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 the 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.

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 miRNA 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-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-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'-AGACUGUCCGAUUCUAAACCL) 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 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 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 leukemia T-cell line Jurkat was kindly provided by Dr. Yoshinobu Nakashiki (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 Invitrogen miRNA Probe Construction kit. The oligonucleotides used for miR-27b and U6 small nuclear RNA (snRNA) were 5'-TTACAGGAGCTCATGCTGCTCTC-3' and 5'-AGAAGATGATTGAGCGCTTCGGCAGCACATATACTAA-3', respectively. *RNAse* protection assays were done using a miVana miRNA Dtection kit according to 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. The protected miRNAs were separated by electrophoresis through 15% polyacrylamide/1× Tris-borate EDTA (TBE)/8 mol/L urea gels with 1% agarose. The 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 pre-miR-27b (pre-miR-27b) were 5'-ACCTCTTCAACAGGGTGAGAGCTTT-3' and 5'-ACCTCTTCAACAGGGTGAGAGCTTT-3', respectively. The forward and reverse primers for human U6 snRNA were 5'-CGCTTTGCGAGCACCATATAC-3' and 5'-TATGGAGGCTTCCAC-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 40 seconds, and for negative control (5'-AGACUGUCCGAUUCUAAACCL) 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 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 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.

**Construction of reporter plasmids.** To construct luciferase reporter plasmids, various target fragments were inserted at the XbaI site, downstream of the luciferase gene in the pGL3-promoter vector. The sequence from +3,358 to +3,881 in the human CYP1B1 gene (5'-CAGACTAGTACCTTACCAGTGCAGAACTTAGCCTTTACCTGTGAA-3') was cloned into the pGL3-promoter recognition element (MRE27b). The fragment containing three copies of the MRE27b, 5'-CATAGTCTAGTCAGACTAGTACCTTACCAGTGCAGAACTTAGCCTTTACCTGTGAA-3' (the matching sequence of miR-27b is italicized), was cloned into the pGL3-promoter vector, resulting in single and double insertions. These plasmids were termed pGL3/1B1MRE8x and pGL3/1B1MRE6x, respectively. A fragment containing the perfect matching sequence with the mature miR-27b, 5'-CATAGTCTAGTCAGACTAGTACCTTACCAGTGCAGAACTTAGCCTTTACCTGTGAA-3' (the matching sequence of miR-27b is italicized), was cloned into the pGL3-promoter vector (pGL3/miR-27b). The region 1 (+3,311 to +4,399) containing the MRE27b (italicized), was cloned into the pGL3-promoter vector, resulting in single and double insertions. These plasmids were termed pGL3/1B1MRE6x and pGL3/1B1MRE8x, respectively. A fragment containing the perfect matching sequence with the mature miR-27b, 5'-CATAGTCTAGTCAGACTAGTACCTTACCAGTGCAGAACTTAGCCTTTACCTGTGAA-3' (the matching sequence of miR-27b is italicized), was cloned into the pGL3-promoter vector (pGL3/miR-27b). The region 1 (+3,311 to +4,399) containing the MRE27b and the region 2 (+3,899 to +4,019) in the human CYP1B1 gene (Fig. 1A) were amplified by PCR using the following primers adapted to the XbaI site: 5'-TTTCTTCAATGCTCAGGTTTGTG-3' and 5'-GAATTGTTGTGTTGTGTTCATGTCC-3'. The PCR products were cloned into the T7- competent Escherichia coli (Invitrogen). MCF-7 cells were cultured in DMEM 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.

**Luciferase assay.** For luciferase assays, various luciferase reporter plasmids (pGL3) were transiently transfected with pRL-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 pRL-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, the luciferase activities were measured with a dual luciferase assay system (Promega, Madison, WI). The luciferase activities were normalized to the Renilla luciferase activity (pRL-TK) to control for variations in transfection efficiency.

**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 (+3,311 to +4,399) and 2 (+3,899 to +4,019). B, sequence of MRE27b is located on +3,358 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’-GAACACGACCTCCCGC-3’ and 5’-AAAGTTATTTAAACAAAGTTTC-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.

SDS-PAGE and Western blot analyses. To prepare microsomes, 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 × 10⁵ and 1 × 10⁶ 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.

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 × 10⁵ and 1 × 10⁶ 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.

**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, high homology was found in the 3’-UTR of 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%).

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 pGL3/1B1MRE5 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

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**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 × 10⁶ 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 × 10⁵ 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).
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. 2F 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 was significantly increased by the transfection of the AsO for miR-27b in HEK293 cells (Fig. 3C). 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). 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).

Reverse 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 regulatory 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 miRNA 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.
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 levels. Highly expressed CYP1B1 in the 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. 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 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 uterine or bladder cancer. It is plausible that the miR-27b would be down-regulated in these cancers. Human CYP1B1 is also expressed in uterine and bladder tissues (36). The regulation of CYP1B1 by miR-27b would occur in these tissues, and the high CYP1B1 levels in uterine 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 +347 to +437 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). 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.

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