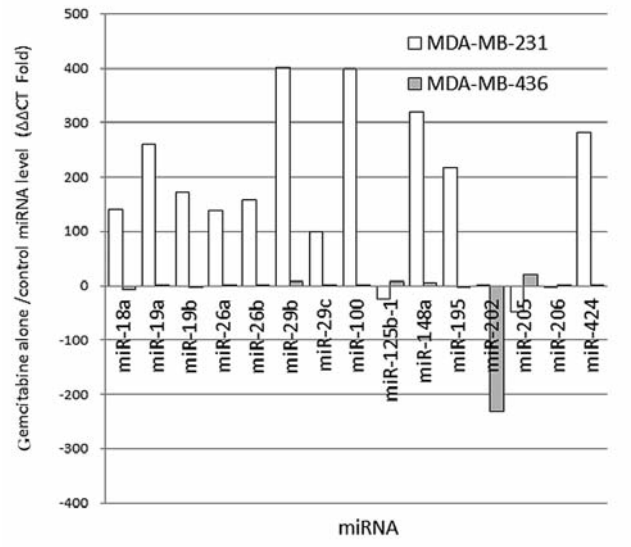

Figure 3. *The addition of gemcitabine-alone increased the miRNA expression levels in MDA-MB-231 cells of miR-18a, -19a, -19b, -26a, -26b, -29b, -29c, -100, -148a, -195, and -424 by more than 100 times.*

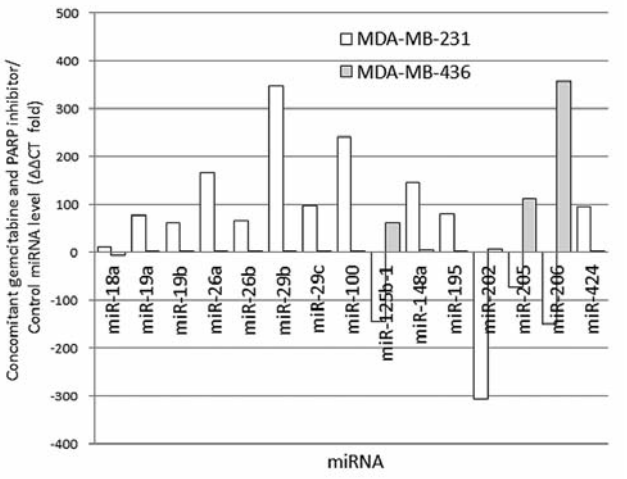

Figure 4. *The miRNA expression levels after the addition of concomitant gemcitabine and PARP1 inhibitor reduced expression of miR-206 in MDA-MB-231 cells but increased it in MDA-MB-436 cells. Results are shown for changes in expression greater than ±100-fold. Combined gemcitabine and PARP1 inhibitor treatment reduced miR-206 expression in MDA-MB-231 cells, but increased it in MDA-MB-436 cells. The expression levels of miRNA –26a, –29b, –100, and -148a increased in MDA-MB-231 cells after the addition of combination of GEM and PARP1 inhibitor.*

in *BRCA1*-mutated cells. An increase in miR-206 regulates a cell cycle-regulatory factor, cyclin D2, and arrests the cell cycle, inhibiting cell proliferation (20, 21). It was suggested that, after the addition of concomitant gemcitabine and PARP1 inhibitor, miR-206 expression was enhanced in BRCA1-mutated cells and the cell cycle was arrested, reducing cancer cells, but the cell cycle continued in the cells carrying *BRCA1* wild-type because miR-206 expression was reduced. Expressions of miR-10a,-26a, -100, and -148a were enhanced by both gemcitabine alone and its combination with PARP1 inhibitor in *BRCA1* wild-type cells. If similar enhancement of the expression by other drugs is clarified, it may be possible to utilize miR-26a, -29b, -100, and -148 expressions as key factors in microRNA-targeting chemotherapy for TNBC. In addition, miR-206 expression was enhanced by the combination with PARP1 inhibitor in *BRCA1*-mutated cells but decreased in *BRCA1* wild-type cells, suggesting that miR-206 plays an important role in PARP1 inhibitor combination therapy for *BRCA1* wild-type breast cancer cells.

Conclusion

The addition of PARP1 inhibitor reduced the miR-206 expression level in *BRCA1* wild-type cells but increased it in *BRCA1*-mutated cells, suggesting that miR-206 serves as a target molecule of PARP1 inhibitor combination therapy for *BRCA1* wild-type TNBC.

References

1 Nandini D, Smith B and Leyland-Jones B: Targeting basal-like breast cancers. Curr Drug Targets *13*: 1510-24, 2012.
2 Cleator S, Heller W and Coombes RC: Triple-negative breast cancer therapeutic options. Lancet Oncol *8*: 235-244, 2007.
3 Helleday T, LO J, Van Gent DC and Engelward BP: DNA double-strand break repair: from mechanistic understanding to cancer treatment. DNA Repair *6*: 923-935, 2007.
4 Rowe BP and Glazer PM: Emergence of rationally designed therapeutic strategies for breast cancer targeting DNA repair mechanisms. Breast Cancer Res *12*: 203, 2010.
5 Alli E, Sharma VB, Sunderesakumar P and Ford LM: Defective Repair of Oxidative DNA Damage in Triple-Negative Breast Cancers Sensitivity to Inhibition of Poly (ADP-Ribose) Polymerase. Cancer Res *69*: 3589-3596, 2009.
6 Shibata H, Miuma S, Saldivar JC and Huebner K: Response of subtype specific human breast cancer-derived cells to PARP and Chk1 inhibition. Cancer Sci *102*: 1882-1888, 2011.
7 Kaelin WG: The concept of synthetic lethality in the context of anticancer therapy. Nat Rev cancer *5*: 689-698, 2005.
8 Farmer H, Mc CabeN, Lord CJ, Tutt AN, Johnson DA, Richardson TB, Santarosa M, Dillon KJ, Hickson I, Knights C, Martin NM, Jackson SP, Smith GC and Ashworth A: Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature *435*: 917-920, 2005.
9 Nowsheen S, Cooper T, Stanley JA and Yang ES: Synthetic lethal interactionsbetween EGFR and PARP inhibition in human triple-negative breast cancer cells. PLoS One *7*: e46614, 2012.
10 Yvette D, Mulligan EA, Wan-Tse V, Thomas HD, Samra K, Suzanne K, Asima M, Gerrit L, Zdenek H, Elizabeth R. P,

Richard JE and Nicola JC: Therapeutic Potential of Poly(ADP-ribose) Polymerase Inhibitor AG014699 in Human Cancers With Mutated or Methylated BRCA1 or BRCA2. J Natl Cancer Inst *103*: 334-346, 2011.
11 Miyoshi Y, Murase K and Oh K: Basal-like subtype and BRCA1 dysfunction in breast cancers. Int J Oncol *13*: 395-400, 2008.
12 Fong PC, Boss DS, Yap TA, Tutt A, Wu P, Mergui- Roelvink M, Mortimer P, Swaisland H, Lau A, O'Connor MJ, Ashworth A, Carmichael J, Kaye SB, Schellens JH and de Bono JS: Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. N Engl J Med *361*: 123-134, 2009.
13 O'Shaughnessy J, Osbome C, Pippen JE, Yoffe M, Patt D, Rocha C, Koo IC, Sherman BM and Bradley C: Iniparib plus chemotherapy in metastatic triple-negative breast cancer. N Engl J Med *364*: 205-214, 2011.
14 Gonzalez-Angulo AM, Timms KM, Liu S, Chen H, Litton JK, Potter J, Lanchbury JS, Stemke-Hale K, Hennessy BT, Arun BK, Hortobagyi GN, Do KA, Mills GB and Meric-Bernstam F: Incidence and outcome of BRCA mutations in unselected patients with triple receptor-negative breast cancer. Clin Cancer Res *17*: 1082-1089, 2011.
15 Ichikawa T, Sato F, Terasawa K, Tsuchiya S, Toi M, Tsujimoto G and Shimizu K: Trastuzumab Produces Therapeutic Actions by Upregulating miR-26a and miR-30b in Breast Cancer Cells *7*: e31422, 2012.
16 L Lezina, N Purmessur, AV Antonov, T Ivanova, E Karpova, K Krishan, M Ivan, V Aksenova, D Tentler, AV Garabadgiu, G Melino and NA Barlev: miR-16 and miR-26a target checkpoint kinases Wee1and Chk1 in response to p53 activation by genotoxic stress. Cell Death and Disease *4*: e953, 2013.
17 Wang C, Bian Z, Wei D and Zhang JG: miR-29b regulates migration of human breast cancer cells. Mol Cell Biochem *352*: 197-207, 2011.
18 Gebreshber CA and Martinez: miR-100 suppresses IGF2 and inhibits breast tomorigenesis by interfering with proliferation and survival signaling. Oncogene *32*: 3306-3310, 2013.
19 Zhang H, Li Y, Huang Q, Ren X, Hu H, Sheng H and Lai M: MiR-148a promotes apoptosis by targeting Bcl-2 in colorectal cancer. Cell Death Differ *18*: 1702-1710, 2011.
20 Zhou J, Tian Y, Li J, Lu B,Sun M, Zou Y. Kong R, Luo Y, Shi Y, Wang K and Ji G: miR-206 is down-regulated in breast cancer and inhibits cell proliferation through the up-regulation of cyclinD2.Biochem Biophys Res Commun *433*: 207-212, 2013.
21 Kondo N, Toyama T, Sugiura H, Fuji Y and Yamashita H: miR-206 Expression is down-regulated in estrogen receptor alpha-positive human breast cancer. Cancer Res *68*: 5004-5008, 2008.

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