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Identification and Functional Assessment of *BCRP* Polymorphisms in a Korean Population

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ABBREVIATIONS: BCRP, breast cancer resistance protein; SNP, single nucleotide polymorphism; ABC, ATP-binding cassette transporter; bp, base pair(s); PCR, polymerase chain reaction; LD, Linkage disequilibrium; MTX, methotrexate; FACS, fluorescence-activated cell sorting; HIF-1α, hypoxia inducible factor-1α.

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ABSTRACT

The breast cancer resistance protein (BCRP) is a member of the ATP-binding cassette transporters. The aim of the present study was to identify genetic variants of *BCRP* in Koreans and to assess the functional consequences of *BCRP* polymorphisms. Twenty single nucleotide polymorphisms (SNPs), including four nonsynonymous SNPs, were identified by DNA sequencing of the *BCRP* gene in 92 Korean subjects. BCRP V12M, Q141K, P269S, and Q126Stop were detected at frequencies of 23%, 28%, 0.2%, and 1.9%, respectively. These four coding variants were also screened in Chinese and Vietnamese subjects, the allelic frequencies among the three populations were compared, and predictions were made as to the potential frequency of each variant. *In vitro* functional analyses of the P269S protein and the promoter SNP -19031C>T (mutated in the hypoxia inducible factor-1α-binding site) were performed and compared with those of the wild-type. P269S exhibited 35-40% decrease in vesicular uptake of [^{3}H]-estrone-3-sulfate (ES) and [^{3}H]-methotrexate (MTX) compared to the wild-type. The promoter SNP -19031C>T did not affect *BCRP* promoter activity in either the presence or absence of chemical-induced hypoxic stress. Our results suggest that the P269S variant could be a functionally altered variant. Genotyping of this variant in clinical studies is needed to address its phenotypic role. Genetic polymorphisms of *BCRP* were found to be very common in Koreans, as well as in other ethnic groups. Comparative analyses among three Asian populations revealed different frequencies for the four functional *BCRP* variants.

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INTRODUCTION

The breast cancer resistance protein (BCRP/ABCP/MXR/ABCG2) is a member of the plasma membrane transporter family and is an ATP-binding cassette protein. BCRP was first identified in multidrug-resistant breast cancer cell lines that were selected in the presence of doxorubicin (DOX) and verapamil (Doyle et al., 1998). BCRP has also been identified in the human placenta (Allikmets et al., 1998), as well as in cancer cell lines selected in the presence of anti-cancer agents, such as mitoxantrone (Miyake et al., 1999). The *BCRP* gene is located on chromosomal region 4q22, consists of 16 exons that span over 66 kb, and encodes a 72-kDa membrane protein that is composed of 655 amino acids (Allikmets et al., 1998; Doyle et al., 1998; Bailey-Dell et al., 2001).

The normal physiological function of BCRP has not been clearly established. High-level expression of ABCG2 has been observed in certain drug-resistant cell lines and tumors, conferring a special multidrug-resistant phenotype on these cancer cells (Miyake et al., 1999; Litman et al., 2001; Volk et al., 2002). In normal human tissues, the highest expression of BCRP is seen in the placenta (Allikmets et al., 1998), while BCRP is expressed at lower levels in the brain, prostate, testis, liver, small intestine, colon, ovary, kidney, and heart (Doyle et al., 1998). This expression profile suggests that BCRP plays a role in protecting the fetus from toxins (Allikmets et al., 1998; Maliepaard et al., 1999). BCRP is also known to confer resistance to chemotherapeutic agents, such as mitoxantrone, methotrexate, topotecan, SN38, and flavopiridol (Jonker et al., 2000; Robey et al., 2001).

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Several BCRP variants have been reported from direct DNA sequencing of the *BCRP* gene. Among these, some coding variants of *BCRP* exhibit increased resistance to anticancer drug treatment (Honjo et al., 2001; Mitomo et al., 2003). It has been recognized that single nucleotide polymorphisms (SNPs) of *BCRP* cause inter-individual variations in the pharmacokinetics for BCRP substrates (Sparreboom et al., 2004).

According to the current literature, the most frequent *BCRP* polymorphisms detected among different ethnic groups are 34G>A, which codes for V12M, and 421C>A, which codes for Q141K (Zamber et al., 2003). The *BCRP* Q141K SNP has been associated with decreased expression of the BCRP protein in that this genotype produces a reduced transporter activity phenotype (Imai et al., 2002; Kondo et al., 2004). The *BCRP* V12M SNP has been reported as having similar activity to the wild-type in terms of DHEAS, MTX, and PAH transport (Kondo et al., 2004), and porphyrin transport (Ai et al., 2006). However, Mizuari et al. (2004) have shown disrupted membrane localization of the V12M variant, which results in decreased transport activity.

Despite several reports on *BCRP* polymorphisms among various ethnic groups, there have been no reports on *BCRP* SNPs in a Korean population. Therefore, we investigated, for the first time, genetic polymorphisms of the *BCRP* gene in a Korean population, and performed *in vitro* functional characterization of a novel regulatory SNP and a coding SNP (P269S). The functional capability of the P269S variant to take up [^{3}H]-ES and [^{3}H]-MTX has been reported as being comparable to that of the wild-type protein (Kondo et al., 2004). However, in the present study, BCRP P269S from human blood

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samples (not from cell lines) was used for the first time. Functional information on P269S is sparse, as compared to the amount of information that has been collected for other coding variants, such as V12M, Q141K, and the null allele Q126Stop. Since proline is known to be a helix breaker, the three-dimensional structure of the BCRP protein may be affected in the P269S variant. Therefore, we performed a functional study of the P269S variant among the four variants identified in the study (V12M, Q141K, P269S, and Q126Stop). We also compared the allelic frequencies of the *BCRP* variants in Koreans to those in Chinese and Vietnamese populations, and we discuss possible ethnic differences with respect to *BCRP* polymorphisms.

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Materials and Methods

Subjects. In all, 275 Korean subjects were recruited for the *BCRP* genotyping study. Genomic DNA samples from 159 Vietnamese, 192 Chinese, 100 African-American, and 100 Caucasian subjects were obtained from the DNA Repository of the Pharmacogenomics Research Center, Inje University (Lee et al., 2005). All of the participants were healthy according to their medical histories, physical examinations, and routine laboratory tests. All participants provided written informed consent for the present study, which was approved by the Institutional Review Board of Busan Paik Hospital (Busan, Korea).

DNA Purification and Sequencing. For direct DNA sequencing and pyrosequencing of the *BCRP* gene, genomic DNA was isolated from whole blood cells using the Qiagen DNA Extraction Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Genomic DNA samples from 92 of the 275 Korean subjects were directly sequenced for the identification of novel sequence variants, and the DNA samples from the remaining 183 subjects were screened for the identified variants by pyrosequencing. Specific primers were designed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to amplify 16 exons of *BCRP*, the proximal promoter region, and the 3'-untranslated region. PCR was performed in a reaction volume of 20 μl in the presence of 150 ng of genomic DNA, 0.2 mM dNTPs, 0.2 μM of each primer, 1.5 mM $MgCl_2$, and 1 U *Taq* polymerase (Roche Pharmaceutical Co., Basel, Switzerland). PCR was performed in the GeneAmp PCR 9700 (Applied Biosystems, Foster City, CA), with an initial denaturation step of 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s,

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annealing at 54.0-64.7°C for 30 s, and extension at 72°C for 30 s. A final termination of elongation step was performed at 72°C for 5 min. The primer sequences and annealing temperatures used are listed in Table 1. The amplified products were directly sequenced using an automated sequencer with the BigDye Terminator Sequencing Kit (Applied Biosystems).

Genotyping of *BCRP* Variants by Pyrosequencing. In order to determine the allelic frequencies of the genetic variants, further genotyping was performed by pyrosequencing the genomic DNA samples from an additional 183 Korean subjects. All of the subjects were screened for *BCRP* V12M, Q126Stop, Q141K, and P269S. To detect the BCRP variants, partial fragments of *BCRP* gene were amplified using the primer pair, of which one primer was biotinylated at its 5'-end. Sequences of the primers and denaturation temperature for PCR were listed in Table 2. Biontinylated PCR products were immobilized into streptavidin-coated beads (Streptavidin Sepharose™ High Performance, Amersham Biosciences) following the strand separation for the PSQ 96 Sample Preparation kit (Pyrosequencing™ Amersham Biosciences). Briefly, Sepharose bead slurry (3 μl) was mixed with binding buffer (37 μl), PCR product (15 μl) and distilled water (25 μl) incubated at room temperature for 10 min at 1400rpm. The beads were transferred to a filter plate and liquid was removed by vacuum filtration (Multiscreen Resist Vacuum Manifold, Millipore Inc.). The DNA strands were separated in denaturation solution (0.5 M NaOH) for 5 sec. The immobilized template was washed in 10 mM Tris-acetate, pH7.6 solution (washing buffer), and transferred to a PSQ 96 plate, resuspended in annealing buffer (20 mM Tris-acetate, pH7.6) containing sequencing primer as

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in Table 2. And then, sequencing primer was annealed at 80-90°C for 3 min. The sequence was analyzed using a PSQ 96 system with SNP Reagent Kit (Pyrosequencing).

Linkage Disequilibrium (LD) and Haplotype Analysis. In addition to the unphased SNP analysis, the haplotypes and pairwise linkages were analyzed using the SNPAlyze software (Dynacom Co., Yokohama, Japan) and Haploview v3.32 (http://www.broad.mit.edu/mpg/haploview/). The LD for each pair of segregating sites was estimated by the values of ID′I and r-square. From the inferred haplotypes, all haplotypes with frequencies >1% were regarded as common haplotypes.

Expression of Recombinant Wild-type and Variant BCRP Proteins. The cDNAs that encode the R482 and G482 proteins were amplified by PCR using their plasmid constructs as templates (kindly provided by Dr. Douglas D. Ross, Greenebaum Cancer Center, University of Maryland, Baltimore, MD). For subcloning of the BCRP variant and wild-type cDNAs into the baculoviral vector, the BCRP coding region was amplified with the primer pair: 5'-ACGCGTAAGCTTATGTCTTCCAGTAATGTC-3' and 5'-ACGCGTCTCGAGTTAAGAATATTTTTTAAG-3'. The PCR products were ligated into the pFastBac vector (Invitrogen, Carlsbad, CA). The BCRP R482-P269S and G482-P269S variants were generated by an overlap extension procedure using specific primers that introduced amino acid substitutions: 5'-AGGCCTCCTGAGCAGACCCGTGGAACATAAG-3' and 5'-CTTATGTTCCACGGGTCTGCTCAGGAGGCCT-3'. The introduction of

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mutations was verified by DNA sequencing of all the constructs. The entire open reading frame sequences for the BCRP wild-type and coding variants in the pFastBac plasmid were verified by direct sequencing before expression in insect cells. Sf9 cells were maintained at 27°C in Grace's Insect Cell culture medium that contained 3.3 g/L yeastolate, 3.3 g/L lactalbumin hydrolysate, and 10% heat-inactivated FBS. The Sf9 cells were infected with the recombinant baculovirus at a multiplicity of infection of 5 to 6. BCRP expression was under the control of the polyhedron promoter. Cells were harvested 72 h after infection, for the preparation of microsomal fractions. Similar amounts of BCRP protein expression were confirmed by immunoblot analysis. The microsomal fraction (baculosome) from the Sf9 cells that expressed BCRP protein was prepared as described previously (Mizuarai et al., 2004). Cell pellet was diluted with hypotonic beffer (0.5 mM Tris-HEPES, pH7.4, and 0.1 mM EGTA) and homogeneted with a Teflon pestle homogenizer. After centrifugation at 2000 xg, the supernatant was further centrifuged at 100,000 xg for 30 min. The resulting pellet was suspended in buffer A (0.25 M sucrose, 10mM Tris-HEPES, pH7.4), layered over 40% (w/v) sucrose solution, and centrifuged at 100,000 xg for 30 min. The turbid layer at the interface was collected and centrifuged once more in buffer A. Resulting membrane fraction was resuspended in small volume of buffer A. The membrane fraction was separated on a 10% SDS-polyacrylamide gel. The resolved proteins were electrophoretically transferred onto a PVDF membrane at 25 V for 30 min using a semi-dry apparatus (Bio-Rad, Hercules, CA). The membrane was immunoblotted with anti-BCRP antibody (#405 clone

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in Litman et al. 2002). The antibody was kindly provided by Dr. Susan E. Bates at NIH (Molecular Therapeutics Branch, NCI, NIH, Bethesda, USA). The specific expression of BCRP was also confirmed by another anti-BCRP antibody (clone BXP-21 from Alexis Corporation, Lausen, Switzerland). After incubation of the membrane with horseradish peroxidase-conjugated anti-mouse IgG, the BCRP proteins were visualized using the ECL Western Blotting Detection System (Santa Cruz Biotechnology, Santa Cruz, CA). Gel images were analyzed using the MultiGauge ver. 2.2 software (Fuji Photo Film Co., Tokyo, Japan) and the LAS-3000 image analyzer (Fuji Photo Film).

Vanadate-sensitive BCRP ATPase Activity. BCRP baculosome (5 μg of protein per reaction) was incubated in a 40-μl assay mixture that contained 50 mM MOPS-Tris (pH 7.0), 50 mM KCl, 5 mM sodium azide, 2 mM DTT, 0.1 mM EGTA-Tris (pH 7.0), and 1 mM ouabain for 20 min at 37°C in the presence or absence of 1.0 mM vanadate, to eliminate nonspecific phosphatases and ATPases from the vacuolar, mitochondrial or plasma membrane. To determine the specific ATPase activity of recombinant BCRP, 1 μl of 5 mM prazosine and 0.5 mM Hoechst 33342 were added into the reaction mixture as the representative substrate and inhibitor, respectively. The reactions were stopped and the amount of inorganic phosphate (P_i) released was determined immediately by measuring the optical density at 630 and 850 nm. DMSO used for compound dilution exhibited no effect on BCRP ATPase activity. Specific BCRP ATPase activities were calculated by subtracting the vanadate-sensitive P_i value from the total P_i value in the absence of vanadate.

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Analysis of Vesicular Uptake of [^{3}H]-ES and [^{3}H]-MTX. ATP-dependent transport of [^{3}H]-ES and [^{3}H]-MTX into membrane vesicles was measured by rapid filtration. Transport assays were performed at 37°C for 15 min in a 62.5-µl volume that contained 10 µl of membrane vesicles (5 mg/ml), 1 µl of 0.2 M MgATP or 0.2 M MgAMP, 1 µl of [^{3}H]-MTX (1 mCi), and 1 µl of 0.75 mM MTX in transport buffer. In case of [^{3}H]-ES, all components of reaction were same with [^{3}H]-MTX except [^{3}H]-ES. The reaction was stopped on ice and the vesicles were collected by filtration through a Unifilters GF/B 96-well plate (PerkinElmer, Boston, MA). Radioactivity was measured in a scintillation counter (Beckman Scintillation Counter, Beckman Coulter Inc., Fullerton, CA). ATP-dependent uptake of [^{3}H]-ES and [^{3}H]-MTX was calculated by subtracting uptake measured in the presence of AMP from uptake measured in the presence of ATP. The data are presented as relative values to the amount of protein in the membrane vesicles.

DNA Constructs and Site-directed Mutagenesis. The pGL3-BCRP (-1235 to +362) plasmid was used (kindly provided by Dr. Douglas D. Ross). To assay the transcriptional activity of the variant-type promoter (-936C>T, -689A>G and -114C>T), mutations were introduced into pGL3-BCRP (-1235 to +362) using an oligonucleotide-directed mutagenesis system (Transformer Site-Directed Mutagenesis kit; BD Biosciences, San Jose, CA) according to the manufacturer's instructions. The synthetic oligonucleotides used for mutagenesis were as follows: 5′-CGATAAGGTACCGTCGACCGATGC-3′, together with 5′-CCCAAATGTTTGGGTGCATCATGAATAT-3′ for –936C>T, 5′-

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CTAATTTCCTAGGGTGGATGCAGCAGGTAG-3′ for –689A>G, and 5′-CAGCGCGGCAGGACATGTGTGCGCTTTCAG-3′ for –114C>T. The introduction of each mutation was verified by DNA sequencing.

Transient Transfection and Promoter Activity Analysis. The HEK293F cells were plated into a 6-well plate in triplicate at 5×10^5 cells/well, and were cotransfected with 1 μg of the *BCRP* promoter construct and 0.1 μg of the β-galactosidase-expressing plasmid using the FuGENE 6 transfection reagent according to manufacturer's protocol. Cells were harvested after 48 h and analyzed for luciferase and β-galactosidase activities using the Luciferase Reporter Gene Assay and Chlorophenolred-ß-D-galactopyranoside (Roche Applied Science), respectively. Luciferase activity was normalized with respect to β-galactosidase activity. Each set of transfections was performed in triplicate and repeated at least twice. To assess the relative contribution of the –114C>T variation to *BCRP* transcriptional activity under hypoxic conditions, the cells were co-transfected with 400 ng of the HIF-1α-expressing plasmid. The control vectors, ARNT (400 ng) and pCMV–β-galactosidase (100 ng), were used as internal controls for transfection efficiency. At 24 h post-transfection, the cells were incubated in the presence of 100 μM deferoxamine for 24 h. All of the luciferase activities were normalized with respect to β-galactosidase activity.

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Results

Identification of *BCRP* Genetic Variants in a Korean Population. All 16 exons, intron-exon boundary regions, and the promoter region of the *BCRP* gene were sequenced, to identify *BCRP* SNPs in 92 Korean subjects. Twenty *BCRP* SNPs were identified in this study. Five variants were found in the promoter, five in the exons, and ten in the intron regions. The four coding SNPs were: 34G>A coding for V12M, 8191C>T coding for Q126Stop, 8825C>A coding for Q141K, and 21850C>T coding for P269S. For more extensive evaluation of the allelic frequencies of the four *BCRP* variants found in the Korean population, the remaining 183 subjects were screened by pyrosequencing for the presence of V12M, Q126Stop, Q141K, and P269S. The allelic frequencies and locations of the *BCRP* genetic variants identified in the Korean subjects are listed in Table 2. V12M and Q141K were found in 23% and 28% of Koreans, respectively. Q126Stop and P269S were found in 1.9% and 0.2% of Koreans, respectively. The frequencies for the ten intronic SNPs ranged from 10 % to 67%. Recently, Kondo et al. (2004) reported the identification of several BCRP variants, which include A149P, R163K, Q166E, P269S, and S441N, in human cell lines. With the exception of the BCRP P269S variant, these variants were not observed in this study.

Linkage Disequilibrium and Haplotype Structure. LD was analyzed using |D′| values across the common SNPs, which were found with allelic frequencies of >5%. All the SNPs used for the LD analysis exhibited Hardy-Weinberg Equilibrium (HWE). The positions of eleven common *BCRP* SNPs calculated by HWE and their haplotype profiles are shown (Fig. 1A). The LD

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profile of *BCRP* showed that all of the SNP pairs in the segment of 34G>A-238A>G-7430A>G-8825C>A were in complete LD (|D′| = 1) (Fig. 1). In the second segment covering 6 SNPs (38485A>G-40086insA-42288C>T-44072C>T-44997A>G-45235C>T), all of the SNP pairs, with the exception of the 40086insA/42288C>T pair, were to a large extent in LD, with |D′| = 0.9. The *BCRP* haplotypes for 11 common SNPs with frequencies >5% were analyzed using the SNPAlyze program (Table 3). Fourteen haplotypes were predicted to have frequencies >1%. The most frequent allele was the wild-type (26%), followed by the Q141K variant. The haplotype analysis suggests that none of the Q141K-containing haplotypes are linked to the V12M variant. To support this strong linkage, the V12M and Q141K variations were assigned to the same haplotype block among two discrete haplotype blocks of the *BCRP* gene (Fig. 1B).

Discovery and Functional Characterization of New SNPs in the *BCRP* Promoter. Among the five SNPs found in the promoter region of *BCRP*, three SNPs (–19855C>T, –19605A>G, and –19031C>T) were identified as novel variants. The frequencies of these novel SNPs were <1.6% and they appeared on different chromosomes. To evaluate the effects of these variants on transcriptional modulating activity, the BCRP promoter-conjugated-luciferase reporter assay was performed. No significant differences were observed between each of the SNPs and the wild-type promoter (Fig. 2A). *BCRP* expression has been reported to be up-regulated through HIF-1α binding to a proximal hypoxia response element (HRE) at the -114 position (Krishnamurthy

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et al., 2004), and one of the novel *BCRP* SNPs was localized to the 5-bp consensus element of the HRE sequence. Therefore, we investigated whether the variation in the HRE affected the responsiveness of the *BCRP* promoter to hypoxic stress. The wild-type promoter and a variant promoter that included –19031C>T were introduced into HEK293F cells and treated with deferoxamine, a hypoxia-mimetic agent. In the untreated condition, the variant and wild-type promoters showed similar transcriptional activities (Fig. 2B). In the presence of deferoxamine, and with co-transfection of the Hif-1α-expressing plasmid, the transcriptional activities of the variant and wild-type were increased to similar extents. These results suggest that this novel SNP in the HRE core site does not cause any alteration of Hif-1α-mediated transcription. The location of -114C>T relative to HRE core region is indicated in Fig. 2C.

Transporter Activity of the P269S Variant. Two different comparisons of the wild-type and P269S variant proteins were performed. Vesicular uptake of [^{3}H]-MTX and [^{3}H]-ES into the microsomal fraction was measured for the BCRP wild-type and P269S proteins expressed in Sf9 cells. BCRP P269S-expressing microsomes showed about 35-40% decrease in [^{3}H]-MTX and [^{3}H]-ES uptake compared to microsomes that expressed the wild-type BCRP (Fig. 3A and 3B). The expression levels of the wild-type and P269S BCRP proteins were similar, as confirmed by immunoblotting (Fig. 3C). In order to investigate whether the decreased functionality of BCRP P269S was derived from the modulation of substrate-dependent ATPase activity, prazosine-induced ATPase activity was measured in the wild-type and P269S-expressing microsomes from Sf9 cells. The basal ATPase activity in Sf9 cell membranes enriched with wild-

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type or variant BCRP proteins in the absence of prazosine showed no difference between wild-type and variant BCRP membranes. The BCRP ATPase activity in the presence of prazosine of the P269S variant was also the same as that of the wild-type protein, which indicates that the decreased activity of P269S is not related to ATPase activity. The expression levels of the recombinant BCRP proteins were similar, as confirmed by immunoblotting (Fig. 4).

Nonsynonymous Variants of the *BCRP* Gene in other Asian Populations. From the screening of four nonsynonymous variants in other ethnic groups, the allelic frequencies of V12M, Q126Stop, Q141K, and P269S were obtained (Table 4). The frequency of V12M was 10-13% higher in Chinese and Vietnamese subjects than in Koreans, whereas Q141K showed no significant differences among the three Asian ethnic groups. The frequency of the Q126Stop variant in Chinese and Vietnamese was <0.5%, although this null variant exhibited a slightly higher incidence (1.9%) in Koreans than in other ethnic groups. The P269S variant was not observed in 100 Caucasians and 100 African-Americans, which suggests that this variant is a rare variant that is found only in Asians.

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Discussion

BCRP was first identified in cell lines that exhibited resistance to mitoxantrone or adriamycin, in which the over-expression of BCRP conferred protection from the cytotoxicities associated with these drugs (Brangi et al., 1999; Maliepaard et al., 1999; Ross et al., 1999). Many studies of these drug-resistant cell lines have indicated that BCRP confers resistance to mitoxantrone, anthracyclines, topotecan, irinotecan, and SN-38 by pumping out the drugs from the cells (Litman et al., 2000; Zhou et al., 2001).

Recent clinical studies have suggested that the large interindividual variability in drug responses is a consequence of genetic alterations in drug transporter genes. For example, the BCRP Q141K variant has been implicated in the altered pharmacokinetics of diflomotecan (Sparreboom et al., 2004). This *BCRP* polymorphism has also been associated with the susceptibility of renal caricinoma cells to drug treatments (Korenaga et al., 2005) and with the low expression of BCRP in the human placenta (Kobayashi et al., 2005). Since BCRP plays an important role in clinical treatment, genetic polymorphisms of *BCRP* have been widely studied in different ethnic groups (Honjo et al., 2002; Backstrom et al., 2003; Wang et al., 2005).

In the present study, we performed a comprehensive evaluation of *BCRP* polymorphisms in a Korean population. Twenty SNPs were identified in 92 Korean subjects. Among the BCRP-coding variants, V12M and Q14lK were the most common in Koreans, with allelic frequencies of 23% and 28%, respectively. These variants are also found frequently in other ethnic groups, such as Caucasians, Japanese, and Chinese (Zamber et al. 2003; Kobayashi

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et al. 2005). The Q141K variant was the most prevalent coding variant in Caucasians (14%), Japanese (35%), and Chinese (35%), as well as in Koreans (28%), whereas it was not detected in African-Americans or in subjects from Africa north of the Sahara (Zamber et al., 2003). The allelic frequency of V12M in Koreans (23%) is much lower than that reported for Southeast Asian (45%), Pacific Islander (64%), Mexican-Indian (90%), and Hispanic (40%) subjects (Zamber et al., 2003). Another nonsynonymous variant, Q126Stop, was detected as a heterozygous genotype in Koreans (1.9%) and in Japanese subjects (1%) (Kobayashi et al., 2005). P269S was observed exclusively in Koreans and Vietnamese, and not in the Caucasian, African-American, and Chinese populations. Taken together with previously reported studies, our results support the notion that *BCRP* polymorphisms show ethnic differences.

Recently, it has been shown that many genes exhibit a block-like pattern of LD and linkage to each other. The LD pattern observed in the present study shows that there are two discrete blocks of *BCRP* genes in the Korean population (Fig. 1B). These two-block structures of LD have also been reported in a Chinese population (Wang et al., 2004). Most of the SNPs in the individual blocks reveal a high frequency of haplotype structure for the *BCRP* gene in Koreans (Table 3). The wild-type showed the highest frequency at 42%. Haplotypes 4, 8, and 11 indicate that a single promoter SNP (20296A>G) and the 238A>G change accompany the V12M nonsynonymous change in Koreans (Table 3). In haplotypes 2, 7, and 13, Q141K (8825C>A) variation is accompanied by 42288C>T and 44997A>G. These strong linkages also demonstrate the block-like haplotype.structure of BCRP gene. For the

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association studies between haplotypes and phenotypes such as drug resistance or disease susceptibility, haplotype-based approach has been known to be more informative than SNP-based approach. Our results on BCRP haplotypes in Korean population would provide information on genetic markers for association studies relating to BCRP polymorphism in this population.

The identification of genetic polymorphisms is recognized as an important topic, since some SNPs can be used as simple genetic markers, as well as for the prediction of *in vivo* functional phenotypes. In the case of *BCRP*, the 421C>A variation (encodes Q141K) has been reported to play important roles in determining the expression level of the protein (Imai et al., 2002; Kobayashi et al., 2005), drug resistance, and ATPase activity (Mizuarai et al., 2004). The V12M SNP has been reported to be associated with membrane localization (Mizuarai et al., 2004). Increased BCRP expression has been linked to reduced efficacy of chemotherapy in acute myeloid leukemia patients, which implies that BCRP expression is associated with the development of multidrug resistance (Steinbach et al., 2002). In addition, BCRP expression has been suggested as a marker for human hematopoietic stem cell protection (Kim et al., 2002). Recently, *BCRP* expression has been shown to be up-regulated by hypoxia through the HRE, which is required for HIF-1α binding during *BCRP* transcription (Blancher and Harris, 1998; Semenza, 2002; Krishnamurthy et al., 2004). Interestingly, of our three novel SNPs in the promoter region of *BCRP*, we localized -19031C>T SNP (-114 position in relation to transcription start site) to the core HRE region of the *BCRP* promoter. Krishnamurthy et al. (2004) revealed that there are two functional HRE (centered at -194, -115) in proximal

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promoter and one distal nonfunctional HRE at –1094 in BCRP gene. They revealed that HRE at –115 is most important in hypoxic induction of BCRP expression. Since the -19031C>T transition variation is localized at the second position in five-nucleotide core region (AC/TGTG) of HRE centered at -115 and the core consensus region is highly conserved, it is speculated that this -19031C>T SNP may affect *BCRP* transcriptional activation. However, it was found that this variation did not significantly alter BCRP transcription under the chemically induced hypoxic condition as well as under normal condition. This suggests that the second base variation (C/T) in the consensus HRE may not be essential for the binding of HIF-1α to the HRE of BCRP promoter. The other two promoter SNPs found in Koreans also showed normal transactivation activity, which suggest that the regulatory SNPs in the BCRP promoter may not contribute to the inter-individual variation of BCRP expression.

In the present study, we have described the allele frequencies for *BCRP* variants in a Korean population and analyzed the function of the P269S coding variant. Kondo et al. (2004) have reported that the P269S variant has activity similar to that of the wild-type protein. However, in at least three repeated experiments, we showed that P269S shows altered ES and MTX uptake, as compared to the wild-type. Both the previous study and our current study show similar protein levels of P269S variant and wild-type protein expression in the systems used. This discrepancy may be partly explained by the different assay systems used, i.e., baculovirus versus adenovirus systems. For example, in Sf9 cells, the cholesterol to phospholipid ratio is much lower than in mammalian cells (Marheineke et al., 1998). Many studies have shown that the

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lipid composition of membrane alters ABC transporters including P-glycoptotein (Troost et al., 2004). Therefore, it cannot be ruled out the possibility that P269S variation may not change the BCRP activity in mammalian cells. Additional experiments in other assay systems will reveal the function of this variant. Similarly, BCRP V12M has been reported to exhibit the same transport function as the wild-type (Kondo et al., 2004), while another study has shown decreased activity caused by disrupted membrane localization (Mizuari et al., 2004). Genotyping in clinical studies is needed to address the relationships between these genotypes and phenotypes.

The present study is the first report on the identification of allelic variants of the *BCRP* gene in a Korean population. In the 92 Korean subjects, twenty SNPs, including four functional variants, were identified in the present study. We also evaluated the frequency of the four functional variants in two representative Asian populations, Chinese and Vietnamese, to find ethnic difference of BCRP polymorphism. Future clinical studies will elucidate the genotype/phenotype relationships for these *BCRP* polymorphisms and the ethnic variation of drug disposition of BCRP substrates.

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Footnotes

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Figure Legends

Fig. 1. LD block structure and haplotype diversity of the *BCRP* gene. A. Genetic organization of *BCRP* and positions of the identified variations. Eleven common *BCRP* SNPs in HWE and the common haplotype profiles are shown. **B.** Pairwise linkage disequilibrium among the *BCRP* SNPs, as calculated by the Haploview software. The dark black box without a star represents a ID′I value of 1.00, and the dark black boxes that contain a star represent values that are not determinable. The higher ID′I values with strong linkage are indicated by dark coloration. Grey and white colorations indicate weak linkages with low ID′I values.

Fig. 2. *In vitro* functional studies of three genetic variations found in the 5′-flanking region of the *BCRP* gene. A. Plasmids that contained the BCRP-936C>T, -689A>G, and -114C>T variations were co-transfected into HEK293F cells with the β-galactosidase-expressing plasmid pCMV-β -Gal. The relative luciferase activity was measured at 24 h post-transfection. **B.** Plasmids that contained either the BCRP-114C or BCRP-114T variation were co-transfected with the HIF-1α-expressing plasmid. After 24 h of incubation, the cells were treated with 100 μM deferoxamine for an additional period of 24 h. **C.** Sequences surrounding the BCRP-114C>T variant. The underlined bases represent the core sequence of the HIF-1α-binding site. The bolded letter indicates the position of -114C>T. The values shown are mean ± S.D. for at least three different experiments.

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Fig. 3*. In vitro* functional characterization of the BCRP P269S variant.

A. Vesicular uptake of [^{3}H]-MTX in membranes obtained from Sf9 cells that express the wild-type BCRP protein or the BCRP P269S variant protein. Isolated Sf9 membrane fractions were incubated with [^{3}H]-MTX for 15 min, washed to remove un-absorbed substrate, and the radioactivity was measured in a liquid scintillation counter. BCRP P269S exhibited significantly decreased activity, compared with the wild-type, in a two-tailed Student's *t*-test; *, $p < 0.05$. **B.** Vesicular uptake of [^{3}H]-estrone-3-sulfatein membranes obtained from Sf9 cells that express the wild-type BCRP protein or the BCRP P269S variant protein. All procedure was same as [^{3}H]-MTX except substrate. **C.** Expression level of wild-type and variant BCRP proteins in Sf9 cell membranes. Empty baculoviral construct was infected to Sf9 cells as a negative control of specific expression of BCRP proteins (Mock). The same membranes in Fig. 3A and B were immunobloted using anti-BCRP antibody as described in Materials and Method section.

Fig. 4. Prazosine-dependent ATPase activity of the BCRP P269S variant.

A. ATPase activities of Sf9 cells that express the wild-type BCRP protein or the BCRP P269S variant protein. The ATPase activities of the Sf9 cell membranes were determined by measuring vanadate-sensitive inorganic phosphate liberation. **B.** The expression levels of the wild-type and variant BCRP proteins in the Sf9 cells were similar, as confirmed by immunoblotting. Empty

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baculoviral construct was infected to Sf9 cells as a negative control of specific expression of BCRP proteins (Mock). The data shown are the mean ± S.D. for at least three different experiments.

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TABLE 1. Primer sequences used for the amplification of the *BCRP* gene fragment and the annealing temperatures used in the PCR

Name	Region	Primer sequence (5'→3')	Size (bp)	PCR condition (Tm; °C)
BCRP1P	Promoter	F: AACCCAGCTAGGTCAGACGA R: TTTGAGTGGGCACAGCAC	557	60.0
BCRP2P	Promoter	F: TTCCTAGGGTAGATGCAGCAG R: CAGGGACAAGCCAAACACTC	509	60.0
BCRP3P	Promoter	F: GTAGAGGCAGGGTTTCACCA R: AAGTGATTGCGCATGTTCAG	559	60.0
BCRP4P	Promoter	F: CGTGCCTGGCCTCTATGTAT R: CTGACGCAGGCAGATCACT	572	60.0
BCRP5P	Promoter	F: GCCACCACACCCAGTGTAAT R: TGCAAAGTAAAAACAAATCAAAACC	518	64.7
BCRP1E	Exon1	F AGCTCGTCCCCTGGATGT R: CCACCAACCTTTCCAGACAC	516	54.0
BCRP2E	Exon2	F: CTGCTCATTGCCACACATTT R: GCCAAAACCTGTGAGGTTCA	400	54.0
BCRP3E	Exon3	F: GTCTCAAACTCCTGGCCTCA R: GCGTTGCAAATGCTCAATAA	403	54.0
BCRP4E	Exon4	F: TGGATTCAAAGTAGCCATGAGA R: ATTCTCCCTGCCTTTTCACA	402	54.0
BCRP5E	Exon5	F: GGTTCATCATTAGCTAGAACTTTACC R: TGGAAAGCAACCATTTTTGA	403	54.0
BCRP6E	Exon6	F: TCTTACAGGACTGGCACACG R CCTTCCCTACATTCTTACCTGCT	426	54.0
BCRP7E	Exon7	F TCAGGCTGAACTAGAGCAAACA R: AGCACCAAATGGAACAAACA	387	60.0
BCRP8E	Exon8	F: CATGGGAAGAAGAGAGAAAGAAA R: CAAAAACACCAACAGCACTCA	412	60.0
BCRP9E	Exon9	F : GGTGTTAGGGAAGCATCCAA R: TGAAGCAGATGATAACAGAACCA	413	54.0
BCRP10E	Exon10	F: GCCAAGCCATTGAGTGTTTA R: TGGGCAACAGAGCATGAC	386	60.0
BCRP11E	Exon11	F: CCACAACAATCCAAGACTGTG R GTAATCCTCCGGATCCCATC	423	60.0
BCRP12E	Exon12	F: GGTCTAGCCCTGAGGATGTG R: GAGTGCAAAATGGACAGGTG	403	64.7

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BCRP13E	Exon13	F: AGGGTGGTTGGAGAGTGGAT R: AGCAGAGCCCCATTTACAGA	412	60.0
BCRP14E	Exon14	F: TGAGTGTCTTGAGTAAGTGGAGAGA R: GACTCCCCAGCCTTGTGTTA	420	54.0
BCRP15E	Exon15	F: TCTTGATTGCCAGGGAAAAT R: CGCGCACAACTCACTTTATG	404	60.0
BCRP16E	Exon16	F: TGACGGATGCTAGGAATGAA R: CCCATGGTTACTGTCTGAGGA	430	64.7

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TABLE 2. Primer sequences used for the pyrosequencing-based genotyping of functional BCRP variants

SNP[a]	Variant	Primer sequence (5'→3')	Size (bp)	PCR condition (Tm; °C)
34G>A	V12M	5'-Biotin-CTCTCCAGATGTCTTCCAGTAATG-3' 5'-GCCAAAACCTGTGAGGTTCA-3' For sequencing: 5'-AGTGTTCCTTTGTGGTTAC-3'	278	54.0
8191C>T	Q126Stop	5'-Biotin-ACTATCAGCCAAAGCACTTACCC-3' 5'-GTCTTAGCTGCAAGGAAAGATCCA-3' For sequencing: 5'-AATGTAATTCAGGTTACGTG-3'	174	54.5
8825C>A	Q141K	5'-Biotin-GTTGCAAGCCGAAGAGCTG-3' 5'-TGATGTTGTGATGGGCACTC-3' For sequencing: 5'-GACGGTGAGAGAAAACTT-3'	69	54.0
21850C>T	P269S	5'-Biotin-TAGCACCAAATGGAACAAACAC-3' 5'- TGTTTGATAGCCTCACCTTATTGG-3' For sequencing: 5'-GAAGACTTATGTTCCACG-3'	236	54.0

[a] Position is indicated with respect to the start codon (ATG) of the *BCRP* gene; the A in the ATG triplet is designated as +1 and the next base towards the 5′-end is designated as -1.

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TABLE 3. Allelic frequencies of *BCRP* variants identified in Koreans[a]

SNP No.	SNP and position[b]	Position relative to transcription start site	Location	Effect	N	Allelic frequency (%)
1	-20296A>G	-1379	Promoter		92	13
2	-19855C>T	-938	Promoter		92	0.5
3	-19605A>G	-688	Promoter		92	0.5
4	-19031C>T	-114	Promoter		92	1.6
5	-18631C>T	+286	5'UTR		92	2.2
6	34G>A		Exon 2	V12M	275	23
7	238A>G		Intron 2		92	25
8	7430A>G		Intron 3		92	9.8
9	8191C>T		Exon 4	Q126Stop	375	1.9
10	8825C>A		Exon 5	Q141K	275	28
11	21850C>T		Exon 7	P269S	674	0.2
12	26297G>A		Exon 9		92	1.1
13	38485A>G		Intron 11		92	24
14	40086insA		Intron 12		92	0.5
15	40110G>T		Intron 12		92	22
16	42288C>T		Intron13		92	67.4
17	42313T>G		Intron13		92	2.2
18	44072C>T		Intron13		92	23.4
19	44997A>G		Intron14		92	49.5
20	45235C>T		Intron15		92	20.1

[a] The reference sequence used has GenBank accession no. AC084732.

[b] Position is indicated with respect to the start codon (ATG) of the *BCRP* gene; the A in the ATG triplet is designated as +1 and the next base towards the 5′-end is designated as -1.

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TABLE 4. Haplotype distribution of *BCRP* gene in Koreans

SNP		-20296 A>G	34 G>A	238 A>G	7430 A>G	8825 C>A	38485 A>G	40110 G>T	42288 C>T	44072 C>T	44997 A>G	45235 C>T	Frequency (%)
AA change			V12M			Q141K							
Haplotype	1												26.4
	2					■		■	■		■		19.9
	3						■		■	■	■	■	7.8
	4	■	■	■					■				7.6
	5								■				7.4
	6		■	■	■		■		■	■	■	■	5.9
	7					■	■		■	■	■	■	4.7
	8	■	■	■									2.9
	9						■		■	■	■		2.5
	10		■	■					■				1.8
	11	■	■	■			■		■	■	■	■	1.7
	12		■	■	■				■				1.4
	13					■			■		■		1.1
	14		■	■	■								1.1

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TABLE 5. Expected allelic frequencies of BCRP V12M, Q126Stop, Q141K, and P269S variants in different Asian populations

Population	No of Subjects	Allelic Frequency (%) (95%CI)			
		V12M	Q126Stop	Q141K	P269S
Korean	275 - 674 [a]	23 (19.6 - 26.6)	1.9 (0.9 - 2.9)	28 (23.8 - 31.2)	0.2 (0 - 0.4)
Chinese	191	33** (28.5 - 37.9)	0.5 (0 - 1.2)	29 (24.3 - 33.3)	0 (0 - 0.1)
Vietnamese	140	36** (30.8 - 42.0)	0.4 (0 - 1.1)	31 (25.7 - 36.5)	0.7 (0 - 1.7)

[a] The numbers of subjects genotyped for the V12M, Q126Stop, Q141K, and P269S variants were 275, 375, 275, and 674, respectively. **, $p < 0.005$.

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Fig. 1

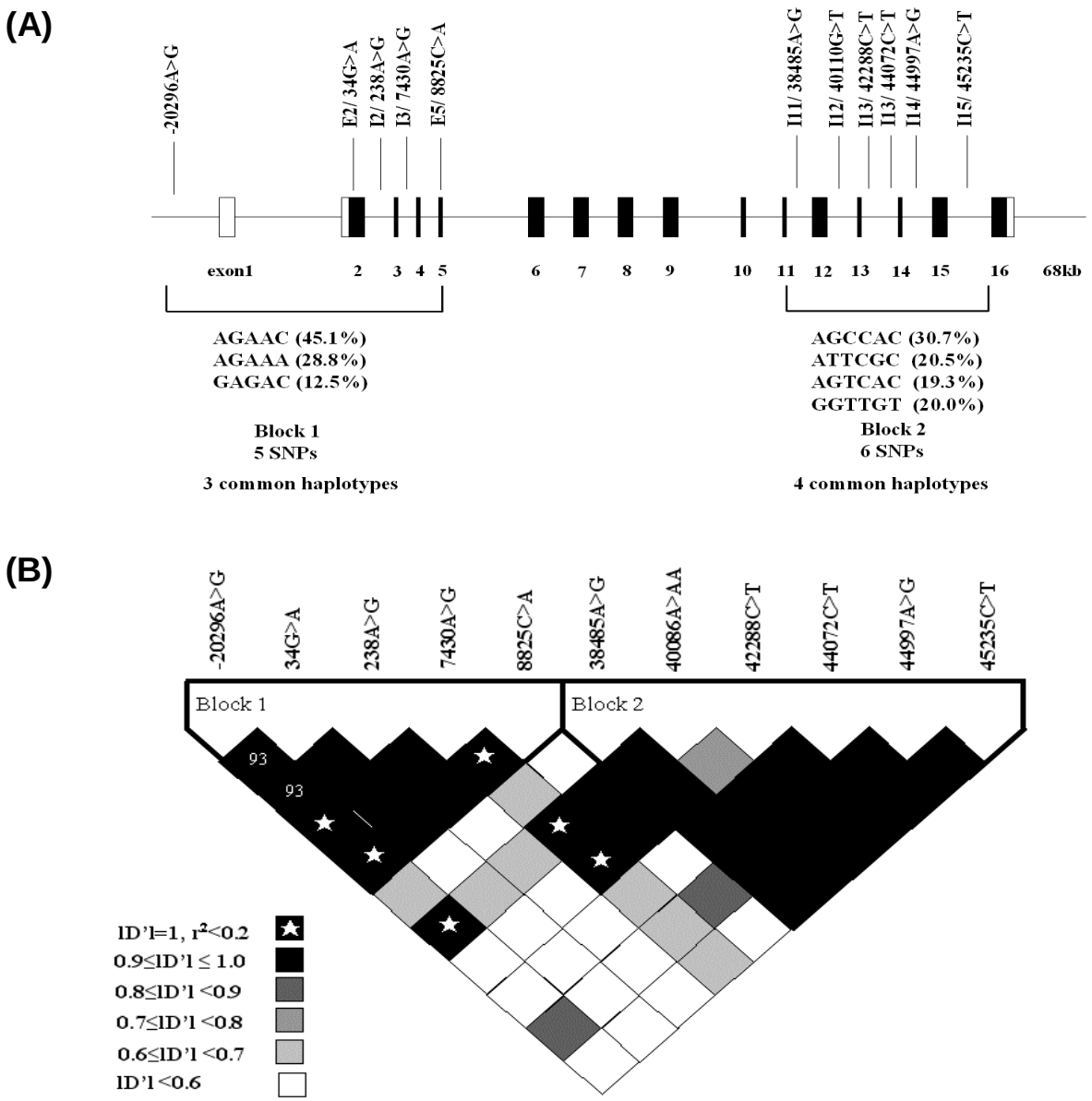

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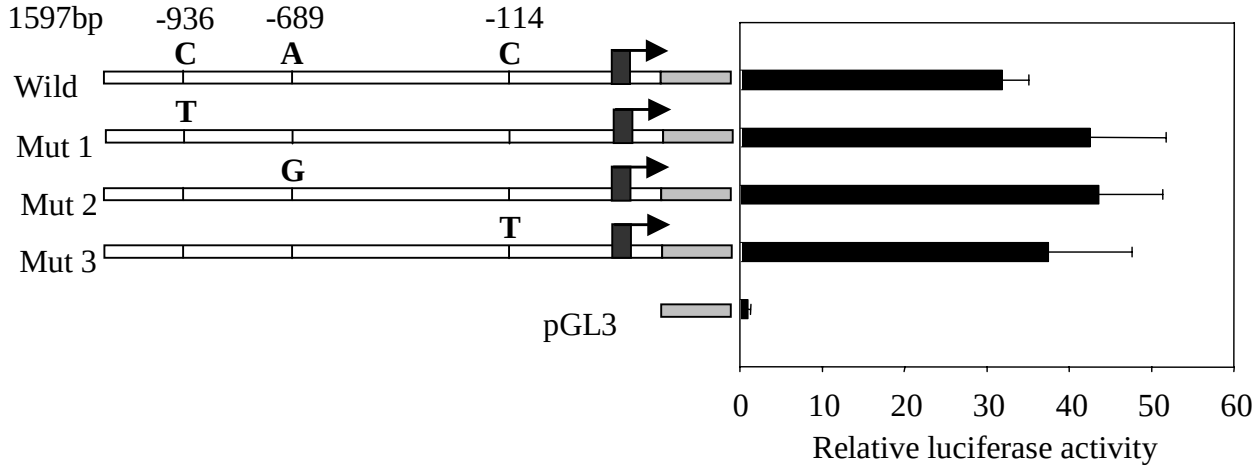
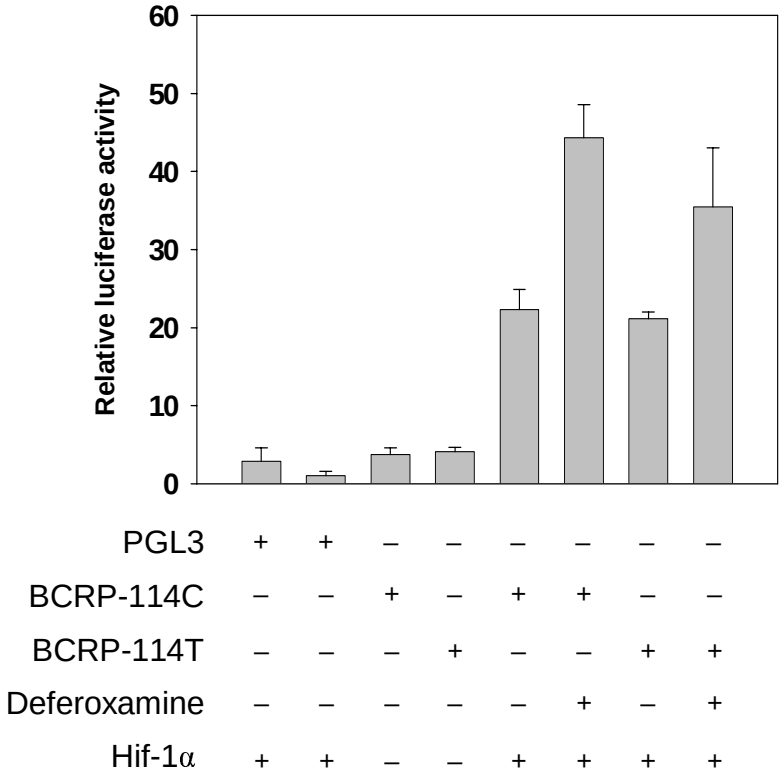
Fig. 2

(A)



(B)



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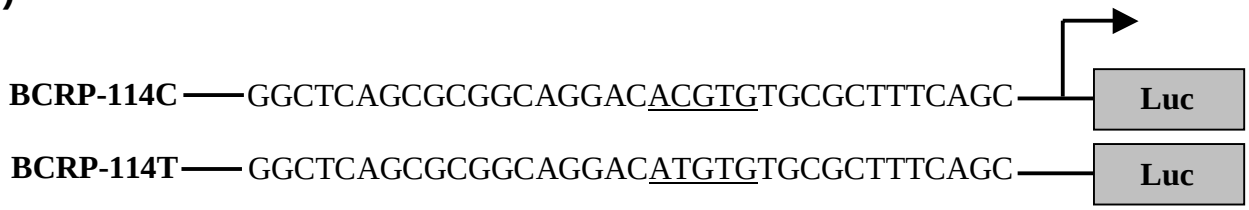

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Fig. 3

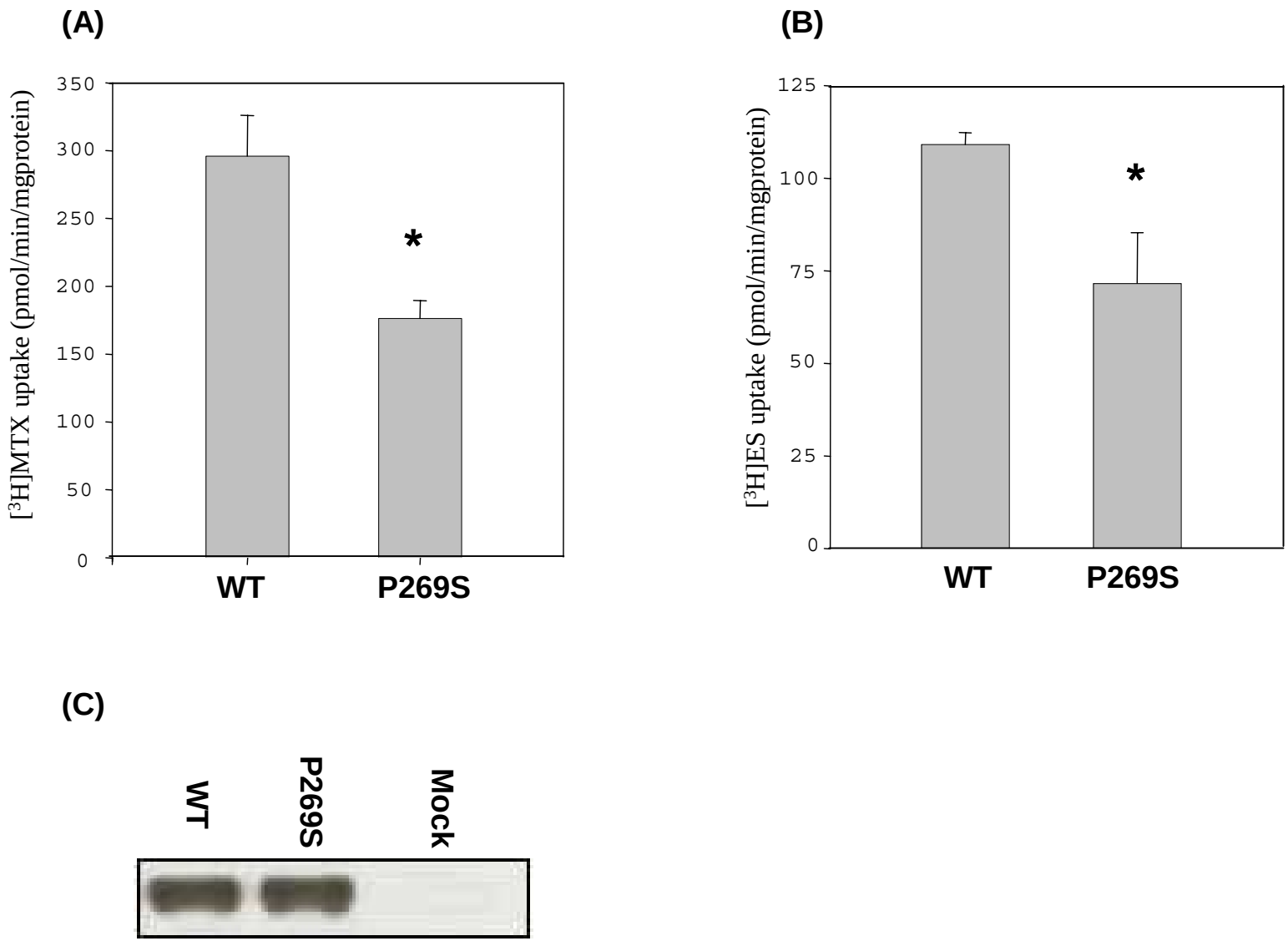

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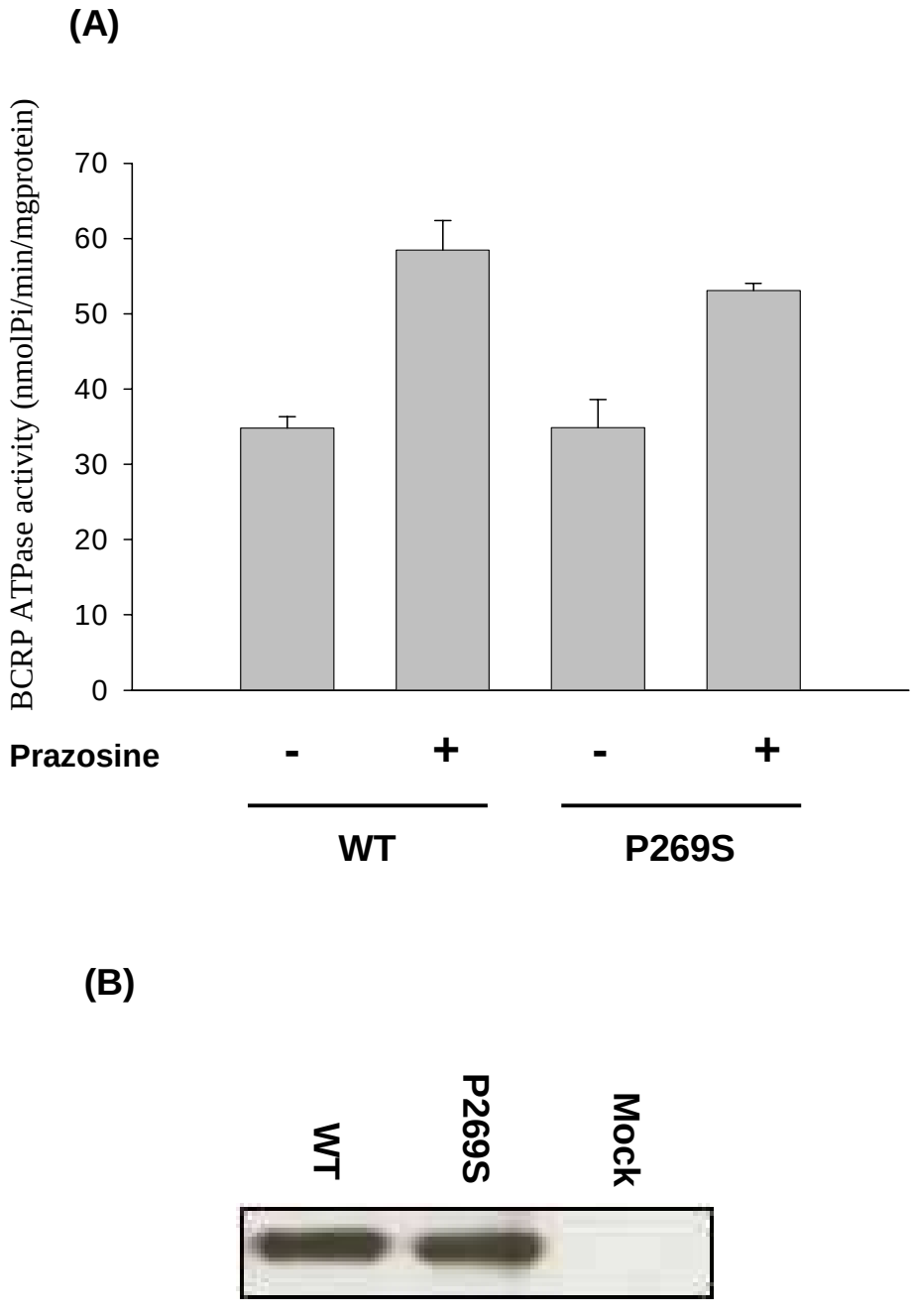
Fig. 4



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