MicroRNA Regulates the Expression of Human Cytochrome P450 1B1

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Abstract

MicroRNAs (miRNA) are small noncoding RNAs that regulate gene expression through translational repression or mRNA cleavage. Here, we found that cytochrome P450 (CYP), a superfamily of drug-metabolizing enzymes, is a target of miRNA. Human CYP1B1, which is highly expressed in estrogen target tissues, catalyzes the metabolic activation of various procarcinogens and the 4-hydroxylation of 17β-estradiol. CYP1B1 protein is abundant in cancerous tissues. We identified a near-perfect matching sequence with miR-27b in the 3′-untranslated region of human CYP1B1. Luciferase assays revealed that the reporter activity of the plasmid containing the miR-27b recognition element was decreased in MCF-7 cells (miR-27 positive) but not in Jurkat cells (miR-27b negative). Exogenously expressed miR-27b could decrease the luciferase activity in Jurkat cells. In MCF-7 cells, the antisense oligoribonucleotide for miR-27b restored the luciferase activity and increased the protein level and enzymatic activity of endogenous CYP1B1. These results suggested that human CYP1B1 is post-transcriptionally regulated by miR-27b. The expression levels of miR-27b and CYP1B1 protein in breast cancerous and adjacent noncancerous tissues from 24 patients were evaluated. In most patients, the expression level of miR-27b was decreased in cancerous tissues, accompanied by a high level of CYP1B1 protein. A significant inverse association was observed between the expression levels of miR-27b and CYP1B1 protein. Thus, the decreased expression of miR-27b would be one of causes of the high expression of CYP1B1 protein in cancerous tissues. This is the first study to show that miRNAs regulate not only essential genes for physiologic events but also drug-metabolizing enzymes. (Cancer Res 2006; 66(18): 9090-8)

Introduction

MicroRNAs (miRNA) have received attention as a new class of small noncoding RNAs regulating the expression of genes that are involved in various biological processes, such as development, cell proliferation, and apoptosis (1, 2). The number of currently known miRNAs in mammalian has risen dramatically, and their total number in humans has been predicted to be as high as 1,000 (3). Primary miRNA transcripts are cleaved by RNase III Drosha in the cell nucleus into 70-nucleotide to 80-nucleotide precursor miRNA (pre-miRNA) hairpins and transported to the cytoplasm, where pre-miRNAs are processed by RNase III Dicer into 19-nucleotide to 25-nucleotide miRNA duplexes. One strand of duplexes is degraded, and the other strand is used as mature miRNA. Mature miRNAs that are incorporated into the RNA-induced silencing complex recognize the 3′-untranslated region (UTR) of the target mRNA and cause translational repression or mRNA cleavage (4). The functional miRNAs have been predicted to control up to 20% to 30% of the genes within the human genome (5, 6). Recently, several studies have reported that the expression profiles of miRNAs were associated with the development of various types of human tumors. For example, human let-7 miRNA is down-regulated in lung cancers and inhibits the growth of lung cancer cells in vitro (7). A recent study indicated that let-7 miRNA controls the post-transcriptional regulation of the RAS oncogene (8). miR-15 and miR-16 were deleted or down-regulated in the majority of B-cell chronic lymphocytic leukemias (9), and the expression levels of miR-143 and miR-145 were decreased in colon cancer tissues as well as in cancer cell lines (10). Some oncogenes have been thought to be the potential target genes of these miRNAs. Thus, the miRNAs may be one of the key regulators of tumorigenesis.

Human cytochrome P450 (CYP) 1B1 is a member of CYP and is mainly expressed in ovary, uterus, and breast (11, 12). CYP1B1 catalyzes the metabolic activation of a variety of procarcinogens and promutagens, including polycyclic aromatic hydrocarbons and aryl amines (12) and metabolism of 17β-estradiol (13–15). Whereas 17β-estradiol contributes to the growth and development of estrogen-dependent cancers, such as breast and endometrial cancers (16), 4-hydroxyestradiol, a catechol metabolite formed by CYP1B1, generates free radicals from reductive-oxidative cycling with the corresponding semiquinone and quinone forms, which cause DNA damage (17, 18). The expression level of CYP1B1 is higher in various types of malignant cancers compared with normal tissues (19). Thus, it is evident that CYP1B1 is associated with cancer. It should be noted that there is no apparent difference in the CYP1B1 mRNA levels between tumor and normal tissues (20, 21). Although there is no direct evidence of lack of association between mRNA and protein of CYP1B1 in panel of human tissues, the phenomena are reminiscent of post-transcriptional regulation. An extremely long 3′-UTR (∼3 kb) is peculiar to CYP1B1 mRNA. This background prompted us to investigate whether human CYP1B1 might be post-transcriptionally regulated by miRNA.

Materials and Methods

Chemicals and reagents. The pGL3-promoter vector, phRL-TK plasmid, pTARGET vector, Tfx-20 reagent, and a dual-luciferase reporter assay system were from Promega (Madison, WI). LipofectAMINE and LipofectAMINE 2000 were purchased from Invitrogen (Carlsbad, CA). The mirVana miRNA Probe Construction kit, mirVana miRNA Detection kit, and Pre-miR miRNA Precursors for miR-27b and for negative control were from Ambion (Austin, TX). Antisense 2′-O-methyl oligoribonucleotides (AsO) for miR-27b (5′-CAGAACUUAGCCACUGUGAAL, in which L is 3′-aminolinker)

and for negative control (5′-AGACUAGCGGUAUCUUAAACCL) were from Dharmacon (Chicago, IL). All primers and oligonucleotides were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Rabbit anti-human CYP1B1 polyclonal antibodies for Western blot analysis and for immunohistochemical analysis were from BD Gentest (Worburn, MA) and Alpha Diagnostic International (San Antonio, TX), respectively. Rabbit anti-human CYP1A1 antibodies and recombinant human CYP1A1 or CYP1B1 expressed in baculovirus-infected insect cells were from BD Gentest. TCDD and G418 were obtained from Cambridge Isotope Laboratories (Cambridge, MA) and Wako Pure Chemicals (Tokyo, Japan), respectively. All other chemicals and solvents were of the highest grade commercially available.

Cells and culture conditions. The human uterine cervix adenocarcinoma cell line HeLa was obtained from Riken Gene Bank (Tsukuba, Japan). The human breast adenocarcinoma cell line MCF-7 and human embryonic kidney cell line HEK293 were obtained from the American Type Culture Collection (Rockville, MD). The human leukemic T-cell line Jurkat was kindly provided by Dr. Yoshinobu Nakanishi (Kanazawa University, Kanazawa, Japan). HeLa cells were cultured in DMEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Invitrogen). MCF-7 cells were cultured in DMEM supplemented with 0.1 mmol/L nonessential amino acid (Invitrogen) and 10% FBS. HEK293 cells were cultured in DMEM supplemented with 4.5 g/L glucose, 10 mmol/L HEPES, and 10% FBS. Jurkat cells were cultured in RPMI 1640 (Nissui Pharmaceutical) supplemented with 10% FBS. These cells were maintained at 37°C under an atmosphere of 5% CO_2-95% air.

RNase protection assay. Total RNA was isolated from the cells using ISOGEN (Nippon Gene, Tokyo, Japan). Antisense RNA probes were synthesized by *mir*Vana miRNA Probe Construction kit. The oligonucleotides used for miR-27b and U6 small nuclear RNA (snRNA) were 5′-TTCACAGTGGCTAAGTTCTGCCTGTCTC-3′ and 5′-AGAAGATTAG-CATGGCCCCTGCGCAAGGCCTGTCTC-3′, respectively. RNase protection assays were done using a *mir*Vana miRNA Detection kit according to the manufacturer's protocol. The antisense RNA probes labeled with [α-^{32}P]UTP using T7 RNA polymerase were hybridized to total RNA (3 μg) at 42°C for 10 hours and then digested by RNase A/T1. The protected miRNAs were separated by electrophoresis through 15% polyacrylamide/1× Tris-borate EDTA (TBE)/8 mol/L urea gels with 1× TBE as the running buffer, and then the miRNAs were detected and quantified with a Fuji Bio-Imaging Analyzer BAS 1000 (Fuji Film, Tokyo, Japan).

Real-time reverse transcription-PCR. The cDNAs were synthesized from total RNAs using ReverTra Ace (Toyobo, Osaka, Japan) according to the manufacturer's protocol. The forward and reverse primers for human precursor miR-27b (pre-miR-27b) were 5′-ACCTCTCTAACAAGGTGCA-GAGCTT-3′ and 5′-ACCTTCTCTTCAGGTGCAGAACTTAG-3′, respectively.
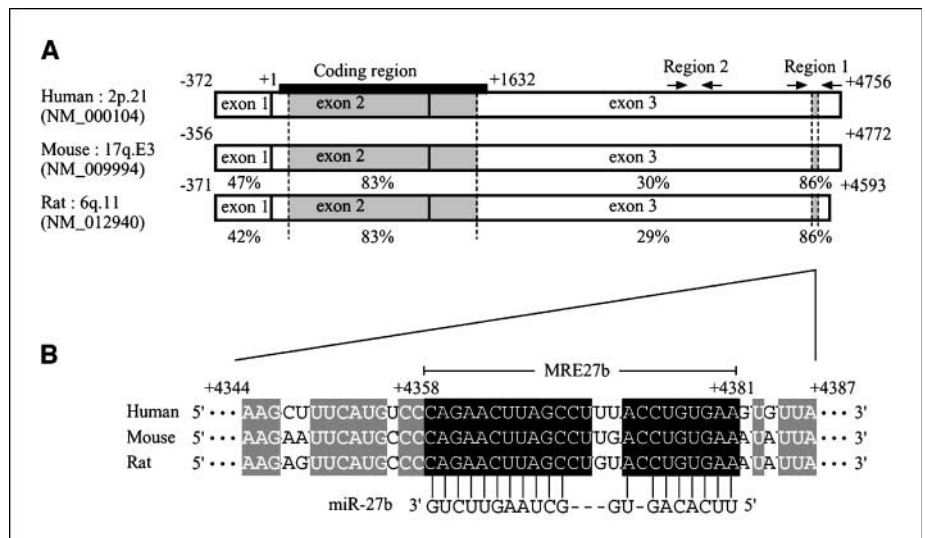
The forward and reverse primers for human U6 snRNA were 5′-CGCTTCGGCAGCACATATACTAA-3′ and 5′-TATGGAACGCTTCAC-GAATTTGC-3′, respectively. The PCR analyses for human pre-miR-27b were done as follows: after an initial denaturation at 95°C for 30 seconds, the amplification was done by denaturation at 95°C for 10 seconds, annealing and extension at 68°C for 20 seconds for 45 cycles. The PCR condition for human U6 snRNA was done as follows: after an initial denaturation at 95°C for 30 seconds, the amplification was done by denaturation at 94°C for 10 seconds, annealing and extension at 62°C for 20 seconds for 45 cycles. PCR was done using the Smart Cycler (Cepheid, Sunnyvale, CA) with Smart Cycler software (version 1.2b).

Construction of reporter plasmids. To construct luciferase reporter plasmids, various target fragments were inserted at the *Xba*I site, downstream of the luciferase gene in the pGL3-promoter vector. The sequence from +4,358 to +4,381 in the human *CYP1B1* gene (5′-CAGAACTTAGCCTT-TACCTGTGAA-3′) was termed miR-27b recognition element (MRE27b). The fragment containing three copies of the MRE27b, 5′-CTAGATTCATGTCC-*CAGAACTTAGCCTTTACCTGTGAA*GTGTTCATGTCC*CAGAACTTAGCCTT-TTACCTGTGAA*GTGTTCATGTCC*CAGAACTTAGCCTTTACCTGTGAA*GTG-3′ (MRE27b is italicized), was cloned into the pGL3-promoter vector, resulting in single and double insertions. These plasmids were termed pGL3/1B1MREx3 and pGL3/1B1MREx6, respectively. A fragment containing the perfect matching sequence with the mature miR-27b, 5′-CTAGA*CAGAACT-TAGCCACTGTGAA*T-3′ (the matching sequence of miR-27b is italicized), was cloned into the pGL3-promoter vector (pGL3/miR-27b). The region 1 (+4,311 to +4,439) containing the MRE27b and the region 2 (+3,899 to +4,019) in the human *CYP1B1* gene (Fig. 1*A*) were amplified by PCR using the following primers adapted to the *Xba*I site: 5′-TTTTCTAGATGTCT-CAGGTTTGTTTT-3′ and 5′-GAATCTAGAATGCAACTATTTGATCT-3′ for region 1 and 5′-GCTCTAGATGCCTCATTATGTCAACCA-3′ and 5′-GCTCTA-GACCTTACCTTTCTTCCATATAAA-3′ for region 2. The pGL3-promoter plasmids containing regions 1 and 2 were termed pGL3/1B1UTR1 and pGL3/1B1UTR2 plasmids, respectively. The complementary sequence of region 1 was also cloned into the pGL3-promoter plasmid (pGL3/1B1UTR1rev). The nucleotide sequences of the constructed plasmids were confirmed by DNA sequencing analyses.

Luciferase assay. For luciferase assays, various luciferase reporter plasmids (pGL3) were transiently transfected with phRL-TK plasmid into MCF-7 and Jurkat cells. Briefly, the day before transfection, the cells were seeded into 24-well plates, and 24 hours later, 380 ng of pGL3 plasmid and 20 ng of phRL-TK plasmid were transfected using Tfx-20 reagent for MCF-7 cells or LipofectAMINE 2000 for Jurkat cells. In some cases, various doses of the precursors for miR-27b or control or the AsOs for miR-27b or control were cotransfected with reporter plasmids. After incubation for 48 hours,

Figure 1. Homology between CYP1B1 mRNAs and the predicted target sequence of miR-27b. *A*, CYP1B1 mRNA in human, mouse, and rat. *Parentheses,* accession numbers. CYP1B1 mRNAs are 5.0 to 5.2 kb in length and consist of three exons. The numbering refers to the ATG in translation starting with A as 1, and the coding region of human CYP1B1 is up to +1,632. *Gray,* highly conserved regions. Homology to each region of human CYP1B1 mRNA. *Arrows,* location and direction of PCR primers for amplification of regions 1 (+4,311 to +4,439) and 2 (+3,899 to +4,019). *B,* sequence of MRE27b is located on +4,358 to +4,381 in the 3′-UTR of human CYP1B1 mRNA. *Gray boxes,* conserved nucleotides; *black boxes,* MRE27b.

the cells were resuspended in passive lysis buffer and then the luciferase activity was measured with a luminometer (Wallac, Turku, Finland) using the dual-luciferase reporter assay system.

Stable expression of recombinant human CYP1B1 in HEK293 cells. A fragment containing the full-length coding region and $3'$-UTR of human *CYP1B1* cDNA (from -21 to $+4,756$) was amplified by PCR using the primers of $5'$-GAAACCGCACCTCCCCG-$3'$ and $5'$-AAAGTATATTAAA-CAAAGTTTC-$3'$. It was subcloned into the pTARGET vector. The nucleotide sequences of the plasmid (pTARGET/CYP1B1) were confirmed by DNA sequencing analyses. HEK293 cells were seeded into six-well plates, and 2 μg of pTARGET/CYP1B1 plasmid were transfected using LipofectAMINE according to the manufacturer's protocols. When the cells reached 60% confluence, they were diluted from 1:10 to 1:200 and subjected to 400 μg/mL G418. The medium was renewed every week, and colonies of stably transfected cells (HEK293/1B1 cells) were isolated and expanded.

Electroporation of AsO and precursor for miR-27b. MCF-7 and HEK293/1B1 cells were washed twice with PBS and resuspended in HEPES-buffered saline [10 mmol/L HEPES (pH 7.3), 140 mmol/L NaCl] with 6 mmol/L glucose at 6×10^5 and 1×10^6 cells per pulse, respectively. A 250-μL aliquot of cells was added to a 0.4-cm gap electroporation cuvette (Bio-Rad, Hercules, CA) with 75, 125, 750, or 1,125 pmol of AsOs or 50, 100, 200, or 400 pmol of precursor and then incubated at $4°C$ for 10 minutes. The cells were then electroporated using a Gene Pulser II (Bio-Rad) at 220 V and 950 μF for MCF-7 cells and at 245 V and 950 μF for HEK293/1B1 cells and grown in the medium for 24 to 96 hours. After the incubation, the protein level and enzymatic activity of CYP1B1 were determined as described below.

SDS-PAGE and Western blot analyses. To prepare microsomes, MCF-7 cells seeded into 10-cm dishes were harvested and homogenized with homogenization buffer [0.1 mol/L Tris-HCl, 0.1 mol/L KCl, 1 mmol/L EDTA (pH 7.4)] and centrifuged at $19,000 \times g$, $4°C$ for 20 minutes. The supernatant was centrifuged at $105,000 \times g$ for 1 hour at $4°C$, and the precipitate was resuspended in TGE buffer [10 mmol/L Tris-HCl, 20% glycerol, 1 mmol/L EDTA (pH 7.4)]. To prepare the whole-cell lysate, HEK293/1B1 cells seeded into 10-cm dishes were harvested and homogenized with lysis solution [8 mol/L urea, 4% CHAPS, 2% Pharmalyte (pH 3-10)] containing protease inhibitors (1 mmol/L DTT, 0.5 mmol/L amidinophenyl methanesulfonyl fluoride hydrochloride, 2 μg/mL aprotinin, 2 μg/mL pepstatin, 2 μg/mL leupeptin). After centrifuging at $12,000 \times g$ for 1 hour at $4°C$, the supernatant was collected. The microsomal protein (30 μg) or whole-cell lysate (10 μg) was separated by 7.5% SDS-PAGE. The gel was transferred onto nitrocellulose membrane and probed with rabbit anti-human CYP1B1 or rabbit anti-human CYP1A1 antibodies. Biotinylated anti-rabbit IgG and Vectastain avidin-biotin complex method (ABC) kit (Vector Laboratories, Burlingame, CA) were used for diaminobenzidine staining.

Enzymatic activity. The enzymatic activity of CYP1B1 was determined using a P450-Glo Assay kit (Promega). After the electroporation, the cells seeded into 24-well plates were treated with 10 nmol/L TCDD for the last 24 hours, and then the medium was replaced with medium containing 20 μmol/L Luciferin $6'$ chloroethyl ether. After incubation for 8 hours at $37°C$ under an atmosphere of 5% CO_2-95% air, 100 μL of the medium were added to 100 μL of Luciferin Detection Reagent. After incubation for 20 minutes at room temperature, the luminescence was measured with a luminometer. The protein concentrations of the cells were determined using Bradford protein assay reagent (Bio-Rad) with γ-globulin as the standard. The enzymatic activity was normalized with the protein content.

Human breast cancerous and adjacent noncancerous tissues. Breast cancerous and adjacent noncancerous tissues were obtained as surgical samples from 24 Japanese patients with primary breast carcinoma. The patients (ages 41-77 years) were nonsmokers and had not undergone chemotherapy. By standard histopathologic criteria, 21 patients were diagnosed as invasive ductal carcinoma and 3 patients as invasive lobular carcinoma. The histologic grade was determined by standard criteria (22) as grade 1 ($n = 1$), grades 1 to 2 ($n = 13$), grade 2 ($n = 9$), and grades 2 to 3 ($n = 1$). The samples were obtained immediately after resection, divided into breast cancerous and adjacent noncancerous tissues, and immediately frozen with liquid nitrogen. The samples were stored at $-80°C$

until use. The expression levels of miR-27b in human breast cancerous and adjacent noncancerous tissues were determined by RNase protection assay and real-time reverse transcription-PCR (RT-PCR) as described above. This study was approved by the Ethics Committee of Kanazawa University. Written informed consent was obtained from all subjects before their participation in this study.

Immunohistochemistry. Immunohistochemical analyses of CYP1B1 were done using formalin-fixed, paraffin-embedded specimens of breast cancerous tissues from 24 patients. The sections were soaked in Antigen Retrieval Citra Solution (BioGenex, San Ramon, CA) at room temperature for 10 minutes and then incubated with anti-human CYP1B1 antibodies at $4°C$ for 16 hours. About the antibodies, no significant cross-reactivity to either human CYP1A1 or CYP1A2 protein has been reported (23). Staining was done using a Vectastain ABC kit. The extent of immunostaining was evaluated by the intensity of staining (score, 1-3), the localization in cytoplasm (score, 1-3), and area of staining (score, 1-4). Based on the combined scores, the samples were divided into three groups as weak (score, 3-5), moderate (score, 6-7), and strong (score, 8-10) staining of CYP1B1 protein. Three independent pathologists judged the results.

Statistical analyses. Data are expressed as mean ± SD of triplicate determinations. Statistical significance was determined by ANOVA and Dunnett multiple comparisons test. Comparison of two groups was made with an unpaired, two-tailed Student's t test. Correlations between the results obtained by RNase protection assay and real-time RT-PCR were determined by Pearson's product-moment method. The statistical significance of differences between the expression level of miR-27b in breast cancerous tissues and adjacent noncancerous tissues was determined by paired, two-tailed Student's t test. The relationships between the immunostained CYP1B1 level and miR-27b level in human breast cancerous tissues were investigated by ANOVA and Tukey method test. A $P < 0.05$ was considered statistically significant.

Results

A miR-27b complementary sequence on the $3'$-UTR of human CYP1B1 mRNA. In the human CYP1B1 mRNA (5.2 kb), the $3'$-UTR is extremely long (~ 3 kb; Fig. 1*A*). When the sequences of the CYP1B1 mRNA were compared between human, mouse, and rat, the homology of the coding region was extremely high (>80%). In addition, high homology was found in the $3'$-UTR near the polyadenylation site of 44 nucleotides in length (from $+4,344$ to $+4,387$). A near-perfect matching sequence with miR-27b was identified (from $+4,358$ to $+4,381$) using the miRNA registry release 7.1 (Fig. 1*B*; ref. 24).[3] This region was termed the miR-27b recognition element (MRE27b). We investigated whether miR-27b might be involved in the regulation of human CYP1B1 expression through the MRE27b.

Expression levels of miR-27b in human cancer cell lines. A RNase protection assay was done to determine the expression level of mature miR-27b in various human cancer cell lines (Fig. 2*A*). The mature miR-27b was detected in HeLa and MCF-7 cells but not in Jurkat and HEK293 cells. The expression level of pre-miR-27b was determined by real-time RT-PCR (Fig. 2*B*). Consistently, HeLa and MCF-7 cells showed significantly high expression of pre-miR-27b compared with Jurkat and HEK293 cells.
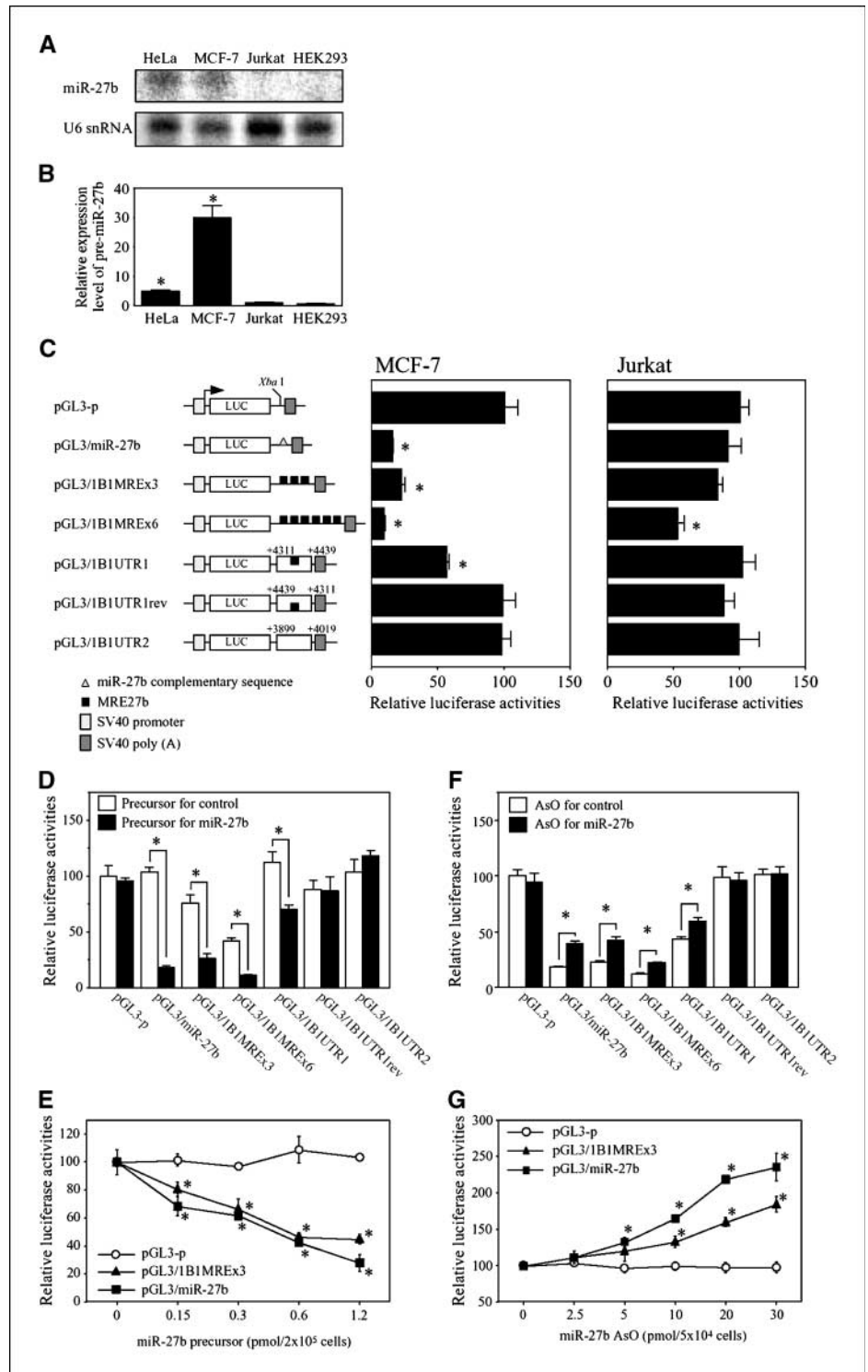
Luciferase assays in MCF-7 or Jurkat cells. Luciferase assays were done using various reporter constructs in MCF-7 and Jurkat cells (Fig. 2*C*). In miR-27b-positive MCF-7 cells, the reporter activity of the pGL3/miR-27b plasmid was significantly lower than that of the pGL3-promoter plasmid. The reporter activities of the pGL3/1B1MREx3 and pGL3/1B1MREx6 plasmids containing multiple
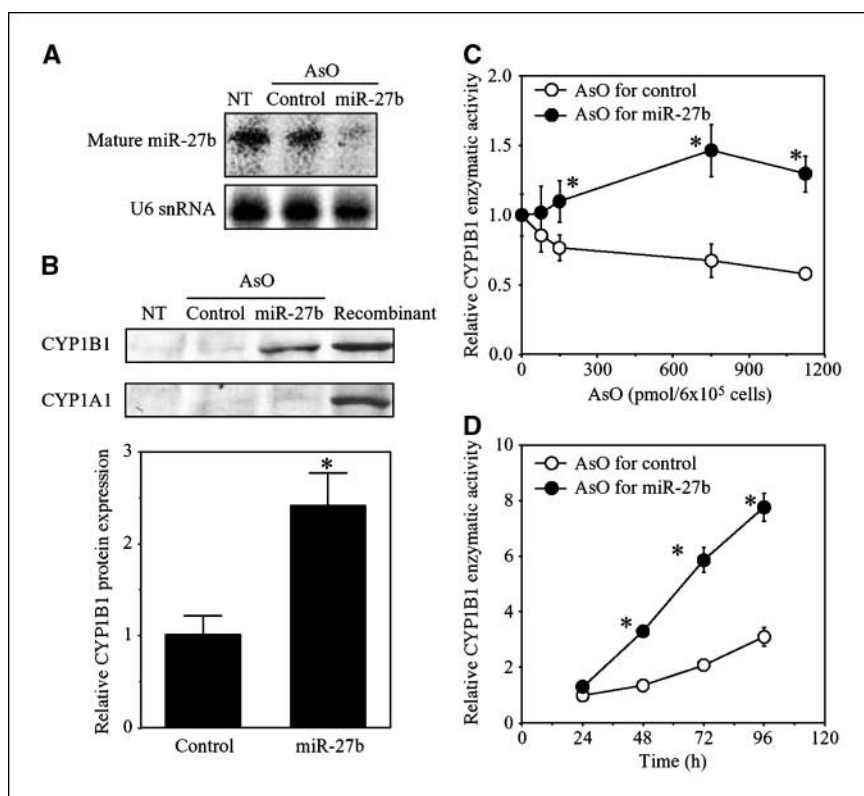
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[3] http://www.sanger.ac.uk/Software/Rfam/mirna/.

copies of MRE27b were also significantly lower than that of pGL3-promoter plasmid. In addition, the pGL3/1B1UTR1 plasmid showed significantly lower reporter activity but the pGL3/1B1UTR1rev and pGL3/1B1UTR2 plasmids did not. In contrast, only the reporter activity of the pGL3/1B1MREx6 plasmid was decreased in miR-27b-negative Jurkat cells. These results suggest that the 3′-UTR of human CYP1B1 represses the activity in association with the expression of miR-27b.

Effects of overexpression or inhibition of miR-27b on luciferase activity. To investigate whether miR-27b might control the luciferase activity, the precursor for miR-27b was exogenously expressed in Jurkat cells (Fig. 2D and E). The overexpression of miR-27b significantly decreased the luciferase activities of the pGL3/miR-27b (17% of control), pGL3/1B1MREx3 (34% of control), pGL3/1B1MREx6 (26% of control), and pGL3/1B1UTR1 (62% of control) plasmids (Fig. 2D). As shown in Fig. 2E, the precursor for

Figure 2. Expression level of miR-27b in various human cancer cell lines and luciferase assays with reporter constructs containing MRE27b in human CYP1B1 in MCF-7 and Jurkat cells. *A,* expression levels of mature miR-27b in HeLa, MCF-7, Jurkat, and HEK293 cells were determined by RNase protection assay. U6 snRNA was used as a loading control. *B,* expression of pre-miR-27b in cells was determined by real-time RT-PCR. Values are the pre-miR-27b level normalized with the U6 snRNA level relative to that in Jurkat cells. *Columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.01$, compared with Jurkat cells. *C,* a series of reporter constructs containing the 3′-UTR of the human *CYP1B1* gene was transiently transfected into MCF-7 or Jurkat cells. Relative luciferase activities were normalized with the *Renilla* luciferase activities. Values are expressed as percentages of the relative luciferase activity of pGL3-promoter plasmid. *Columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.01$, compared with pGL3-promoter. *D,* a series of reporter constructs containing the 3′-UTR of the human *CYP1B1* gene was transiently transfected into Jurkat cells with 1.2 pmol/2×10^5 cells of the precursors for miR-27b or control. *E,* various concentrations of the precursors for miR-27b were transiently transfected into Jurkat cells with luciferase reporter plasmids. *F,* a series of reporter constructs containing the 3′-UTR of the *CYP1B1* gene was transiently transfected into MCF-7 cells with 30 pmol/5×10^4 cells of the AsO for miR-27b or control. *G,* various concentration of the AsO for miR-27b were transiently transfected into MCF-7 cells with luciferase reporter plasmids. Relative luciferase activity was normalized to the *Renilla* luciferase activity. *Points,* mean of three independent experiments; *bars,* SD. *, $P < 0.01$, compared with the precursor for control or the AsO for control (*D* and *F*). *, $P < 0.01$, compared with pGL3-promoter without precursor or AsO (*E* and *G*).

Figure 3. Effects of AsO for miR-27b on the protein level and enzymatic activity of endogenous CYP1B1 in MCF-7 cells. *A* and *B*, AsOs for miR-27b or control (750 pmol/6×10^5 cells) were electroporated into MCF-7 cells. After 72 hours, the cells were harvested and total RNA and microsomes were isolated. *A*, expression levels of mature miR-27b and U6 snRNA were determined by RNase protection assays. *B*, expression levels of CYP1B1 and CYP1A1 protein were determined by Western blot analyses. The recombinant human CYP1B1 protein (50 fmol) and CYP1A1 protein (50 fmol) were used as controls. *Columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.01$, compared with the AsO for control. *C,* various concentrations of the AsOs for miR-27b or control were electroporated into MCF-7. After 72 hours, the CYP1B1 enzymatic activities were determined by P450-Glo assay. Values are expressed relative to the activity without AsO. *Points,* mean of three independent experiments; *bars,* SD. *, $P < 0.01$, compared with the AsO for control. *D,* after the electroporation with 750 pmol/6×10^5 cells of the AsOs for miR-27b or control, the MCF-7 cells were incubated for 24 to 96 hours and then the enzymatic activities were measured. Values are expressed relative to the activity with AsO for 24 hours. *Points,* mean of three independent experiments; *bars,* SD. *, $P < 0.01$, compared with the AsO for control.

miR-27b decreased the luciferase activities of pGL3/miR-27b and pGL3/1B1MREx3 plasmids in a concentration-dependent manner.

To investigate the effect of inhibition of endogenous miR-27b on the luciferase activity, AsO for miR-27b was transfected in MCF-7 cells (Fig. 2*F* and *G*). The transiently transfected AsO for miR-27b significantly increased the luciferase activities of the pGL3/miR-27b (2.1-fold of control), pGL3/1B1MREx3 (1.8-fold of control), pGL3/1B1MREx6 (1.8-fold of control), and pGL3/1B1UTR1 plasmids (1.4-fold of control; Fig. 2*F*). As shown in Fig. 2*G*, the AsO for miR-27b increased the luciferase activities of pGL3/miR-27b and pGL3/1B1MREx3 plasmids in a concentration-dependent manner. These results suggest that miR-27b recognized the MRE27b on the human CYP1B1 mRNA and regulated the expression post-transcriptionally.

Effects of inhibition of miR-27b on protein level and enzymatic activity of endogenous CYP1B1 in MCF-7 cells. We investigated the effects of the inhibition of miR-27b on the protein level and enzymatic activity of endogenous CYP1B1. A RNase protection assay revealed that the endogenous miR-27b level was greatly decreased by the transfection of the AsO for miR-27b in MCF-7 cells (Fig. 3*A*). As shown in Fig. 3*B*, the CYP1B1 protein level was significantly increased by the transfection of the AsO for miR-27b. That there was no change of the CYP1A1 protein level indicated that the effects of the AsO for miR-27b were specific for CYP1B1 protein. The effects of the AsO for miR-27b on the enzymatic activity of CYP1B1 were examined by a P450-Glo assay. The enzymatic activity of CYP1B1 was increased by the electroporation of the AsO for miR-27b in MCF-7 cells in concentration- and time-dependent manners (Fig. 3*C* and *D*).
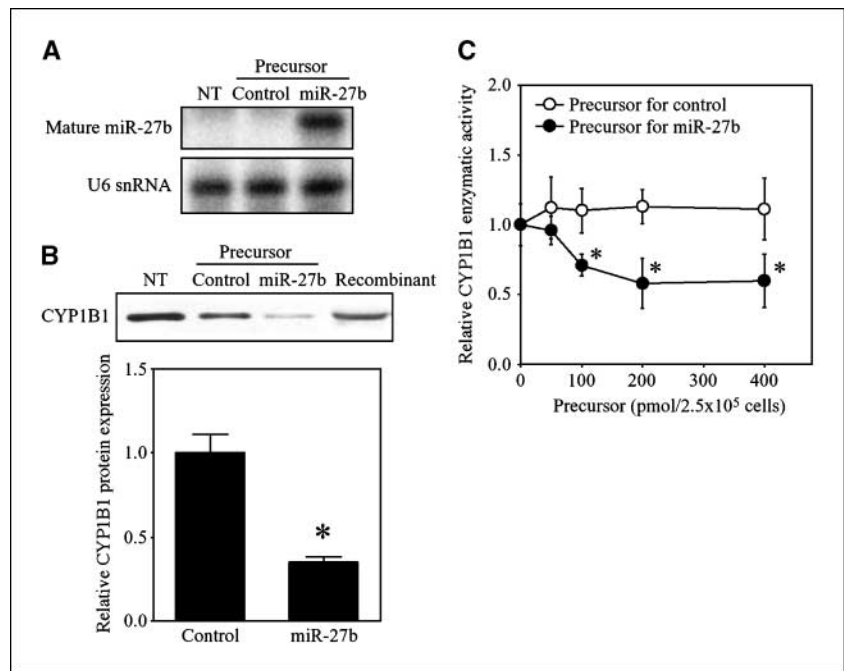
Effects of overexpression of miR-27b on protein level and enzymatic activity of exogenous CYP1B1 in HEK293 cells. HEK293/1B1 cells were used to investigate the effects of overexpression of miR-27b on the protein level and enzymatic activity

of CYP1B1. A RNase protection assay revealed that the miR-27b level was greatly increased by the transfection of the precursor for miR-27b in HEK293/1B1 cells (Fig. 4*A*). As shown in Fig. 4*B*, the CYP1B1 protein level was significantly decreased by the transfection of the precursor for miR-27b. P450-Glo assays showed that the enzymatic activity of CYP1B1 was decreased by the electroporation of the precursor for miR-27b in HEK293/1B1 cells in a concentration-dependent manner (Fig. 4*C*). These results suggest that miR-27b regulates the protein level and enzymatic activity of CYP1B1.

CYP1B1 protein level in human breast cancer. To investigate whether miR-27b affects the CYP1B1 expression *in vivo*, the expression levels of CYP1B1 protein in breast cancerous tissues from 24 patients were determined by immunohistochemistry (Fig. 5). All of breast cancers showed positive immunoreactivity for CYP1B1, and in each case, CYP1B1 was specifically localized to cancer cells. In most samples, the CYP1B1 protein was localized in the cytoplasm, but in some samples, the nuclei were also stained. The extent of staining varied among samples (Fig. 5*A* to *C*). According to the scoring, samples from 6, 11, and 7 patients were categorized to groups I (weak staining), II (moderate staining), and III (strong staining), respectively. No staining was observed in normal rabbit IgG (Fig. 5*D*).

Inverse association between expression level of miR-27b and CYP1B1 protein in human breast cancer. RNase protection assays require abundant RNA compared with real-time RT-PCR. Because the RNA quantities obtained from human samples were limited, we investigated whether the pre-miR-27b level determined by real-time RT-PCR can substitute for the mature miR-27b levels determined by RNase protection assay. Breast cancerous and adjacent noncancerous tissues from 11 patients were used for RNase protection assay and real-time RT-PCR analysis. Figure 6*A*

Figure 4. Effects of precursor for miR-27b on protein level and enzymatic activity of exogenous CYP1B1 in HEK293/1B1 cells. *A* and *B,* precursors for miR-27b or control (200 pmol/2.5 × 10⁵ cells) were electroporated into HEK293/1B1 cells. After 48 hours, the cells were harvested and total RNA and whole-cell lysate were isolated. *A,* expression levels of mature miR-27b and U6 snRNA were determined by RNase protection assays. *B,* expression levels of CYP1B1 protein was determined by Western blot analysis. Recombinant human CYP1B1 protein (500 fmol) was used as a control. *Columns,* mean of three independent experiments; *bars,* SD. *, P < 0.001, compared with the precursor for control. *C,* various concentrations of the precursors for miR-27b or control were electroporated into HEK293/1B1 cells. After 48 hours, the CYP1B1 enzymatic activities were determined by P450-Glo assay. Values are expressed relative to the activity without precursor. *Points,* mean of three independent experiments; *bars,* SD. *, P < 0.05, compared with the precursor for control.
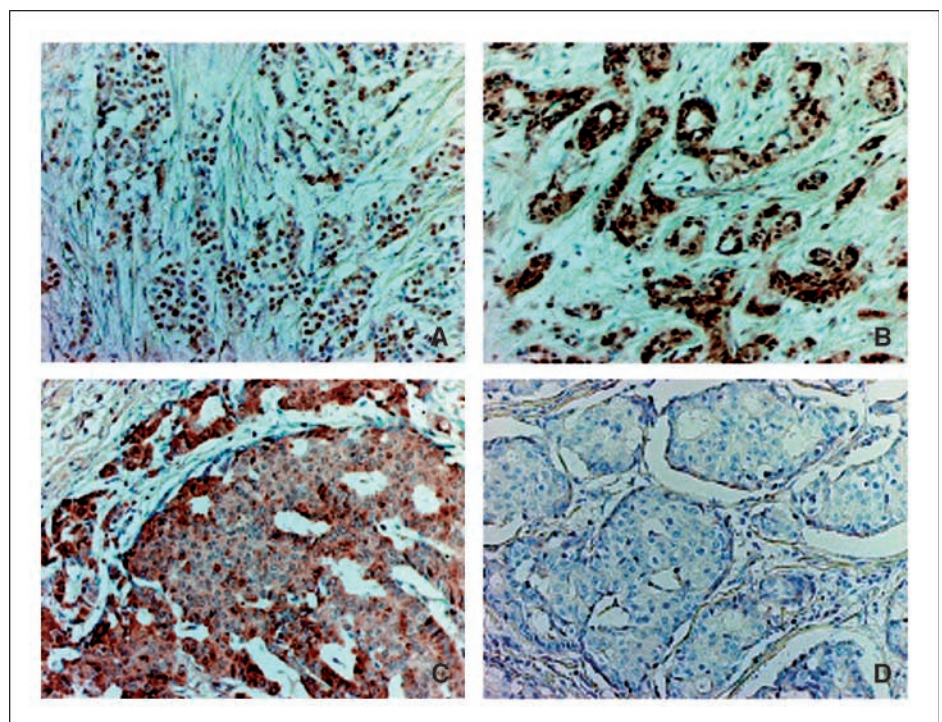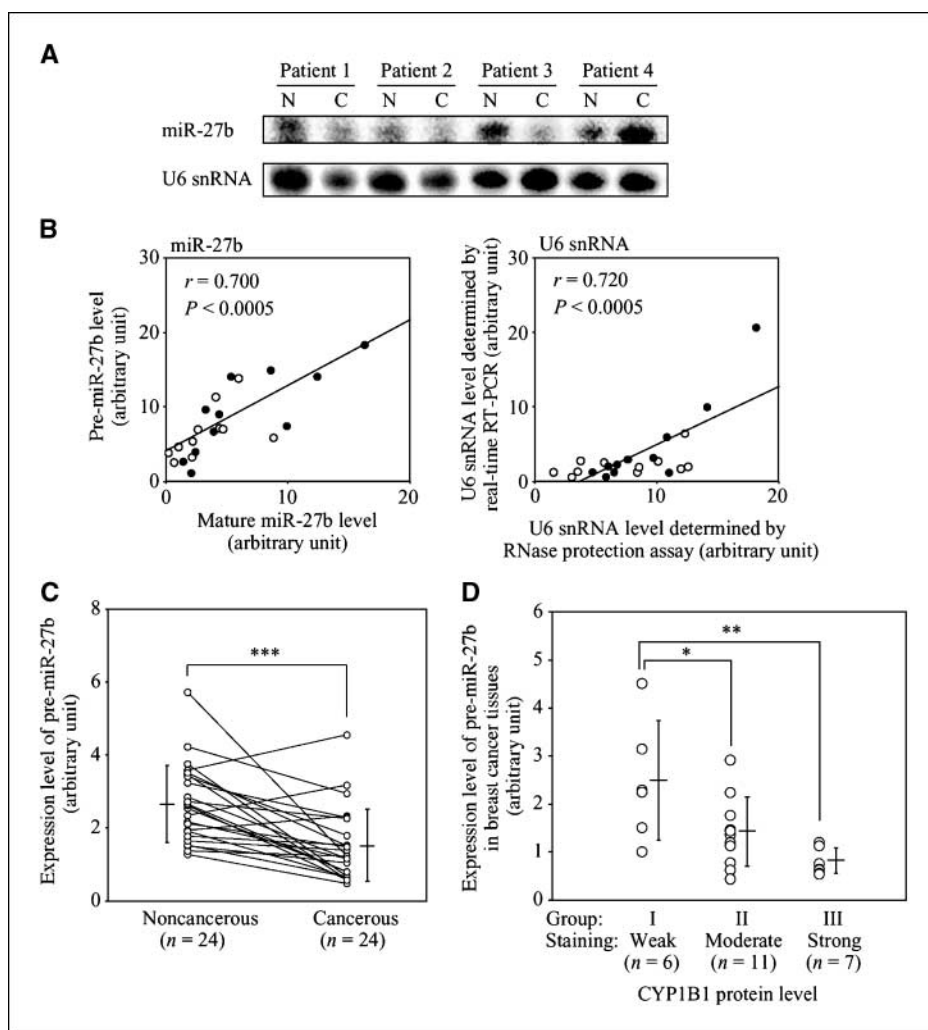
shows a typical autoradiogram of the mature miR-27b levels in four patients. In three of four patients, the mature miR-27b levels in the cancer tissues were lower than those in noncancerous tissues. A significant correlation ($r = 0.700$; $P < 0.0005$) was observed between the mature miR-27b levels and pre-miR-27b levels (Fig. 6B). In addition, a significant correlation ($r = 0.720$; $P < 0.0005$) was observed between the U6 snRNA levels determined by the RNase protection assay and those determined by real-time RT-PCR. Accordingly, we evaluated the miR-27b level normalized with U6

snRNA in breast cancerous and adjacent noncancerous tissues from 24 patients by real-time RT-PCR. Consequently, it was clearly shown that the expression level of pre-miR-27b in cancerous tissues (0.48-4.55, 1.52 ± 0.99) was significantly ($P < 0.0005$) lower than that in noncancerous tissues (1.28-5.71, 2.66 ± 1.06; Fig. 6C).

We investigated the relationship between the miR-27b level and CYP1B1 protein level in human breast cancers (Fig. 6D). The expression levels of pre-miR-27b in each group were as follows: group I (2.49 ± 1.25), group II (1.43 ± 0.72), and group III

Figure 5. Immunohistochemical staining of CYP1B1 protein in breast cancerous tissues. Immunohistochemical analyses were done in human breast cancerous tissues using anti-human CYP1B1 antibodies. *A,* grades 1 to 2 invasive ductal carcinoma. CYP1B1 immunostaining is weakly detected in the nucleus (score 3). *B,* grades 1 to 2 invasive ductal carcinoma. CYP1B1 immunostaining is moderately observed in the cytoplasm and nucleus (score 6). *C,* grade 2 invasive ductal carcinoma. Strong immunostaining of CYP1B1 in the cytoplasm was observed (score 8). *D,* there was no staining with normal rabbit IgG. Original magnification, ×200.

Figure 6. Expression of miR-27b in human breast cancerous tissues and relationship with CYP1B1 protein level. *A,* expression levels of mature miR-27b in breast cancerous (*C*) and adjacent noncancerous (*N*) tissues from four patients were determined by RNase protection assay. U6 snRNA was used as a loading control. *B, left,* correlation was observed between the mature miR-27b levels and pre-miR-27b; *right,* correlation was observed between the expression levels of U6 snRNA determined by RNase protection assay and real-time RT-PCR. Breast cancerous (●) and noncancerous tissues (○) from 11 patients were analyzed. *C,* comparison between the expression levels of pre-miR-27b in breast cancerous and adjacent noncancerous tissues obtained from 24 patients. Expression level of pre-miR-27b was determined by real-time RT-PCR and normalized with U6 snRNA level. *Horizontal bars,* mean; *bars,* SD. ***, $P < 0.0005$, paired Student's *t* test. *D,* inverse association between the expression levels of miR-27b and CYP1B1 protein in human breast cancer. According to the scoring of CYP1B1 immunohistochemistry, 24 samples were divided to three groups: group I (weak staining, 6 samples), group II (moderate staining, 11 samples), and group III (strong staining, 7 samples). *Y axis,* expression level of pre-miR-27b normalized with U6 snRNA in breast cancerous tissues. *Horizontal bars,* mean; *bars,* SD, *, $P < 0.05$; **, $P < 0.01$.

(0.82 ± 0.27). Significant differences were observed between groups I and II ($P < 0.05$) or III ($P < 0.01$). Thus, an inverse association was observed between the expression level of miR-27b and the CYP1B1 protein level in human breast cancer. In contrast to the CYP1B1 protein level, no relationship was observed between the CYP1B1 mRNA level and the miR-27b level in human breast tissues (data not shown). These results suggested that CYP1B1 is post-transcriptionally regulated by miR-27b. The decreased expression of miR-27b would be one of the causes of the high expression of CYP1B1 protein in cancerous tissues.

Discussion

miRNAs are a recently discovered family of short noncoding RNA whose final product is a ∼22-nucleotide functional RNA molecule. They play important roles in the regulation of target genes by binding to complementary regions of transcripts to repress their translation or regulate degradation. As many as 1,000 miRNA genes are thought to exist in the human genome (3). Some miRNAs are reported to be associated with physiologic functions, such as differentiation, development, and disease. Because miRNAs have only very recently received attention, the target genes of miRNAs are not completely understood yet. In the present study, we examined whether human CYP1B1, which is a member of CYP and catalyzes the metabolism of procarcinogens and estradiol, might be a target of miRNA.

We identified MRE27b in the 3′-UTR in CYP1B1 mRNA. Luciferase assays showed that the endogenous and exogenous miR-27b negatively regulated the activity through MRE27b. The AsO for miR-27b could restore the protein level and enzymatic activity of endogenous CYP1B1, whereas the precursor for miR-27b decreased the protein expression and enzymatic activity of exogenous CYP1B1. These results clearly indicated that the expression of human CYP1B1 is post-transcriptionally regulated by miR-27b. It is well known that CYP1B1 is transcriptionally regulated, as we (25–27) and others (28–30) previously reported the involvement of aryl hydrocarbon receptor, Sp1, estrogen receptor, and steroidogenic factor-1. Thus, in addition to the transcriptional regulation, we found that the post-transcriptional regulation is also responsible for the CYP1B1 expression. The sequences of mRNA around MRE27b are highly conserved among species (Fig. 1). Therefore, the regulation by miR-27b may occur in other species.

miR-27b is highly expressed in human normal breast (31). Recent studies have reported that the miRNA expression levels are changed with the development of tumors, such as those of lung cancer (7), chronic lymphocytic leukemias (9), colorectal neoplasia (10), large cell lymphoma (32), and glioblastoma (33). Thus, many miRNAs are differentially expressed in different cancers. The

miRNAs are generally down-regulated but sometimes up-regulated in cancers. In the present study, we found that the miR-27b level is decreased in breast cancerous tissues compared with noncancerous tissues. Immunohistochemical analyses revealed the high expression of CYP1B1 protein in breast cancerous tissues in accordance with previous studies (19, 34). The high expression of CYP1B1 protein in cancer cells would result from the decreased expression of miR-27b. The patients in the present study were both estrogen receptor–positive and progesterone receptor–positive. No association was observed between the levels of these receptors and the miR-27b or CYP1B1 level (data not shown). Furthermore, the miR-27b or CYP1B1 level was not associated with the presence or absence of lymph node metastasis (10 of 24 patients had lymph node metastasis). Thus, the biopathologic features or tumor stage of breast cancer would not affect the inverse association between the miR-27b and CYP1B1 levels. Highly expressed CYP1B1 in breast cancer would enhance the metabolism of 17β-estradiol. Whereas 17β-estradiol contributes to the growth and development of estrogen-dependent cancers, such as breast and endometrial cancers (16), 4-hydroxyestradiol formed by CYP1B1 causes DNA damage (17, 18). Thus, the abnormal expression of CYP1B1 would be related to the development of estrogen-dependent cancer.

More than half of the human miRNA genes are located at sites known to be involved in cancers, such as fragile sites, minimal regions of loss of heterozygosity, minimal regions of amplification, or common break point regions. Such locations support the notion that some miRNAs are involved in tumorigenesis. Calin et al. (35) reported that the gene coding miR-27b is located on the locus that is deleted in some cancers, such as urothelial or bladder cancer. It is plausible that the miR-27b would be down-regulated in these cancers. Human CYP1B1 is also expressed in urothelial and bladder tissues (36). The regulation of CYP1B1 by miR-27b would occur in these tissues, and the high CYP1B1 levels in urothelial or bladder cancer (36) might be due to the decreased miR-27b level.

The gene coding miR-27b is located on human 9q22.1, clustering with miR-23b and miR-24-1. Because these miRNAs are components of the same transcriptional unit (gi|4105182; ref. 37), the expression profiles of these miRNAs are considered to be in parallel. A moderate pairing with miR-24-1 is found at the neighborhood of MRE27b from +4,347 to +4,370 on the human *CYP1B1* gene, although the pairing probability is lower than that of miR-27b (miR-24-1, the score is 144 and the energy is −15.4 kcal/mol; miR-27b, the score is 158 and the energy is −29.5 kcal/mol; ref. 38).[4] miR-27a, which is a paralogous miRNA of miR-27b, has one nucleotide mismatch with the miR-27b and its pairing is possible (the score is 151 and the energy is −25.9 kcal/mol). Thus, the possibility that miR-24-1 or miR-27a may regulate the CYP1B1 expression cannot be excluded.

CYP1B1 is also expressed in eye tissue (39). Mutations or genetic polymorphisms of CYP1B1 are associated with primary congenital glaucoma (40, 41), and structural defect in eyes has been found in cyp1b1 knockout mice (42), indicating that CYP1B1 is responsible for eye development. At present, there is no information about the expression of miRNAs in eye tissue. The possibility that CYP1B1 level might be modulated by miR-27b in eye tissue in relation with eye development is worth pursuing in the future.

In conclusion, the results presented here suggested that human CYP1B1 is post-transcriptionally regulated by miR-27b, which would serve as a possible mechanism for the high expression of CYP1B1 protein in cancerous tissues. The silencing mechanism by miRNA might be one of the key factors regulating the cell-specific expression as well as individual differences in the expression of CYPs.

Acknowledgments

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[4] http://www.microrna.org.

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