Biochemical and Biophysical Research Communications 430 (2013) 1312–1318

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Functional characterization of genetic variations in the *MDR3* promoter

Geun Hye Jang, Tae Hee Kim, Yunseo Choe, Ahrong Ham, Ji Ha Choi *

Department of Pharmacology, School of Medicine, Ewha Womans University, Seoul, Republic of Korea

ARTICLE INFO

Article history:
Received 1 December 2012
Available online 19 December 2012

Keywords:
MDR3
Promoter
Haplotype
NF-Y
Polymorphism
Transcription

ABSTRACT

Multidrug resistance 3 (MDR3) is present on the canalicular membrane of the hepatocyte and plays an important role in protecting the liver from bile acids. In this study, we characterized the transcriptional effects of four common haplotypes and four polymorphic variants in the promoter region of *MDR3* that were identified in 126 DNA samples from Koreans. We measured the luciferase activities of the four *MDR3* promoter haplotypes using *in vitro* reporter assays. Among them, two haplotypes showed a significant decrease in reporter activity compared to the reference. One of the mechanisms by which these haplotypes might decrease *MDR3* transcriptional activity was determined: one of the polymorphisms that are present in haplotype 3, g.-1584C>T, was associated with a significant reduction in the promoter activity of *MDR3,* and the transcription factor NF-Y was predicted to bind to the promoter in the region of g.-1584C>T. Electrophoretic mobility shift assays showed that the g.-1584C allele exhibited greater binding to NF-Y than did the g.-1584T allele. Through the measurement of promoter activity after the overexpression of NF-Y, we found that NF-Y can act as a transcriptional activator of *MDR3*. These data suggest that the reduced transcriptional activity of g.-1584C>T results from a reduction in the binding affinity of the activator NF-Y to the *MDR3* promoter region. Our study suggests that two common haplotypes of *MDR3* can regulate the transcriptional rate of *MDR3* and that NF-Y may be one of the transcriptional factors involved in this regulation.

1. Introduction

The liver is the one of the major organs responsible for the elimination of drugs and other xenobiotics from the body [1]. Human multidrug resistance 3 (MDR3, *ABCB4*) belongs to the family of ATP-binding cassette transporters and shows 80% amino acid homology to MDR1 (*ABCB1*) [2,3]. MDR3 is present on the canalicular membrane of the hepatocyte and translocates phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane [4]. Because this translocation is an essential process for the excretion of bile acids, it is widely accepted that MDR3 plays an important role in protecting the liver from bile acids. It was known that defects in MDR3 expression due to genetic variants can cause a rare hepatic disease, progressive familial intrahepatic cholestasis type 3 (PFIC3) [5–8]. Several studies found that some genetic variations of *MDR3* have the association with the susceptibility to hepatobiliary diseases such as drug-induced liver injury, and biliary cirrhosis [9–10].

In spite of the clinical importance of MDR3 transporter, there were few studies to investigate the expression or function of each *MDR3* variant. Most previous functional analysis of *MDR3* genetic variations have been restricted to nonsynonymous mutations identified in PFIC3 patients. For example, a nonsynonymous mutation, p.I541F, found in a PFIC3 patient showed decreased transport activity owing to reduced membrane expression [8].

Smit et al. [11] cloned and characterized the promoter region of *MDR3*. Their study showed that the *MDR3* promoter is composed of GC-rich sequences and contains multiple putative SP1 binding sites. Another study reported that there is a highly conserved farnesoid X receptor (FXR) response element in the distal region of the *MDR3* promoter, and that transcription of *MDR3* can be trans-activated by FXR [12]. Recently, genetic variations in the *MDR3* promoter were identified by direct sequencing using large DNA samples by two separate groups [13–15]. Furthermore, the functions of a few variants were evaluated by *in vitro* reporter assays [13]. However, no variant showed a significant change in function compared to the reference in their study.

The goal of the current study was to functionally characterize common haplotypes of the *MDR3* promoter region in Koreans and to determine the mechanism by which *MDR3* variants alter promoter activity. We screened 126 genomic DNA samples from healthy Koreans to identify variants in the *MDR3* promoter. We then investigated the function of each haplotype using *in vitro* reporter assays and gel shift assays. Our study provides information about the mechanisms responsible for the transcriptional

* Corresponding author. Address: Department of Pharmacology, School of Medicine, Ewha Womans University, 911-1 Mok-dong, Yangcheon-gu, Seoul 158-710, Republic of Korea. Fax: +82 2 2653 8891.
E-mail address: jihachoi@ewha.ac.kr (J.H. Choi).

0006-291X/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.12.041

regulation of *MDR3* and identifies common functionally important haplotypes that may contribute to interindividual variations in the expression level of this transporter.

2. Materials and methods

2.1. Genetic analysis of MDR3 variants

This study was approved by the Institutional Review Board of Ewha Medical Center, Seoul, Korea. One hundred and twenty-six 126 genomic DNA samples were collected from healthy individuals from the DNA bank of the Korea Pharmacogenomics Research Network at Seoul National University, Seoul, Korea. All subjects provided a written informed consent, and the health status of each was evaluated by routine physical examinations and laboratory tests. All subjects enrolled in this study had an East-Asian ethnic background. To identify genetic variants in the promoter region of *MDR3*, a polymerase chain reaction (PCR) fragment (−2127 to +36 bp from the translational start site) was amplified and directly sequenced by an automated genetic analyzer (Applied Biosystems). Haplotype assembly was performed using the program Haploview 4.2 (developed by the Broad Institute). Nucleotide location numbers were assigned from the translational start site according to the *MDR3* mRNA sequence (GenBank accession number; NM_018849.2).

2.2. Construction of MDR3 plasmid and its variants

To construct reporter plasmid containing the *MDR3* promoter region, 1687 bp of the *MDR3* gene was amplified from a genomic DNA sample from an individual with haplotype 1 as defined in Table 3. Subsequently, a second PCR was performed using primers containing recognition sites for the restriction endonucleases NheI and HindIII (Table 1). The amplified 1675 bp product was inserted into the pGL4.11b[*luc*2] vector (Promega Corporation), and the DNA sequences were confirmed by direct sequencing. Genetic variants in the promoter region were introduced into the pGL4.11b-MDR3 vector using the QuikChange® II Site-Directed Mutagenesis Kit (Agilent Technologies) with the primers listed in Table 1, and DNA sequences were confirmed by direct sequencing.

2.3. Measurement of MDR3 promoter activity in vitro

The reporter plasmids containing the reference or variants of *MDR3* were transfected into HepG2 (human liver carcinoma), HEK-293T (human embryonic kidney), HCT-116 (human colon carcinoma), and HeLa (human cervix adenocarcinoma) cell lines using Lipofectamine LTX and Plus reagents (Invitrogen Corporation). 30 h after transfection, reporter activities were measured using the Dual luciferase® reporter assay system (Promega) according to the manufacturer's protocol and quantified using the luminometer (Terner Biosystems). The amount of transfected plasmid was equalized by adding pGL4.74 renilla vector. The firefly luciferase to renilla luciferase ratios were determined and defined as the relative luciferase activity. To examine the effect of NF-Y on the basal promoter activity of *MDR3*, reference or variant reporter plasmids were co-transfected with increasing amounts of NF-Y-pcDNA3.1(+) vector (1–5 ng). The NF-Y gene (BC039244) was purchased (Thermo Scientific Open Biosystems) and inserted into the pcDNA3.1(+) vector.

2.4. Electrophoretic mobility shift assay (EMSA) of MDR3

Nuclear protein extracts were obtained from HepG2 cells. Nuclear protein extracts (25 μg) were incubated with ^{32}P-labeled oligonucleotide (2×10^5 counts/min) for 30 min at room temperature. The reaction mixtures were loaded on a 6% nondenaturing polyacrylamide gel and electrophoresed for 70 min at 80 V. The dried gel was exposed to CP-BU film (Agfa) for 16 h at −80 °C. For the shift assay, 2.1 μl of a mixture of NF-YA (sc-7712, Santa

Table 1
Oligonucleotide primers used in the construction of *MDR3* reporter plasmids or *NF-Y* plasmid and electrophoretic mobility shift assay (EMSA).

Primes for MDR3 promoter cloning	
First PCR (−1700 to −14)	
Sense	5′-TTG GCC TCG CCT GAA CTT TTA CTA-3′
Antisense	5′-GAA ACC ACA GCC TCA GAA CCA AGT-3′
Second PCR (−1698 to −24)[a]	
Sense (NheI site)	5′-CTA **GCT AGC** GGC CTC GCC TGA ACT TTT-3′
Antisense (HindIII site)	5′-CCC **AAG CTT** CCT CAG AAC CAA GTA CAC CCT CT-3′
Primers for MDR3 mutagenesis PCR[b]	
g.-1584C>T	5′-CCT AAT AAC ACC CTT ATT TTA TAG AT**T** CAA TGA CTG AGT CAA GAA TTA CAT-3′
g.-1031C>T	5′-ACT CAA ATA GGT GGT AGG AG**T** AGA GAC AAT TCA ATA CAG AC-3′
g.-1014A>G	5′-GAG CAG AGA CAA TTC AAT AC**G** GAC AGA AGT CTT AGA TGA GA-3′
g.-682A>C	5′-GAC CAG AAC TGG GG**C** TGC GGA AGC AAG AG-3′
g.-495C>G	5′-CCG AGG CTC CAG **G**CT GAT CTC GGT C-3′
g.-414C>T	5′-GGC GCC CCG G**T**G GCA AGA GCG-3′
g.-395C>G	5′-CGG CAG GCT G**G**G CCC CTG GCC-3′
g.-378T>C	5′-TGG CCC GCG CC**C** AGC CTG GGG AG-3′
g.-186A>G	5′-AGA GGC CCT GCC AG**G** CAC GCG CGA GGT TC-3′
Primers for NF-Y cloning[a]	
Sense (NheI site)	5′**GCT AGC** CCA GAG TGG ACA GGA ATC TCA C-3′
Antisense (KpnI site)	5′-**GGT ACC** ACC TTG ATC AGC TCC ATC ACA T-3′
Primers for EMSA	
Reference (g.-1584C)[b]	5′-TTA TAG AT**C** CAA TGA CTG AGT C-3′
Variant (g.-1584T)[b]	5′-TTA TAG AT**T** CAA TGA CTG AGT C-3′
Consensus NF-Y[c]	5′-ACT TTT AA **C CAA T** CA GAA AAA T-3′

[a] The restriction endonuclease sites are marked by bold-faced letters with underlines.
[b] The SNP sites are marked by bold-faced letters with underlines.
[c] The consensus sequence of *NF-Y* is marked by bold-faced letters with underlines [18].

Table 2
Frequencies of *MDR3* genetic variations in promoter region.

rs number	Variant	Minor allele frequency	Cs number	Variant	Minor allele frequency
rs3747806	g.-1921T>C	0.020	rs4148808	g.-1014A>G	0.258
rs45464696	g.-1603A>T	0.002	rs2071645	g.-495C>G	0.276
rs4148805	g.-1584C>T	0.258	rs55679062	g.-414C>T	0.071
rs4148806	g.-1484T>C	0.016	rs55976025	g.-395C>G	0.071
-	g.-1169T>C	0.004	rs2071646	g.-378T>C	0.325
rs4148807	g.-1031C>T	0.258			

Data were obtained from DNA samples from 126 unrelated Korean individuals.
The position of the variant is based upon the translational start site.

Cruz Biotechnology), NF-YB (sc-7711X, Santa Cruz Biotechnology), and NF-YC (sc-7714X, Santa Cruz Biotechnology) antibodies or 1 μl of MZF-1 (sc-46178X, Santa Cruz Biotechnology) antibody were incubated with the nuclear extract for 30 min at room temperature prior to the binding reaction. The intensity of each band on the film was measured using ImageJ (National Institutes of Health).

2.5. Statistical analysis

P values for the luciferase assay were calculated using one-way analysis of variance followed by Dunnett's two-tailed test. To compare the effects of NF-Y on the *MDR3* promoter between the reference and the variant g.-1584C>T, a paired *t*-test was performed. *P* values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. Genetic variations of MDR3 in the promoter region

We identified 11 variants in the promoter region of *MDR3* in 126 DNA samples from Korean populations; nine of them were polymorphic (minor allele frequency >1%) (Table 2). One of the *MDR3* variants, g.-1169T>C (from the translational start site), was first identified in this study, and we did not observe the g.-1510T>G, g.-1020C>G, and g.-186A>G variants previously identified in another Korean populations [15]. Table 3 shows the frequency distributions of the common (frequency >5%) haplotypes in the *MDR3* promoter region. Haplotype 1 (H1), which consists of all the major alleles of 7 common *MDR3* variants, showed the highest frequency and followed by the haplotype 2 (H2) containing only one genetic variant, g.-378T>C.

3.2. Promoter activity of MDR3 variants in vitro

To characterize the functional effects of the promoter variants, we constructed a reference (H1) MDR3-pGL4.11b[*luc2*] reporter plasmid, and the reporter assay was performed 30 h after the transfection of the reporter plasmid into various cells, such as HepG2, HEK293T, HCT116, and HeLa cells. Among the four cell lines tested, the MDR3 reference-HepG2 cells showed the highest promoter activity (Fig. 1A). The promoter activity of MDR3 reference-HCT116 cells was comparable with that of MDR3 reference-HepG2 cells. HepG2 cells were used in further reporter assays to examine the promoter activities of the variants (Fig. 1B and C), because the origin of these cells is the human liver, in which MDR3 is highly expressed. Haplotype 1 was used as a reference for the functional analysis. The promoter activities of haplotypes 2 (H2) and 3 (H3) showed decreased reporter gene expression by 16% and 46%, respectively, compared to that of the reference. All variants, such as g.-1584C>T, g.-1031C>T, g.-1014A>G, and g.-495C>G that are present in haplotype 3 showed 27–30% decreased promoter activity, compared to that of the reference (Fig. 1B). In addition, we measured the promoter activity of two other *MDR3* variants, g.-682A>C and g.-186A>G (Fig. 1C), which are present in very low frequencies in Caucasians or Koreans, respectively [15]. Both are located in untranslated exons that can affect the mRNA stability of *MDR3*. One of them, g.-682A>C, showed significantly increased promoter activity, compared to the reference (Fig. 1C). Both variants were not observed in our samples.

3.3. Effect of the variant g.-1584C>T on the binding affinity of NF-Y within the MDR3 promoter region

To investigate the mechanisms by which *MDR3* variants might cause a reduction in transcriptional activity, we identified transcription factors that could bind to the promoter region of *MDR3* in the vicinity of each variant that is present in haplotype 2 or 3 using TFSearch 3.1 (Real-World Company Partnership). It was predicted that the only one variant, g.-1584C>T, might affect the binding affinity of a transcriptional factor. In other words, TFSearch 3.1 predicted that nuclear factor-Y (NF-Y) would have a higher binding affinity to the g.-1584C allele, than to the g.-1584T allele. NF-Y consists of three subunits such as NF-YA, NF-YB and NF-YC resulting in a trimer that binds to DNA with high specificity [16]. This prediction, if validated, would explain the reduced transcriptional activity of the variant, i.e., in the presence of the variant, NF-Y would activate transcription less potently.

To functionally validate this prediction, we performed electrophoretic mobility shift assays (EMSAs) to determine whether NF-Y could bind to the promoter region containing the g.-1584C>T site. Labeled oligonucleotides (2×10^5 counts/min, reference g.-1584C: lanes 1–3 and 8; variant g.-1584T: lanes 4–6 and 9, NF-Y consensus: lane 7, Fig. 2A) were incubated with nuclear protein ex-

Table 3
Frequencies of common *MDR3* haplotypes in the promoter region.

ID	g.-1584 C>T	g.-1031 C>T	g.-1014 A>G	g.-495 C>G	g.-414 C>T	g.-395 C>G	g.-378 T>C	Frequency
H1	C	C	A	C	C	C	T	0.352
H2	C	C	A	C	C	C	**C**	0.319
H3	**T**	**T**	**G**	**G**	C	C	T	0.252
H4	C	C	A	C	**T**	**G**	T	0.052

The minor alleles are marked in bold-faced letters with underlines.
Data were obtained from DNA samples from 126 unrelated Korean individuals.

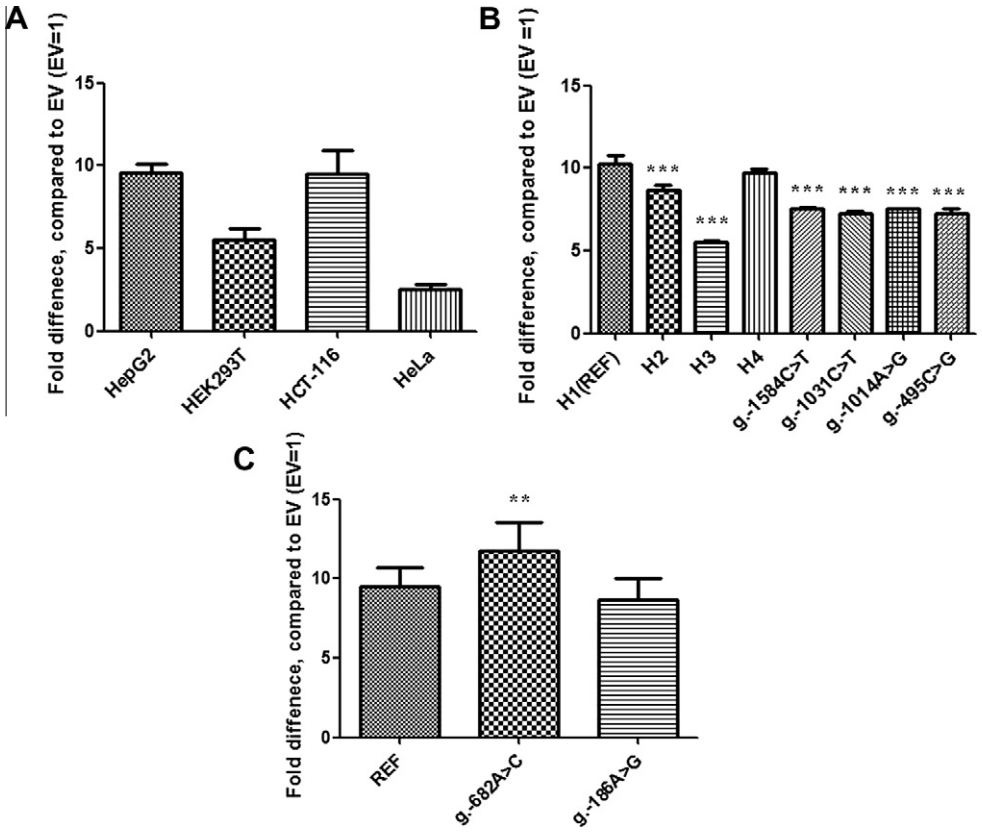

Fig. 1. Luciferase activities of the *MDR3* promoter haplotypes or variants. Luciferase activities were measured 30 h after the transfection of *MDR3* reference reporter plasmids (H1) into various kinds of cell lines (A) or reporter plasmids containing the major *MDR3* haplotypes, genetic variants in the haplotype 3 (B) or two known rare variants (C) into HepG2 cells. The reporter activity of each construct was compared with that of the empty vector (pGL4.11b[*luc2*]). The data shown represent the mean ± SD from triplicate wells in a representative experiment. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. reference promoter activity.

tracts (25 μg) obtained from HepG2 cells. Both the reference and variant probes formed DNA-protein complexes, although the intensity of the complex with the variant probe was decreased 52%, compared to that of the reference (lanes 1 and 4, Fig. 2A). The supershift in the presence of antibodies against NF-Y confirmed that NF-Y was present in the complex (lanes 7–9, Fig. 2A). To support the result from the supershift assay, we performed another supershift assay using non-NF-Y-specific antibody. A supershift was not observed with myeloid zinc finger-1 (MZF-1) antibody (lane 5, Fig. 2B). In the competition assay, various concentrations of unlabeled NF-Y consensus oligonucleotides competed with the NF-Y probes in a dose-dependent manner (lanes 2–3, Fig. 2B).

3.4. Effect of NF-Y on MDR3 promoter activity

The effect of NF-Y on the promoter activity of *MDR3* was examined by conducting a luciferase assay following over-expression of the NF-Y transcription factor. To examine the effect of NF-Y on the promoter activity of *MDR3*, reference (H1) or variant (g.-1584T) reporter constructs with increasing amounts of plasmids containing NF-Y were co-transfected in HepG2 cells. We observed that NF-Y caused a dose-dependent increase in *MDR3* promoter activity and that the effects of NF-Y were larger in the presence of the reference, g.-1584C, than of the variant, g.-1584T (Fig. 3).

4. Discussion

MDR3 is highly expressed on the canalicular membrane of the hepatocytes, and mediates the excretion of bile acids [3]. Functional changes in the activity or expression level of MDR3 caused by genetic variants were known as a major cause of PFIC3. Though there are few known substrates of the MDR3 transporter, it is believed that MDR3 may be involved in the transport of a broad array of drugs, because MDR3 shows high amino acid homology to MDR1 that plays an important role in transporting of various clinical drugs such as digoxin and paclitaxel [17]. Therefore, genetic variants of this transporter could result in changes in the pharmacokinetics or pharmacodynamics of drugs that are substrates of the transporter.

This study was performed to identify and functionally characterize common haplotypes in the promoter region of *MDR3* in the Korean population. To identify genetic variations, the promoter region of *MDR3,* containing about −2.1 kb from the translation start site (GenBank accession number; AC005068.2), was sequenced directly using a large number of samples. There were 11 variants and nine of them were polymorphic (Table 2). Recently, Lang et al. [15] established the genetic variability and haplotype structure of *MDR3* by screening using 159 DNA samples consisting of 64 Caucasian, 47 Japanese, and 48 Korean populations. They identified 18 genetic variations within the same region of the *MDR3* promoter as in our study. Three rare variants, such as g.-1510T>G, g.-1020C>G, and g.-186A>G, which were singleton in Koreans in their study, were not observed in our study. On the other hand, two rare variants, g.-1921T>C and g.-1169T>C, which showed 2% and 0.4% frequencies, respectively, in our study, were not observed in their Korean populations. In particular, g.-1169T>C was reported for the first time in this study. In the case of g.-1921T>C, this might be able to affect mRNA stability because it is located in the untranslated exon 3 [15].

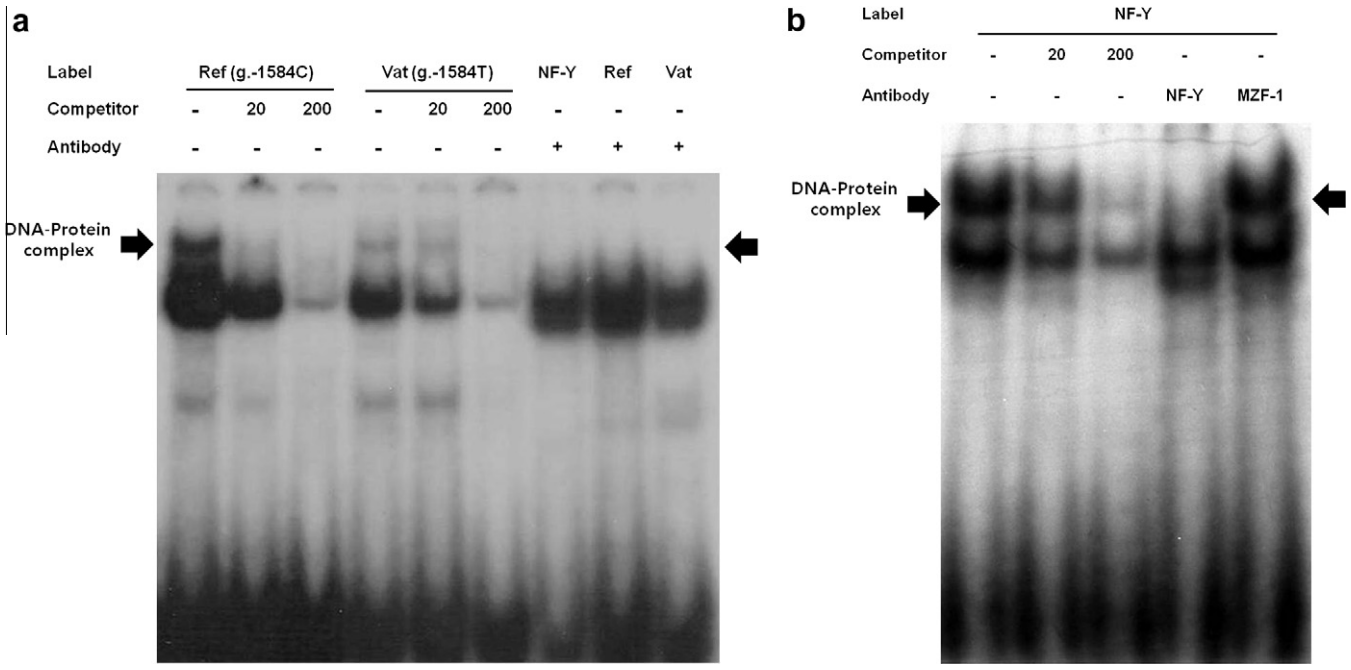

Fig. 2. Electrophoretic mobility shift analysis of the MDR3 reference and the variant g-1584C>T. (A) Labeled oligonucleotides (MDR3 reference: lanes 1–3 and 8; variant: lanes 4–6 and 9; NF-Y consensus: lane 7) were incubated with nuclear protein extracts (25 ug). The arrow indicates the position of the DNA–protein complex. Competition reactions and supershift assays were performed using different amounts (20-fold [lanes 2, 5] and 200-fold [lanes 3, 6] molar excess) of unlabeled MDR3 reference (lanes 2–3) or variant (lanes 5–6) oligonucleotides, and a mixture of three different kinds of antibodies against NF-Y, respectively (lanes 7–9). (B) Labeled NF-Y consensus oligonucleotides were incubated with nuclear protein extracts (25 μg). Competition reactions were performed using different amounts (20-fold [lane 2] and 200-fold [lane 3] molar excess) of unlabeled NF-Y consensus oligonucleotides. Supershift assays were performed using a mixture of three different kinds of antibodies against NF-Y (lane 4) or antibody against MZF-1 (lanes 5).

Haplotypes 2 and 3 showed a significant decrease in promoter activity in reporter assays (Fig. 1B). In a previous study, the promoter activities of two vectors containing a combination of several variants in the *MDR3* promoter were measured using the *in vitro* luciferase assay [13]. Although both vectors showed decreased promoter activities compared to that of the reference in their study, statistical significance was not observed. Transcription factor binding site (TFBS) analysis suggest that the transcription factor NF-Y could bind to the region encompassing g.-1584C>T, one of the variants that are present in haplotype 3, and that the binding affinity of this transcription factor would be variant-dependent. In other words, NF-Y would have a higher binding affinity for the g.-1514C allele than for g.-1514T allele.

NF-Y has been known as a CCAAT box-binding protein that binds to the DNA sequence YYRRCCAATCAG [18]. Because CCAAT motif is one of the common promoter elements, NF-Y is involved in transcriptional regulation of a large number of genes and plays an important physiological role in tumor suppressor gene expression, and stem cell proliferation and so on [19,20]. We observed that *MDR3* contains a CCAAT box with the sequence AGATCCAATGAC, which would be recognized by NF-Y. The variant g.-1584C>T has the sequence, AGATTCAATGAC, which contains a single nucleotide change in the CCAAT box and is not as good match as the reference. Therefore, we predicted that NF-Y would show a binding preference to the reference DNA sequence as compared to the variant sequence. The results of EMSAs supported our hypothesis. The intensity of the DNA-NF-Y complex was decreased in the presence of g.-1584C>T by 52% (Fig. 2A). Previous studies found that NF-Y mediates the transcriptional activation of *MDR1* by UV irradiation or drugs such as trichostatin A and sodium butyrate [21,22]. We also observed that NF-Y could act as an activator of *MDR3* transcription. In particular, the effect of NF-Y on luciferase activity was larger in the presence of the reference, g.-1584C, than the variant, g.-1584T (Fig. 3). This suggests that NF-Y binds more avidly to the reference, g.-1584C.

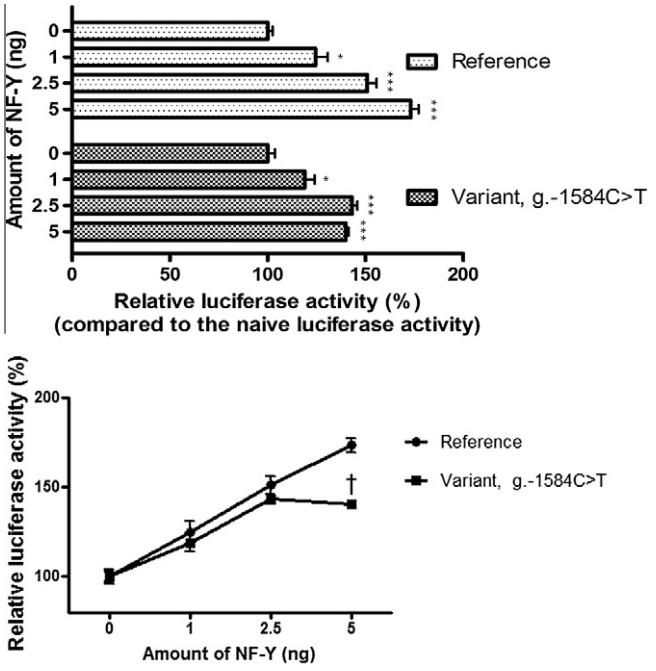

Fig. 3. Effect of NF-Y on *MDR3* promoter activity. Luciferase activities were measured 30 h after co-transfection of reference or variant reporters and various amounts of NF-Y plasmids into HepG2 cells. The reporter activity of each construct was compared with that of empty vector (pGL4.11b[*luc2*]). The data shown represent mean ± SD from triplicate wells in a representative experiment. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. naive promoter activity and $^{\dagger}P < 0.01$ vs. reference promoter activity.

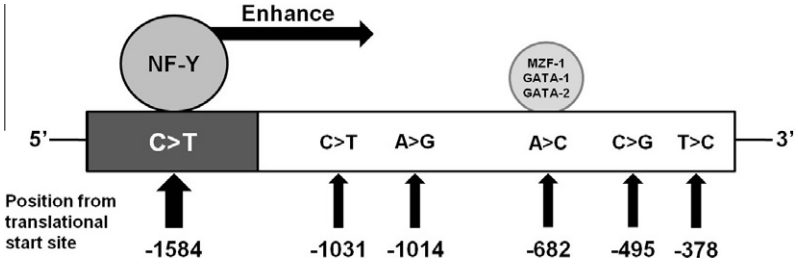

Fig. 4. Schematic interaction of the *MDR3* promoter with transcription factors that are proposed to be involved in *MDR3* regulation. The transcription factor NF-Y can bind to the site containing the variant g-1584C>T; in particular, it has a higher binding affinity for the g-1584C allele. NF-Y functions as an activator of *MDR3* transcription. Other variants, which showed significantly different luciferase activities compared to the reference, are included in the schematic, although the mechanisms by which these *MDR3* variants may cause changes in transcriptional activities were not determined in this study. In the case of g-682A>C, GATA-1, GATA-2, and MZF-1 bind to the *MDR3* promoter region containing g-682A>C. However, the binding affinity for each transcriptional factor was comparable between the g-682A and g-682C alleles.

In addition, we measured the promoter activity of two known *MDR3* variants, g.-682A>C and g.-186A>G, that are located in untranslated exon −1 and exon 1, respectively. In a previous study, both were found at very low frequencies in Caucasian or Korean populations, respectively [15]. However, we did not observe both variants in this study. In the luciferase assay, the g.-682A>C variant showed increased activity by 23.5% compared to that of the reference, while the activity of g.-186A>G was comparable to that of the reference (Fig. 1C). TFBS analyses suggested that several transcription factors, such as GATA-1, GATA-2, and MZF-1 could bind to the region encompassing g.-682A>C, and that each would have a higher binding affinity in the presence of the g.-682A allele than of g.-682C. To validate it, we performed EMSAs and confirmed that all these transcriptional factors bind to the *MDR3* promoter region containing g.-682A>C. However, the binding affinity for each transcriptional factor was comparable between the reference and variant in our study (data not shown). Here we can suggest that one rare variant of *MDR3* found in Caucasian, g.-682A>C may regulate the transcription of *MDR3*.

Recently, genetic analysis of *MDR3* was performed using DNA samples from three patients having itraconazole, the known inhibitor of MDR3,-induced cholestatic liver injury [23]. Four *MDR3* variants were found in two of the patients. Among them, two variants were synonymous mutations and the others were g.-1584C>T and g.-1014A>G, which showed decreased promoter activities in our study. Therefore, we suggest that these two variants may be associated with a susceptibility to drug-induced cholestatic liver injury.

In conclusion, we characterized genetic variants in the promoter region of *MDR3* and found that two common haplotypes in the Korean populations, H2 and H3 showed significantly decreased promoter activities. One of the mechanisms by which these *MDR3* haplotypes may decrease transcriptional activities was determined in this study: one of polymorphisms that are present in H3, g.-1584C>T, is associated with a significant reduction in the promoter activity of *MDR3*. The mechanism appeared to be related to the reduced binding of the transcription factor, NF-Y, which acts as an activator of transcription, to the region containing the variant g.-1584C>T (Fig. 4). Our studies revealed that the two common haplotypes in the promoter of *MDR3* result in changes in the transcriptional activity of *MDR3*. The presence of either of these haplotypes may be an important predisposing factor for drug-induced liver injury, or may affect the pharmacokinetics or drug response of various drugs that are substrates of MDR3. To confirm our suggestion, further study will be necessary to investigate the effect of *MDR3* haplotypes on the expression of *MDR3 in vivo*.

Acknowledgments

This study was supported by National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) [2010-0003262]; and a grant of the National Project for Personalized Genomic Medicine, Ministry for Health & Welfare, Republic of Korea [A111218-PG03]. We thank Dr. I. Jang and Dr. J. Cho for giving us a great help to get genomic samples from the DNA bank of Korea Pharmacogenomics Research Network at Seoul National University, Seoul, Korea.

References

[1] Y. Shitara, T. Horie, Y. Sugiyama, Transporters as a determinant of drug clearance and tissue distribution, Eur. J. Pharm. Sci. 27 (2006) 425–446.
[2] A.M. Van der Bliek, F. Baas, T. Ten Houte, P.M. Kooiman, T. Van der Velde-Koerts, The human mdr3 gene encodes a novel P-glycoprotein homologue and gives rise to alternatively spliced mRNAs in liver, EMBO J. 6 (1987) 3325–3331.
[3] R.P. Oude Elferink, C.C. Paulusma, Function and pathophysiological importance of ABCB4 (MDR3 P-glycoprotein), Pflugers Arch. 453 (2007) 601–610.
[4] S. Ruetz, P. Gros, Phosphatidylcholine translocase: a physiological role for the mdr2 gene, Cell 77 (1994) 1071–1081.
[5] J.F. Deleuze, E. Jacquemin, C. Dubuisson, D. Cresteil, M. Dumont, S. Erlinger, O. Bernard, M. Hadchouel, Defect of multidrug-resistance 3 gene expression in a subtype of progressive familial intrahepatic cholestasis, Hepatology 23 (1996) 904–908.
[6] J.M. de Vree, E. Jacquemin, E. Sturm, D. Cresteil, P.J. Bosma, J. Aten, J.F. Deleuze, M. Desrochers, M. Burdelski, O. Bernard, R.P. Oude Elferink, M. Hadchouel, Mutations in the MDR3 gene cause progressive familial intrahepatic cholestasis, Proc. Natl. Acad. Sci. USA 95 (1998) 282–287.
[7] D. Degiorgio, C. Colombo, M. Seia, L. Porcaro, L. Costantino, L. Zazzeron, D. Bordo, D.A. Coviello, Molecular characterization and structural implications of 25 new ABCB4 mutations in progressive familial intrahepatic cholestasis type 3 (PFIC3), Eur. J. Hum. Genet. 15 (2007) 1230–1238.
[8] J.L. Delaunay, A.M. Durand-Schneider, D. Delautier, A. Rada, J. Gautherot, E. Jacquemin, T. Aït-Slimane, M. Maurice, A missense mutation in ABCB4 gene involved in progressive familial intrahepatic cholestasis type 3 leads to a folding defect that can be rescued by low temperature, Hepatology 49 (2009) 1218–1227.
[9] C. Lang, Y. Meier, B. Stieger, U. Beuers, T. Lang, R. Kerb, G.A. Kullak-Ublick, P.J. Meier, C. Pauli-Magnus, Mutations and polymorphisms in the bile salt export pump and the multidrug resistance protein 3 associated with drug-induced liver injury., Pharmacogenet. Genomics 17 (2007) 47–60.
[10] Y. Ohishi, M. Nakamura, N. Iio, S. Higa, M. Inayoshi, Y. Aiba, A. Komori, K. Omagari, H. Ishibashi, K. Tsukamoto, Single-nucleotide polymorphism analysis of the multidrug resistance protein 3 gene for the detection of clinical progression in Japanese patients with primary biliary cirrhosis, Hepatology 48 (2008) 853–862.
[11] J.J. Smit, C.A. Mol, L. van Deemter, E. Wagenaar, A.H. Schinkel, P. Borst, Characterization of the promoter region of the human MDR3 P-glycoprotein gene, Biochim. Biophys. Acta 1261 (1995) 44–56.
[12] L. Huang, A. Zhao, J.L. Lew, T. Zhang, Y. Hrywna, J.R. Thompson, N. de Pedro, I. Royo, R.A. Blevins, F. Peláez, S.D. Wright, J. Cui, Farnesoid X receptor activates transcription of the phospholipid pump MDR3, J. Biol. Chem. 278 (2003) 51085–51090.
[13] C. Pauli-Magnus, T. Lang, Y. Meier, T. Zodan-Marin, D. Jung, C. Breymann, R. Zimmermann, S. Kenngott, U. Beuers, C. Reichel, R. Kerb, A. Penger, P.J. Meier, G.A. Kullak-Ublick, Sequence analysis of bile salt export pump (ABCB11) and multidrug resistance p-glycoprotein 3 (ABCB4, MDR3) in patients with intrahepatic cholestasis of pregnancy, Pharmacogenetics 14 (2004) 91–102.
[14] C. Pauli-Magnus, R. Kerb, K. Fattinger, T. Lang, B. Anwald, G.A. Kullak-Ublick, U. Beuers, P.J. Meier, BSEP and MDR3 haplotype structure in healthy Caucasians, primary biliary cirrhosis and primary sclerosing cholangitis, Hepatology 39 (2004) 779–791.
[15] T. Lang, M. Haberl, D. Jung, A. Drescher, R. Schlagenhaufer, A. Keil, E. Mornhinweg, B. Stieger, G.A. Kullak-Ublick, R. Kerb, Genetic variability,

haplotype structures, and ethnic diversity of hepatic transporters MDR3 (ABCB4) and bile salt export pump (ABCB11), Drug Metab. Dispos. 34 (2006) 1582–1599.
[16] M.C. Motta, G. Caretti, G.F. Badaracco, R. Mantovani, Interaction of the CCAAT-binding trimer NF-Y with nucleosomes, J. Biol. Chem. 274 (1999) 1326–1333.
[17] C. Funk, The role of hepatic transporters in drug elimination, Expert Opin. Drug Metab. Toxicol. 4 (2008) 363–379.
[18] T. Inoue, J. Kamiyama, T. Sakai, Sp1 and NF-Y synergistically mediate the effect of vitamin D(3) in the p27(Kip1) gene promoter that lacks vitamin D response elements, J. Biol. Chem. 274 (1999) 32309–32317.
[19] N. Lützner, J. De-Castro Arce, F. Rösl, Gene expression of the tumour suppressor LKB1 is mediated by Sp1, NF-Y and FOXO transcription factors, PLoS. One 7 (2012) e32590.
[20] G. Bungartz, H. Land, D.T. Scadden, S.G. Emerson, NF-Y is necessary for hematopoietic stem cell proliferation and survival, Blood 119 (2012) 1380–1389.
[21] S. Jin, K.W. Scotto, Transcriptional regulation of the MDR1 gene by histone acetyltransferase and deacetylase is mediated by NF-Y, Mol. Cell. Biol. 18 (1998) 4377–4384.
[22] Z. Hu, S. Jin, K.W. Scotto, Transcriptional activation of the MDR1 gene by UV irradiation. Role of NF-Y and Sp1, J. Biol. Chem. 275 (2000) 2979–2985.
[23] T. Yoshikado, T. Takada, T. Yamamoto, H. Yamaji, K. Ito, T. Santa, H. Yokota, Y. Yatomi, H. Yoshida, J. Goto, S. Tsuji, H. Suzuki, Itraconazole-induced cholestasis: involvement of the inhibition of bile canalicular phospholipid translocator MDR3/ABCB4, Mol. Pharmacol. 79 (2001) 241–250.