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# Human CYP24 Catalyzing the Inactivation of Calcitriol Is Post-Transcriptionally Regulated by miR-125b

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## ABSTRACT

Human vitamin $D_3$ hydroxylase (CYP24) catalyzes the inactivation of 1$\alpha$,25-dihydroxyvitamin $D_3$ (calcitriol), which exerts antiproliferative effects. CYP24 has been reported to be overexpressed in various cancers in which microRNA levels are dysregulated. In silico analysis identified a potential miR-125b recognition element (MRE125b) in the 3′-untranslated region of human CYP24 mRNA. We investigated whether CYP24 is regulated by miR-125b. In luciferase assays using a reporter plasmid containing MRE125b, transfection of the antisense oligonucleotide (AsO) for miR-125b increased the reporter activity in KGN cells, and transfection of precursor miR-125b decreased the reporter activity in MCF-7 cells. The endogenous CYP24 protein level was also increased by AsO for miR-125b in KGN cells and was decreased by precursor miR-125b in MCF-7 cells. These results suggested that human CYP24 is regulated by miR-125b. Immunohistochemical analysis revealed that the CYP24 protein levels in human breast cancer were higher than in adjacent normal tissues, without an accompanying CYP24 mRNA increase. On the other hand, the expression levels of miR-125b in cancer tissues were significantly ($P < 0.0005$) lower than those in normal tissues. It is noteworthy that the CYP24 protein levels in cancer tissues were inversely associated with the cancer/normal ratios of the miR-125b levels, indicating that the decreased miR-125b levels in breast cancer tissues would be one of the causes of the high CYP24 protein expression. In conclusion, this study clearly demonstrates that miR-125b post-transcriptionally regulates the CYP24, which serves as a possible mechanism for the high CYP24 expression in cancer tissues.

Human CYP24 is a key enzyme involved in the inactivation of 1$\alpha$,25-dihydroxyvitamin $D_3$ [1,25$(OH)_2D_3$; calcitriol]. 1,25$(OH)_2D_3$ is typically considered a regulator of calcium homeostasis, but it has now received much interest for its antitumor activity (Deeb et al., 2007). For ensuring the appropriate biological effects of 1,25$(OH)_2D_3$, the balance between bioactivation and inactivation is critical. CYP24 has been reported to be overexpressed in various tumor cells (Deeb et al., 2007). Because CYP24 limits the biological activity of the vitamin D signaling system, the overexpression may abrogate the vitamin D-mediated growth control. In fact, it has been reported that the overexpression of CYP24 is associated with poor prognosis and overall reduced survival in patients with esophageal cancer (Mimori et al., 2004). As for the cause of the CYP24 overexpression, an amplification of the chromosomal region 20q13.2, where the *CYP24* gene is located, in human breast cancer was reported (Kallioniemi et al., 1994; Albertson et al., 2000). Albertson et al. (2000) found that the relative levels of CYP24 mRNA were higher in breast cancers with the amplification, although the number of samples was only three. Townsend et al. (2005) also reported increased CYP24 mRNA levels in breast cancers. On the other hand, de Lyra et al. (2006) reported that there was no difference in the CYP24 mRNA levels between breast cancer and normal tissues. In contrast, Anderson et al. (2006) reported that CYP24 mRNA was down-regulated in breast cancer relative to normal tissues. Although we cannot compare these expression profiles because the genetic background is heterogeneous in different breast cancer cells (Hicks et al., 2006), the overexpression of CYP24 protein is not necessarily associated with the increased CYP24 mRNA level. This background allowed us to postulate the

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**ABBREVIATIONS:** miRNA, microRNA; 1,25$(OH)_2D_3$, 1$\alpha$,25-dihydroxyvitamin $D_3$; MRE125b, miR-125b recognition element; AsO, antisense oligonucleotide; UTR, untranslated region; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; RT-PCR, reverse transcription-polymerase chain reaction; VDR, vitamin D receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

involvement of a post-transcriptional mechanism in CYP24 regulation.

MicroRNAs (miRNAs) are endogenous ~22-nucleotide noncoding RNAs that regulate gene expression through the translational repression or degradation of target mRNAs (Bartel, 2004). The human genome may contain up to 1000 miRNAs, and 30% of human mRNAs are predicted to be targets of miRNAs (Lewis et al., 2005). Accumulating evidence has revealed an important role of miRNAs in cancer (Medina and Slack, 2008). In various cancers, the miRNA levels are dysregulated (Lu et al., 2005). In the present study, we investigated whether miRNAs may be involved in the regulation of the human CYP24 expression.

## Materials and Methods

**Chemicals and Reagents.** $1,25(OH)_2D_3$ and corticosterone were purchased from Wako Pure Chemical Industries (Osaka, Japan). $25(OH)D_3$ and $24,25(OH)_2D_3$ were from Funakoshi (Tokyo, Japan). The pGL3-promoter vector, phRL-TK plasmid, Tfx-20 reagent, and a dual-luciferase reporter assay system were purchased from Promega (Madison, WI). LipofectAMINE 2000 and LipofectAMINE RNAiMAX were from Invitrogen (Carlsbad, CA). PremiR miRNA precursors for miR-125b-1 and negative control 2 were from Ambion (Austin, TX). Antisense locked nucleic acid/DNA mixed oligonucleotides (AsO) for miR-125b (5′-TCACAAGTTAGGGTCTCAGGGA-3′, underlined letters show locked nucleic acid) and for negative control (5′-AGACTAGCGGTATCTTAAACC-3′) were from Greiner Japan (Tokyo, Japan). All primers and oligonucleotides were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Goat anti-human CYP24 polyclonal antibodies and Alexa Fluor 680 donkey anti-goat IgG were from Santa Cruz Biotechnology (Santa Cruz, CA) and Invitrogen, respectively. All other chemicals and solvents were of the highest grade available commercially.

**Cells and Culture Conditions.** The human breast adenocarcinoma cell lines MCF-7 and MDA-MB-435 and the human embryonic kidney (HEK) cell line 293 were obtained from the American Type Culture Collection (Manassas, VA). The human ovarian granulosa-like tumor cell line KGN (Nishi et al., 2001) and the human hepatoma cell line HepG2 were obtained from Riken Gene Bank (Tsukuba, Japan). MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.1 mM nonessential amino acid (Invitrogen) and 10% fetal bovine serum (FBS; Invitrogen). MDA-MB-435 cells and HepG2 cells were cultured in DMEM supplemented with 10% FBS. HEK293 cells were cultured in DMEM supplemented with 4.5 g/l glucose, 10 mM HEPES, and 10% FBS. KGN cells were cultured in a 1:1 mixture of DMEM and Ham's F-12 medium (Nissui Pharmaceutical) supplemented with 10% FBS. These cells were maintained at 37°C under an atmosphere of 5% $CO_2$/95% air.

**Northern Blot Analysis and Real-Time RT-PCR for Mature miR-125b.** Total RNA (20 $\mu$g) isolated from the cells using ISOGEN (Nippon Gene, Tokyo, Japan) was separated on 15% denaturing polyacrylamide gels containing 8 M urea. The RNA was then electrophoretically transferred to Zeta-Probe GT Genomic Tested Blotting Membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were probed with $^{32}$P-labeled DNA probe for miR-125b (5′-TCA CAA GTT AGG GTC TCA GGG A-3′), and then the miRNAs were detected and quantified with a Fuji Bio-Imaging Analyzer BAS 1000 (Fuji Film, Tokyo, Japan).

For quantification of mature miR-125b, polyadenylation and reverse transcription were performed using an NCode miRNA First-Strand cDNA Synthesis Kit (Invitrogen) according to the manufacturer's protocol. The forward primer for miR-125b was 5′-TCC CTG AGA CCC TAA CTT GTG A-3′, and the reverse primer was the supplemented universal quantitative polymerase chain reaction primer. The real-time polymerase chain reaction was performed using the Smart Cycler (Cepheid, Sunnyvale, CA) with Smart Cycler software (version 1.2b) as follows: after an initial denaturation at 95°C for 30 s, the amplification was performed by denaturation at 95°C for 10 s and annealing and extension at 60°C for 10 s for 45 cycles.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analyses for CYP24 Protein and Real-Time RT-PCR for CYP24 mRNA.** Whole-cell lysates were prepared by homogenization with lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40) containing protease inhibitors (0.5 mM *p*-amidinophenyl methanesulfonyl fluoride hydrochloride, 2 $\mu$g/ml aprotinin, and 2 $\mu$g/ml leupeptin). The protein concentrations were determined using Bradford protein assay reagent (Bio-Rad). The whole-cell lysates (20 $\mu$g) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P transfer membrane (Millipore Corporation, Billerica, MA). The membrane was probed with goat anti-human CYP24 antibodies and Alexa Fluor 680 donkey anti-goat IgG antibodies. The band densities were quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences, Cambridge, UK).

The cDNAs were synthesized from total RNAs using ReverTra Ace (Toyobo, Osaka, Japan). The forward and reverse primers for CYP24 mRNA were 5′-CAG CAA ACA GTC TAA TGT GG-3′ and 5′-AGC ATA TTC ACC CAG AAC TG-3′, respectively. The real-time RT-PCR analysis was performed as follows: after an initial denaturation at 95°C for 30 s, the amplification was performed by denaturation at 94°C for 4 s and annealing and extension at 62°C for 20 s for 45 cycles. The CYP24 mRNA levels were normalized with GAPDH mRNA determined by real-time RT-PCR as described previously (Tsuchiya et al., 2004).

**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-promoter vector. The sequence from +1575 to +1592 in the human *CYP24* gene (5′-TCA TAT CCA ACT CAG GGA-3′) was termed miR-125b recognition element (MRE125b). The fragment containing three copies of the MRE125b, 5′-CTA GAT TTG CTA ACA TCA TAT CCA ACT CAG GGA AGC GGA TTT GCT AAC ATC ATA TCC AAC TCA GGG AAG CGG ATT TGC TAA CAT CAT ATC CAA CTC AGG GAA GCG GAT-3′ (MRE125b is underlined), was cloned into the pGL3-promoter vector (pGL3/MRE3). The complementary sequence was also cloned into the pGL3-promoter vector (pGL3/MRE3rev). A fragment containing the perfect matching sequence with the mature miR-125b, 5′-CTA GAT CAC AAG TTA GGG TCT CAG GGA T-3′ (the matching sequence is underlined), was cloned (pGL3/c-miR-125b). A fragment containing the sequence from +1529 to +1609 was cloned, resulting in single (pGL3/UTR1) and double (pGL3/UTR2) forward insertions and a single reverse insertion (pGL3/UTR1rev). The nucleotide sequences of the constructed plasmids were confirmed by DNA sequencing analyses.

**Luciferase Assay.** Various pGL3 plasmids were transiently transfected with phRL-TK plasmid into KGN and MCF-7 cells. In

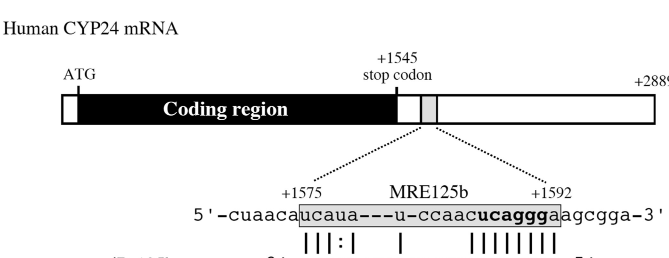


**Fig. 1.** Predicted target sequence of miR-125b in the human CYP24 mRNA. The numbering refers to the ATG in translation starting with A as 1, and the coding region is up to +1545. Sequence of MRE125b (gray box) is located on +1575 to +1592 in the 3′-UTR of human CYP24 mRNA. Boldface letters represent seed sequence.

brief, the day before transfection, the KGN cells ($8 \times 10^4$ cells/well) and MCF-7 cells ($4 \times 10^4$ cells/well) were seeded into 24-well plates. After 24 h, 450 ng of pGL3 plasmid and 50 ng of phRL-TK plasmid were transfected using Tfx-20 reagent. To KGN cells, 3.5 pmol of AsOs for miR-125b or control was cotransfected using LipofectAMINE 2000; to MCF-7 cells, 0.25 pmol of precursors for miR-125b-1 or control was cotransfected using Tfx-20 reagent. After incubation for 48 h, the cells were resuspended in passive lysis buffer, and then the luciferase activity was measured with a Wallac luminometer (PerkinElmer Life and Analytical Sciences, Turku Finland) using the dual-luciferase reporter assay system.

**Assessment of the Endogenous CYP24 Expression Level in KGN and MCF-7 Cells.** To investigate the effects of miR-125b on the expression of endogenous CYP24 protein, 50 nM precursors or 50 nM AsOs for miR-125b or control were transfected into KGN ($4 \times 10^5$ cells/well) and MCF-7 cells ($2.5 \times 10^5$ cells/well), respectively, on six-well plates using LipofectAMINE RNAiMAX. After 72 h, total RNA was isolated using ISOGEN, and the mature miR-125b levels were determined by Northern blot analysis. Whole-cell lysate was prepared, and the CYP24 protein level was determined by Western blot analysis. The CYP24 protein levels were normalized with the $\beta$-actin protein levels determined with rabbit anti-human $\beta$-actin antibodies (BioVision, Mountain View, CA) and IRDye 680 goat anti-rabbit IgG antibodies (LI-COR Biosciences). To determine the CYP24 enzymatic activity, $24,25(OH)_2D_3$ formation from $25(OH)D_3$ was measured. The KGN and MCF-7 cells seeded on six-well plates were transfected with AsO and precursor as described above. After 24 h, the cells were treated with 50 nM $1,25(OH)_2D_3$ for 24 h to induce CYP24 expression. The cells were then incubated with $25(OH)D_3$ in the medium supplemented with 3% FBS for 18 h. To the collected medium, corticosterone was added as an internal standard. The medium was extracted with 4 volumes of chloroform/methanol (3:1). The organic phase was recovered and dried. The resulting residue was dissolved with 50% acetonitrile and was subjected to high-performance liquid chromatography. The column used was an YMC-Pack ODS-A (6.0 × 300 mm, 5 $\mu$m) column (YMC, Tokyo, Japan), and the column temperature was 35°C. The mobile phase was 55% acetonitrile containing 0.2% acetic acid (A) and 90% acetonitrile (B). The condition for elution was as follows: 0% B (0–40 min); 0 to 100% B (40–50 min); 100% B (50–60 min); and 100 to 0% B (60–65 min). Linear gradients were used for all solvent changes. The flow rate was 1.0 ml/min. The eluent was monitored at 265 nm. The retention times of corticosterone, $24,25(OH)_2D_3$, and $25(OH)D_3$ were 9, 41, and 61 min, respectively. The quantification of the metabolite was performed by comparing the high-performance liquid chromatography peak height with that of an authentic standard with reference to the internal standard.

**Human Breast Cancer and Adjacent Normal Tissues.** This study was approved by the Ethics Committee of Kanazawa University (Kanazawa, Japan). Written informed consent was obtained from patients before their participation in this study. Breast cancer and adjacent normal tissues were obtained as surgical samples from 14 Japanese patients with primary breast carcinoma. The patients (42–77 years old) had not undergone chemotherapy. Thirteen patients with invasive ductal carcinoma and one patient with invasive lobular carcinoma were participants. The histological grade was determined by standard criteria as grade 1–2 ($n = 9$), grade 2 ($n = 4$), and grade 2–3 ($n = 1$). The samples were obtained immediately after resection, divided into breast cancer and adjacent normal tissues, and immediately frozen with liquid nitrogen. The samples were stored at −80°C until use. The expression levels of mature miR-125b were determined by real-time RT-PCR and were normalized with the

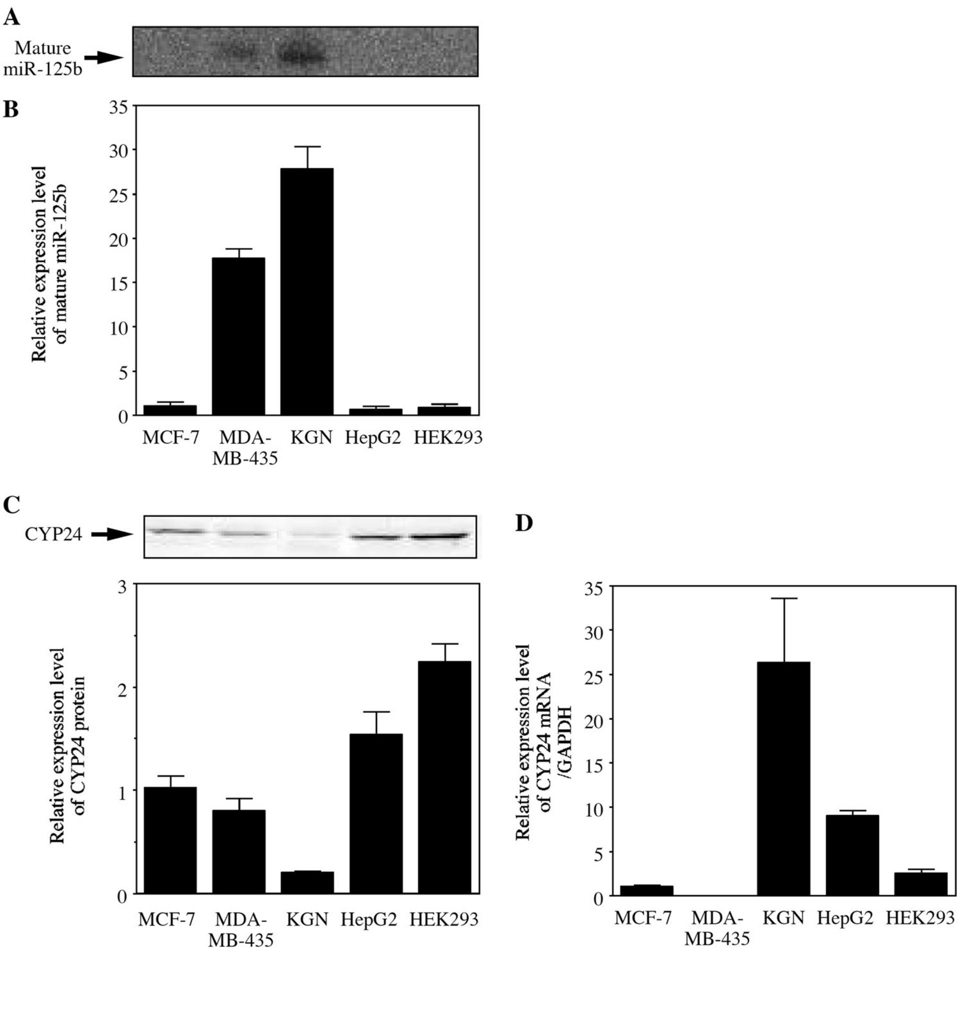


**Fig. 2.** Expression levels of miR-125b and CYP24 in various human cell lines. A, the expression levels of mature miR-125b in MCF-7, MDA-MB-435, KGN, HepG2, and HEK293 cells were determined by Northern blot analyses. B, the expression levels of mature miR-125b were determined by real-time RT-PCR. C, the expression levels of CYP24 protein were determined by Western blot analyses. D, the expression levels of CYP24 mRNA were determined by real-time RT-PCR. The expression levels were normalized with the expression level of GAPDH as a control. Values are expressed relative to the values in MCF-7 cells. Data are the mean ± S.D. of triplicate determinations.

18S rRNA levels determined by real-time RT-PCR as follows: the forward and reverse primers were 5′-GGC CCT GTA ATT GGA ATG AGT C-3′ and 5′-GAC ACT CAG CTA AGA GCA TCG-3′, respectively. After an initial denaturation at 95°C for 30 s, the amplification was performed by denaturation at 94°C for 10 s, with annealing and extension at 68°C for 20 s for 30 cycles.

**Immunohistochemistry.** Immunohistochemical analyses of CYP24 were performed using formalin-fixed, paraffin-embedded specimens of breast cancer tissues from 14 patients. The sections were soaked in Liberate Antibody Binding Solution (Polysciences, Warrington, PA) at room temperature for 10 min and then incubated with anti-human CYP24 antibodies at 4°C for 16 h. Staining was performed using a VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA). The extent of immunostaining in cancer cells was evaluated by the intensity of staining, dividing the samples into three groups (low, medium, and high levels).

**Statistical Analyses.** Data are expressed as mean ± S.D. of triplicate determinations or three independent experiments. Statistical significance was determined by analysis of variance and Dunnett's multiple comparisons test. Comparison of two groups was made with an unpaired, two-tailed Student's $t$ test. Correlation analysis was performed by Spearman's rank method. The statistical significance of difference between the expression level of miR-125b in breast cancer and normal tissues was determined by paired, two-tailed Student's $t$ test. The relationship between the CYP24 protein level and the cancer/normal ratio in miR-125b level was investigated by analysis of variance and Tukey method test. A value of $P < 0.05$ was considered statistically significant.

## Results

**miR-125b Interacts with the 3′-UTR of Human CYP24 mRNA.** Computational predictions using the microRNA targets web site (http://www.targetscan.org/) indicate that miR-125b shares complementarity with a sequence in the 3′-UTR of the CYP24 mRNA at +1575 to +1592 (energy, −21.6 kcal/mol) (Fig. 1). This region was termed the miR-125b recognition element (MRE125b). The seed sequence (nucleotides 2–7) of miR-125b was perfectly matched with the predicted binding site of the CYP24 mRNA. In this study, we investigated whether miR-125b might be involved in the regulation of human CYP24 expression through the MRE125b.

**miR-125b and CYP24 Are Differentially Expressed in Human Cell Lines.** The expression levels of mature miR-125b in MCF-7, MDA-MB-435, KGN, HepG2, and HEK293 cells were determined by Northern blot analysis. KGN and MDA-MB-435 cells showed a clear band of mature miR-125b, but the other cell lines did not (Fig. 2A). We also performed real-time RT-PCR analysis using NCode miRNA First-Strand cDNA Synthesis Kit (Fig. 2B) to detect the mature miR-125b because this method is more sensitive than Northern blot analysis. Extremely low levels of the mature miR-125b were detected in MCF-7, HepG2, and HEK293 cells. The CYP24 protein levels were determined by Western blot analyses (Fig. 2C), and the CYP24 mRNA levels were determined by real-time RT-PCR (Fig. 2D). The CYP24 protein levels were not positively correlated ($R_s = -0.100$, $P = 0.950$) with the CYP24 mRNA levels, indicating the post-transcriptional regulation of CYP24. It is noteworthy that a trend of inverse association between the CYP24 protein levels and the mature miR-125b levels was found ($R_s = -0.900$, $P = 0.083$).

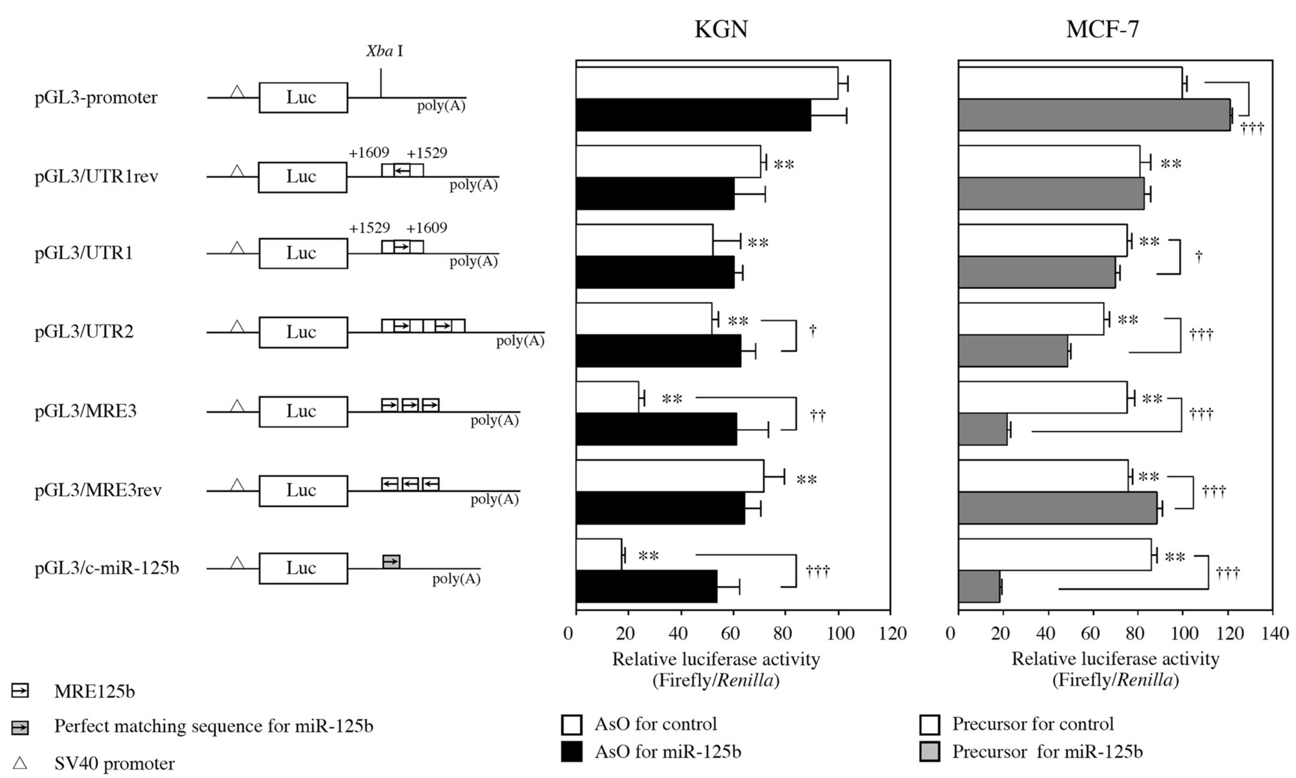


**Fig. 3.** Luciferase assays with reporter constructs containing MRE125b in human CYP24 in KGN and MCF-7 cells. A series of reporter constructs containing the 3′-UTR of the human CYP24 was transiently transfected into KGN cells with the AsO for miR-125b or control, or into MCF-7 cells with the precursors for miR-125b or control. The firefly luciferase activity for each construct was normalized with the *Renilla reniformis* luciferase activities. Values are expressed as percentages of the relative luciferase activity of pGL3-promoter plasmid. Each column represents the mean ± S.D. of three independent experiments. **, $P < 0.01$, compared with pGL3-promoter by analysis of variance and Dunnett test. †, $P < 0.05$; ††, $P < 0.01$; †††, $P < 0.005$ compared with the control by Student's $t$ test.

**MRE125b in CYP24 Is a Target of Post-Transcriptional Repression by miR-125b.** To investigate whether MRE125b is functional in the regulation by miR-125b, luciferase assays were performed. The pGL3/MRE3 plasmid containing three copies of the MRE125b was used because it is known that the multiplicity of the binding site allows efficacious detection of the effects of miRNA. The pGL3/UTR1 plasmid was used to investigate whether the intact 3′-UTR sequence of CYP24, including MRE125b, can be recognized by miRNA. The pGL3/UTR2 plasmid was used to confirm whether the multiplicity could intensify the effects of miRNA. The pGL3/c-miR-125b plasmid containing the perfect matching sequence with the mature miR-125b was used as a positive control. These plasmids were transfected into the KGN cells showing the highest expression of miR-125b (Fig. 2A). We first confirmed that the luciferase activity of the pGL3/c-miR-125b plasmid was significantly ($P < 0.01$) lower than that of the control pGL3-promoter and was significantly ($P < 0.005$) restored by the transfection of AsO for miR-125b (Fig. 3). The reporter activity of the pGL3/MRE3 plasmid was also significantly lower than that of the control plasmid, and it was significantly ($P < 0.01$) restored by the transfection of AsO for miR-125b. The reporter activities of the pGL3/UTR1 and pGL3/UTR2 were also significantly lower than that of the control plasmid, and the activity of pGL3/UTR2 was significantly ($P < 0.05$) restored by the transfection of AsO for miR-125b. Next, to investigate the effect of the overexpression of miR-125b on the luciferase activity, the precursor for miR-125b was transfected in MCF-7 cells in which the endogenous miR-125b level was low. The overexpression of miR-125b significantly decreased the luciferase activities of the pGL3/c-miR-125b, pGL3/MRE3, pGL3/UTR1, and pGL3/UTR2 plasmids. These results suggest that miR-125b recognized the MRE125b on the human CYP24 mRNA and regulated the expression.

**Endogenous CYP24 Levels Are Regulated by miR-125b.** We investigated the effects of inhibition of miR-125b on the CYP24 protein level and enzymatic activity in KGN cells. Northern blot analysis confirmed that the endogenous miR-125b level was prominently decreased by the transfection of the AsO for miR-125b (Fig. 4A). As shown in Fig. 4B, the CYP24 protein level was significantly ($P < 0.005$) increased (1.4-fold) by the transfection of the AsO for miR-125b. The 25(OH)$D_3$ 24-hydroxylase activity was also increased by the transfection of the AsO, although the difference was statistically insignificant (Fig. 4C). We next investigated the effects of overexpression of miR-125b on the CYP24 protein level and enzymatic activity in MCF-7 cells. Northern blot analysis confirmed that the mature miR-125b level was prominently increased by the transfection of the precursor for miR-125b (Fig. 4A). As shown in Fig. 4B, the CYP24 protein level was significantly ($P < 0.05$) decreased (70% of control) by the transfection of the precursor for miR-125b. The 25(OH)$D_3$ 24-hydroxylase activity was also significantly ($P < 0.05$) decreased by the transfection of the precursor (Fig. 4C). These results suggest that miR-125b regulates the endogenous CYP24 level.

**CYP24 Protein Levels Are Inversely Associated with miR-125b Levels in Human Breast Cancer.** To investigate whether miR-125b affects the CYP24 expression in vivo, we used breast cancer tissues from 14 patients. The expression levels of CYP24 protein in breast cancer were determined by immunohistochemistry (Fig. 5A). All of the breast tissues showed cytoplasmic immunoreactivity for CYP24. The CYP24 protein levels were higher in cancer tissues than in adjacent normal tissues. The extent of CYP24 staining in cancer tissues varied among individuals. No staining was observed in normal goat IgG. In regard to the CYP24 mRNA, the levels normalized with GAPDH mRNA were lower in cancer tissues than in normal tissues (data not shown). This was due to the increased levels of GAPDH mRNA in the cancer tissues. When the non-normalized CYP24 mRNA levels in the cancer and normal tissues were compared, there was no difference. In addition, no difference was observed in

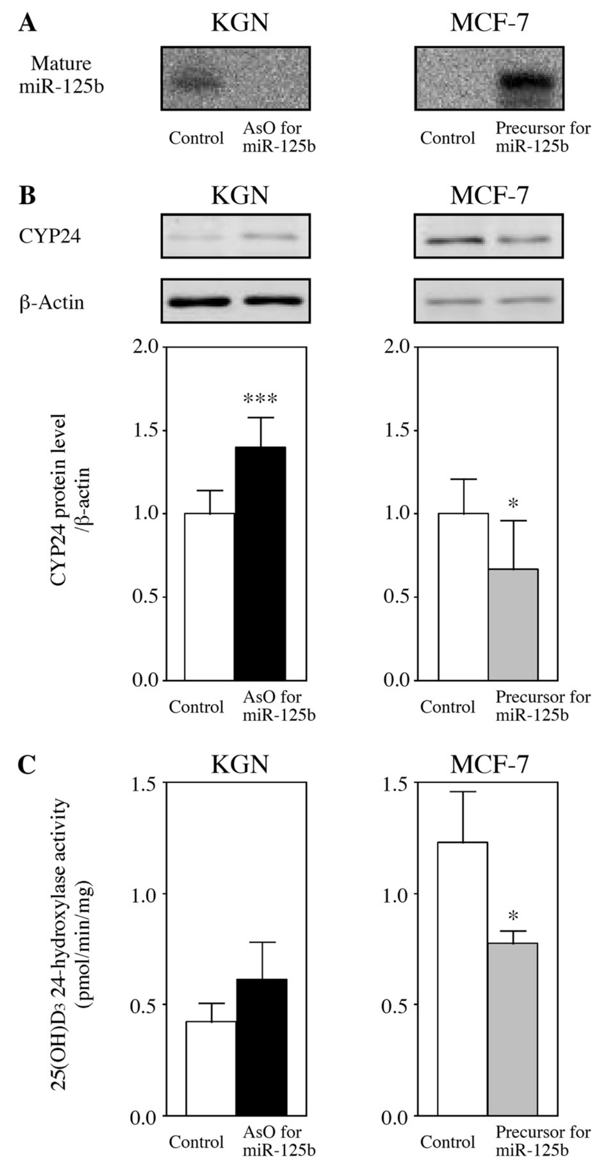


**Fig. 4.** Effects of miR-125b on the endogenous CYP24 protein level in KGN or MCF-7 cells. AsOs for miR-125b or control (2.5 pmol/4 × $10^5$ cells) were transfected into KGN cells and precursors for miR-125b or control (84 pmol/1.68 × $10^5$ cells) were transfected into MCF-7 cells. After 72 h, total RNA and whole-cell lysate were prepared. A, the expression levels of mature miR-125b were determined by Northern blot analysis. B, the expression levels of CYP24 protein were determined by Western blot analysis. C, the CYP24 enzymatic activity was determined using 25(OH)$D_3$ as a substrate as described under *Materials and Methods*. Each column represents the mean ± S.D. of three independent experiments. *, $P < 0.05$; ***, $P < 0.005$ compared with the control.

the CYP24 mRNA levels normalized with the 18S rRNA levels in the cancer and normal tissues (data not shown). Thus, the higher levels of CYP24 protein in cancer tissues would not be due to the increased CYP24 mRNA levels. As shown in Fig. 5B, the mature miR-125b levels normalized with the 18S rRNA levels in the cancer tissues were significantly ($P < 0.0005$) lower than those in normal tissues, in agreement with previous studies (Calin et al., 2004; Iorio et al., 2005). It is noteworthy that an inverse association was observed between the CYP24 protein levels in breast cancer tissue and the cancer/normal ratio of mature miR-125b levels (Fig. 5C). These results suggest that the decrease of miR-125b is one of the causes of the high expression of CYP24 protein in breast cancer tissues. No association was observed between the levels of CYP24 or miR-125b and the biopathological features (estrogen receptor and progesterone receptor levels and the presence or absence of lymph node metastasis) and the tumor stage of breast cancer (data not shown). Thus, the pathological characteristics would not affect the inverse association between the CYP24 and miR-125b levels.

## Discussion

In the present study, we investigated whether human CYP24, which catalyzes the inactivation of $1,25(OH)_2D_3$, might be a target of miRNA. In silico analysis identified MRE125b in the 3′-UTR in CYP24 mRNA. The luciferase assay revealed that the endogenous and exogenous miR-125b negatively regulated the activity through MRE125b. Consistent with previous reports (Tsuchiya et al., 2006; Wang et al., 2006), the repression was increased by multiplicity of the binding site. The AsO for miR-125b restored the luciferase activity. These results suggest that the MRE125b is functionally recognized by miR-125b. The overall complementarity of MRE125b for miR-125b is low, but the seed sequence of miR-125b was perfectly matching. Therefore, the seed-sequence matching and accessibility of CYP24 would allow access to miR-125b. Furthermore, the endogenous CYP24 protein level was increased by the inhibition of miR-125b and was decreased by the overexpression of miR-125b. These results clearly indicate that human CYP24 is post-transcriptionally regulated by miR-125b.

It has been reported that human CYP24 is regulated by transcription factors such as vitamin D receptor (VDR) (Chen and DeLuca, 1995), pregnane X receptor (Pascussi et al., 2005), constitutive androstane receptor (Moreau et al., 2007), and silencing mediator for retinoid and thyroid hormone receptors (Konno et al., 2009). A recent study also reported that the DNA methylation status affects the basal and VDR-dependent promoter activity of CYP24 (Chung et al., 2007; Novakovic et al., 2009). Cui et al. (2009) recently reported

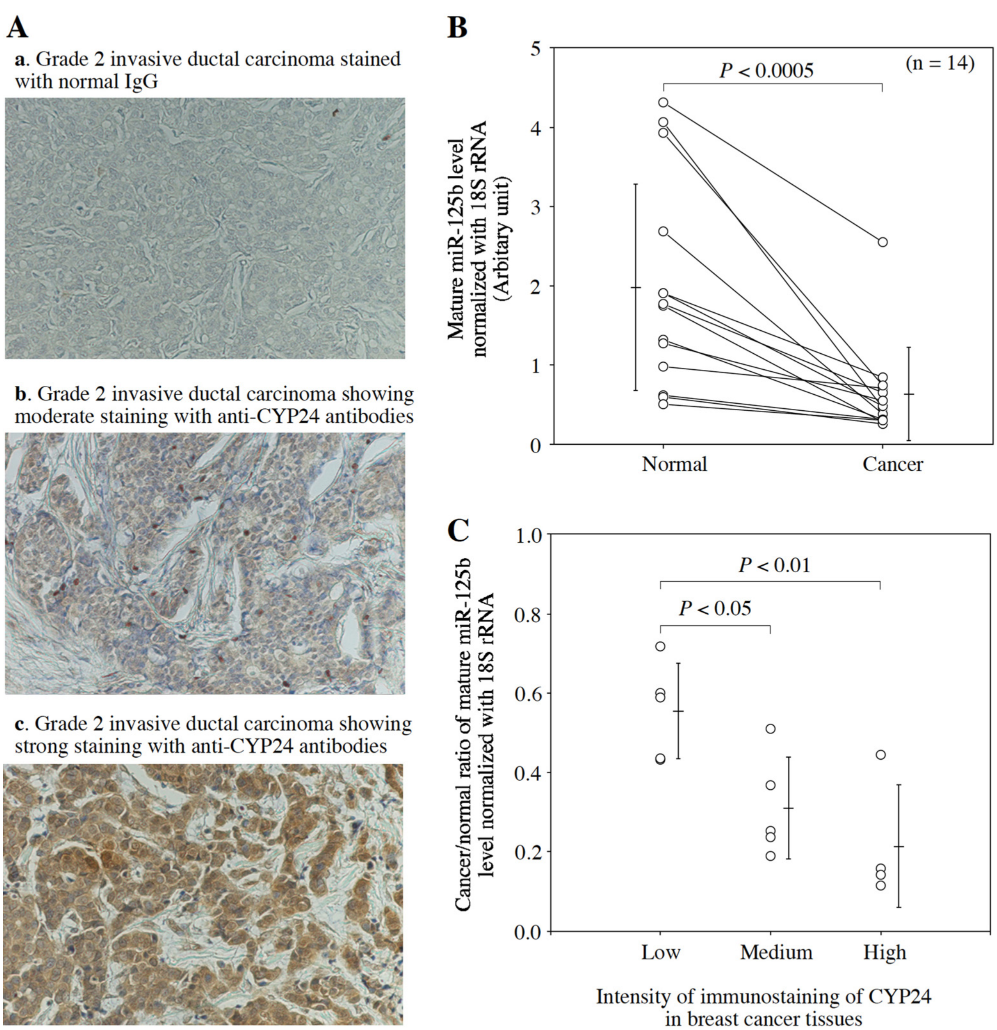


**Fig. 5.** Expression levels of CYP24 protein and miR-125b in human breast cancer tissues. A, immunohistochemical staining of CYP24 protein in breast cancer tissues. a, grade 2 invasive ductal carcinoma with normal IgG showed no staining. b, grade 2 invasive ductal carcinoma with anti-human CYP24 showed medium staining. c, grade 2 invasive ductal carcinoma with anti-human CYP24 showed high staining. B, the expression levels of mature miR-125b in breast cancer tissues and adjacent normal tissues obtained from 14 patients were determined by real-time RT-PCR. The expression levels were normalized with the 18S rRNA level. Horizontal and vertical bars represent the mean ± S.D. C, the relationship between the CYP24 protein levels in breast cancer tissues and the cancer/normal ratio of mature miR-125b levels.

that the extracellular signal-regulated kinase signaling pathway is involved in the $1,25(OH)_2D_3$-mediated CYP24 induction. In addition to these transcriptional regulation mechanisms, we found that the CYP24 expression is post-transcriptionally regulated by miRNA. Thus, this study provides new insight into the regulation mechanisms of human CYP24.

This is the first report, to our knowledge, demonstrating by immunohistochemistry that CYP24 protein is elevated in human breast cancer tissues. The increased expression of CYP24 would be due to the decreased expression of miR-125b in human breast cancer tissues. Mature miR-125b is formed by two precursors, miR-125b-1 and miR-125b-2, which are located in chromosomes 11q24.1 and 21q11.2, respectively (http://microrna.sanger.ac.uk/sequences/). It has been reported that the chromosome regions 11q23–24 (Negrini et al., 1995) and 21q11–21 (Yamada et al., 2008) are frequently deleted in breast cancers. This could be one of the mechanisms of the down-regulation of miR-125b in breast cancer.

We have found that human VDR is also regulated by miR-125b (Mohri et al., 2009). Therefore, the up-regulation of VDR in breast cancer by the down-regulation of miR-125b may partly contribute to the up-regulation of CYP24. The increase of CYP24 level would attenuate antitumor activity of $1,25(OH)_2D_3$. In contrast, the increase of VDR level would augment the antitumor activity of $1,25(OH)_2D_3$. In our recent study (Mohri et al., 2009), we investigated the effects of miR-125b on the antiproliferative effects of $1,25(OH)_2D_3$ by evaluating the growth of MCF-7 cells. We found that the cell growth was significantly inhibited by $1,25(OH)_2D_3$; however, the inhibited cell growth was prominently diminished by the overexpression of miR-125b (Mohri et al., 2009). Accordingly, the effects of miR-125b could be stronger for VDR than for CYP24 in this breast cancer cell line. A possible explanation for this observation is that the CYP24 protein level in the MCF-7 cells might be too low to inactivate the exogenously added $1,25(OH)_2D_3$.

The miR-125b has been considered to be a tumor suppressor gene, because it can suppress the expression of *ERBB2* and *ERBB3* oncogenes (Scott et al., 2007). In addition, it has been reported that miR-125b inhibited the cell proliferation of human breast cancer cells (Scott et al., 2007), hepatocellular carcinoma cells (Li et al., 2008), and thyroid carcinoma cells (Visone et al., 2007). In contrast, Lee et al. (2005) reported that inhibition of miR-125b resulted in the decrease of growth of human prostate cancer cells. Because the miR-125b expression differently changes in human tumors and that the miR-125b is down-regulated in breast, ovarian, and bladder cancers but is up-regulated in pancreas and stomach cancers (Volinia et al., 2006), it can be hypothesized that the miR-125b acts in different ways depending on the cellular context. Mizuno et al. (2008) have recently reported that miR-125b is involved in osteoblastic differentiation through the regulation of cell proliferation. Because we found the role of miR-125b in controlling the level and action of $1,25(OH)_2D_3$, it is also interesting to know the function of miR-125b for normal calcium and bone homeostasis in the future.

In the miRNA field, rapid progress has been made in the previous half-decade. The roles of miRNA in biological processes such as cell proliferation, development, and apoptosis as well as in various diseases such as cancer, cardiovascular diseases, and Alzheimer's disease have become recognized (Erson and Petty, 2008; Garofalo et al., 2008). Information concerning the targets of miRNA is increasing. However, among a large number of cytochrome P450 isoforms, the currently known isoforms that were identified as targets of miRNA are only human CYP1B1 (Tsuchiya et al., 2006), rat CYP2A3 (Kalscheuer et al., 2008), and human CYP24 in this study. Further studies are required to understand the contribution of miRNAs to the regulation of drug-metabolizing enzymes in relation to their physiological roles.

In conclusion, we found that human CYP24 is post-transcriptionally regulated by miR-125b, which would serve as a possible mechanism for the high CYP24 expression in cancer tissues. This study could provide new insight into the regulatory mechanism of human CYP24.

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