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 1B1 (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 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 miRNA 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-translationally regulated by miRNA.

Materials and Methods

Chemicals and reagents. The pGL3-promoter vector, pBIK-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′-CAGAACCUGCCACUGUGAA, in which I is 3′-aminolinker)
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and for negative control (5'-AGACUAGCGGUAAUCUUAAACCL) were from Dharmaco (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 Test (Woburn, 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 Nakashini (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% CO₂, 95% air.

RNAse protection assay. Total RNA was isolated from the cells using ISOGEN (Nippon Gene, Tokyo, Japan). Antisense RNA probes were synthesized by using the manufacturer’s protocol. The antisense RNA probes labeled with 32P]UTP using T7 RNA polymerase were hybridized to total RNA. The protected RNA probes were labeled with [α-32P]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. Total RNA was isolated from the cells using ISOGEN (Nippon Gene, Tokyo, Japan). Antisense RNA probes were synthesized by using the manufacturer’s protocol. The antisense RNA probes labeled with [α-32P]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. Total protected miRNAs were separated by electrophoresis through 15% polyacrylamide/8 mol/L urea gels with 1% Tris-borate EDTA (TBE)/8 mol/L urea gels with 1% Tris-borate EDTA (TBE). The protected miRNAs were detected and quantified with a FujiBio-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 CYP1B1 mRNA were 5'-CTTCCAGTGGCTAAGTTCTGCCTGTCTC-3' and 5'-AGAAGATTAGGATCCGCCCCTGGCCAGCGGCTGTC-3', respectively. The forward and reverse primers for human U6 snRNA were 5'-CGTTCCGGCAGCATACTACTA-3' and 5'-TATGGAAAGGCTTGG-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 into the XbaI site, downstream of the luciferase gene in the pGL3-promotor vector. The sequence from +3385 to +3591 in the human CYP1B1 gene (5'-CAGAACATTTGGCT- TACCTGGA-3') was cloned into the pGL3-promotor vector (pGL3/miR-27b). The fragment containing three copies of the MRE27b, 5'-CAGACACTTGACACTTGACACTTGACACTTG-3' (the matching sequence of miR-27b is italicized), was cloned into the pGL3-promotor vector, resulting in single and double insertions. These plasmids were termed pGL3/ 1B1MRE3x and pGL3/1B1MRE6x, respectively. A fragment containing the perfect matching sequence with the mature miR-27b, 5'-CAGACACTTGACACTTGACACTTG-3' (the matching sequence of miR-27b is italicized), was cloned into the pGL3-promotor vector (pGL3/miR-27b). The region 1 (+4311 to +4439) containing the MRE27b and the region 2 (+3899 to +4019) in the human CYP1B1 gene (Fig. 1A) were amplified by PCR using the following primers adapted to the XbaI site: 5'-TTTCTAGATGCTCAGGTTTTGTTT3' and 5'-GAATCTAGAATGCAACTATTTGATCT3'. The forward and reverse primers for human CYP1A1 mRNA were 5'-ACCTCTCTAACAAGGTGCA- GAGCTT-3' and 5'-ACCTATCTCAGGTTGACAACTTAG-3', respectively. The forward and reverse primers for human U6 snRNA were 5'-CGTTCCGGCAGCATACTACTA-3' and 5'-TATGGAAAGGCTTGG-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).

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, 100 ng of pGL3 plasmid and 20 ng of phRL-TK plasmid were transfected using 1μl-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,
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′-GAAGCCACCTCTCCGGG-3′ and 5′-AAATGTTATAAAACAAAGTTGTT-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®/C2 reagent according to the manufacturer’s protocols. When the cells reached 60% confluency, 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/C1B1 cells) were isolated and expanded.

**Electroporation of AsO and precursor for miR-27b.** MCF-7 and HEK293/C1B1 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 × 10^6 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 AsO5 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/C1B1 cells and grown in the medium for 24 to 96 hours. After the incubation, the protein levels 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 × g, 4°C for 20 minutes. The supernatant was centrifuged at 105,000 × 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/C1B1 cells seeded into 10-cm dishes were harvested and homogenized with lysis solution [8 mol/L urea, 4% CHAPS, 2% 20 mM sodium phosphate (pH 3-10)] containing protease inhibitors (1 mmol/L DTT, 0.5 mmol/L amiodinophenyl methanesulfonyl fluoride hydrochloride, 2 µg/mL aprotinin, 2 µg/mL pepstatin, 2 µg/mL leupeptin). After centrifuging at 12,000 × 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 mmol/L TCD for the last 24 hours, and then the medium was replaced with medium containing 20 µmol/L Luciferin 6′-chloetholyl ether. After incubation for 8 hours at 37°C under an atmosphere of 5% CO2-95% 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 antibody 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. 1A). 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, 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. 1B; ref. 24). 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. 2A). 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. 2B). 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. 2C). 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 pGL3/1B1MRE3 and pGL3/1B1MRE6 plasmids containing multiple
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
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. 2A 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. 2F). As shown in Fig. 2G, 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. 3A). As shown in Fig. 3B, 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. 3C 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. 4A). As shown in Fig. 4B, 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. 4C). 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. 5A 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. 5D).

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 6A
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
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 and CYP1B1 levels. Highly expressed CYP1B1 in breast cancer would not affect the inverse association between node metastasis (10 of 24 patients had lymph node metastasis). Thus, the biopathologic features or tumor stage of breast cancer (36) might be due to the decreased miR-27b level.

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.

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