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ORIGINAL PAPER

Screening for herbal medicines that affect *ZIC1* gene methylation in colorectal cancer

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Abstract The zinc finger of the cerebellum (*ZIC1*) is a tumor suppressor gene that is silenced through promoter hypermethylation in colorectal cancer. This study was conducted to evaluate the efficacy of herbal medicines to demethylate the *ZIC1* gene in colorectal cancer. We evaluated the effects of 30 herbal medicines on global DNA methylation, including the *ZIC1* gene, in SW620 cells by a commercial DNA methylation assay. After the first round of screening, candidate herbal medicines were selected based on *ZIC1* gene expression by reverse transcription-polymerase chain reaction and methylation-specific PCR analysis. The efficacy of the selected candidate medicines on DNA demethylation was then determined by methylation-specific PCR analysis and pyrosequencing. Of the extracts evaluated, Vitex rotundifolia was associated with *ZIC1* re-expression in SW620 cells. Of the herbal medicines studied, V. rotundifolia is the best candidate for the demethylation of *ZIC1*. Additional studies should be conducted to determine whether V. rotundifolia and other herbal medicines represent novel therapeutic avenues that can be utilized for the prevention or treatment of colorectal cancer.

Keywords *ZIC1*, Epigenetic regulation, Colorectal cancer, *Vitex rotundifolia*

Colorectal cancer (CRC) is a major cause of cancer morbidity and mortality. Approximately one-third of CRC patients die from the disease[1]. Nearly 50% of all people with CRC will have a local recurrence or develop distant metastases. The median survival time for these patients is 4-22 months[2]. A current estimate is that 15-30% of CRCs may have a major hereditary component, given the occurrence of CRC in first- or second-degree relatives[3,4]. The etiology of CRC from a polyp to malignant tumor has been explained by pathways involving epigenetic alteration or accumulation of genetic alterations[5]. Genetic alterations involve mutations in oncogenes and tumor suppressor genes. Genetic alterations, cancer initiation and promotion can occur through epigenetic regulation[6,7]. Epigenetic gene regulation involves reversible alteration of genomic patterns and chromatin organization through histone modification and DNA methylation[8,9]. DNA methylation is a well-understood postsynthetic modification of DNA that frequently results in gene silencing. DNA methylation is a mark on genomic DNA created by the addition of a methyl group to the 5-carbon position of cytosine, predominantly in a 5′-CpG-3′ sequence context[10,11]. CpG islands are located in the promoter regions of many genes[12,13].

The *ZIC* gene family plays an important role in the development of the neural crest and the cerebellum in vertebrates[14-18]. In addition, *ZIC* family proteins can bind to GC-rich regions in target genes[18]. Despite its role in neural development, *ZIC1* participates in the oncogenesis of undifferentiated nervous system tumors[19] and has recently been described as a tumor suppressor gene silenced through promoter hypermethylation in CRC[20]. *ZIC1* can function as a repressor of downstream targets of bone morphogenetic protein and sonic hedgehog (Shh) and also plays a role in the Notch signaling pathway during neural tube development[17,18].

The aim of the present work was to elucidate the epigenetic regulation of the *ZIC1* gene involved in the

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Table 1. Global methylation effect of herbal medicines in SW620 cells.

Herbal medicines	DNA methylation (%)	Herbal medicines	DNA methylation (%)
Control (SW620)	100	*Oryza sativa*	143
5-aza-2-deoxicytidine	56	*Astragalus complanatus*	138
Chrysanthemum	127	*Verbena officinalis*	133
Cynanchum atratum	117	*Notopterygium incisum*	113
Artemisia anomala	173	*Tussilago farfara*	170
Cannabis sativa	135	*Ophiopogon japonicus*	107
Ephedra sinica	109	*Morinda officinalis*	108
Trichosanthes kirilowii	109	*Angelica daburica*	134
Curcuma longa	162	*Vitex rotundifolia*	140
Sophora subprostata	125	*Sinapis alba*	103
Lycium chinense	79	*Gentiana scabra*	76
Celosia argentea	90	*Lonicera japonica*	164
Santalum album	118	*Magnolia liliflora*	163
Cnidium monnieri	95	*Trichosanthes kirilowii*	99
Alisma orientalis	57	*Tribulus terrestris*	94
Eclipta prostata	88	*Luffa cylindrica*	90

Values represent mean ± SD. (n=3). Each herbal medicine was applied at 10 μg/mL. Global DNA methylation was normalized by control (non-treated cells, 100%) for all data sets.

development or progression of CRC. Accordingly, we evaluated the efficacy of a selection of herbal medicines to demethylate the *ZIC1* gene in a CRC cell line.

Global DNA methylation

We determined the content of 5-hmC in SW620 CRC cells treated with 30 herbal medicines or a demethylation agent (5-aza-2-deoxycitidine). DNA was isolated from a total of 32 samples (30 samples and a positive and negative control). Global DNA methylation data are summarized in Table 1. The effects on global DNA methylation in the herbal-treated SW620 cells were less than that of the non-treated SW620 cells (control) with 9 herbal medicines: *Lycium chinense, Celosia argentea, Cnidium monnieri, Alisma orientalis, Eclipta prostate, Gentiana scabra, Trichosanthes kirilowii, Tribulus terrestris*, and *Luffa cylindrica* (Table 1). However, the effects on global DNA methylation in the herbal-treated SW620 cells were greater than those of the non-treated control cells (SW620 cells) with 21 herbal medicines: *Curcuma longa* (CL), *Astragalus complanatus, Vitex rotundifolia* (VRE), *Chrysanthemum, Verbena officinalis, Oryza sativa, Cynanchum atratum, Notopterygium incisum, Artemisia anomala, Tussilago farfara, Cannabis sativa, Ophiopogon japonicas, Ephedra sinica, Morinda officinalis, Trichosanthes kirilowii, Angelica daburica, Sophora subprostata, Sinapis alba, Santalum album, Magnolia liliflora*, and *Lonicera japonica* (LJ) (Table 1).

Validation of DNA methylation of the *ZIC1* gene via methylation specific-PCR

CRC cells treated with each herbal medicine, with three

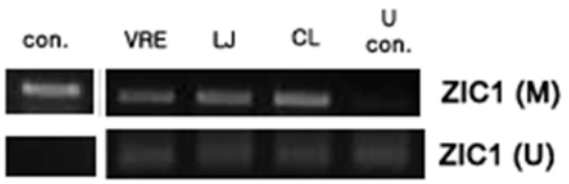

Figure 1. Promoter methylation contributes to *ZIC1* downregulation in a colorectal cancer cell line. The methylation status of the *ZIC1* promoter CpG island was detected by methylation-specific PCR in herbal-medicine-treated colorectal cancer cells. Abbreviations: U, unmethylated DNA; M, methylated DNA; Con: non-treated SW620, VRE: *Vitex rotundifolia*, LJ: *Lonicera japonica*, CL: *Curcuma longa*, U con, unmethylated control (5-aza-2-deoxycytidine).

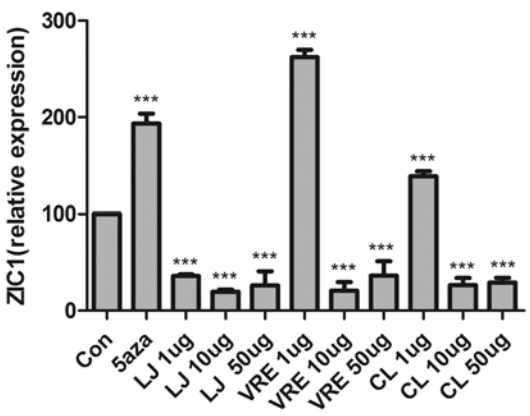

Figure 2. *ZIC1* gene expression in herbal-medicine-treated colorectal cancer cells. Analysis of *ZIC1* mRNA expression in herbal-medicine-treated SW620 cells. β-actin mRNA amplification was used as a control and to assess RNA integrity. Abbreviations: 5-aza: 5-aza-2-deoxycytidine, VRE: *Vitex rotundifolia*, LJ: *Lonicera japonica*, and CL: *Curcuma longa*. $P < 0.001$.

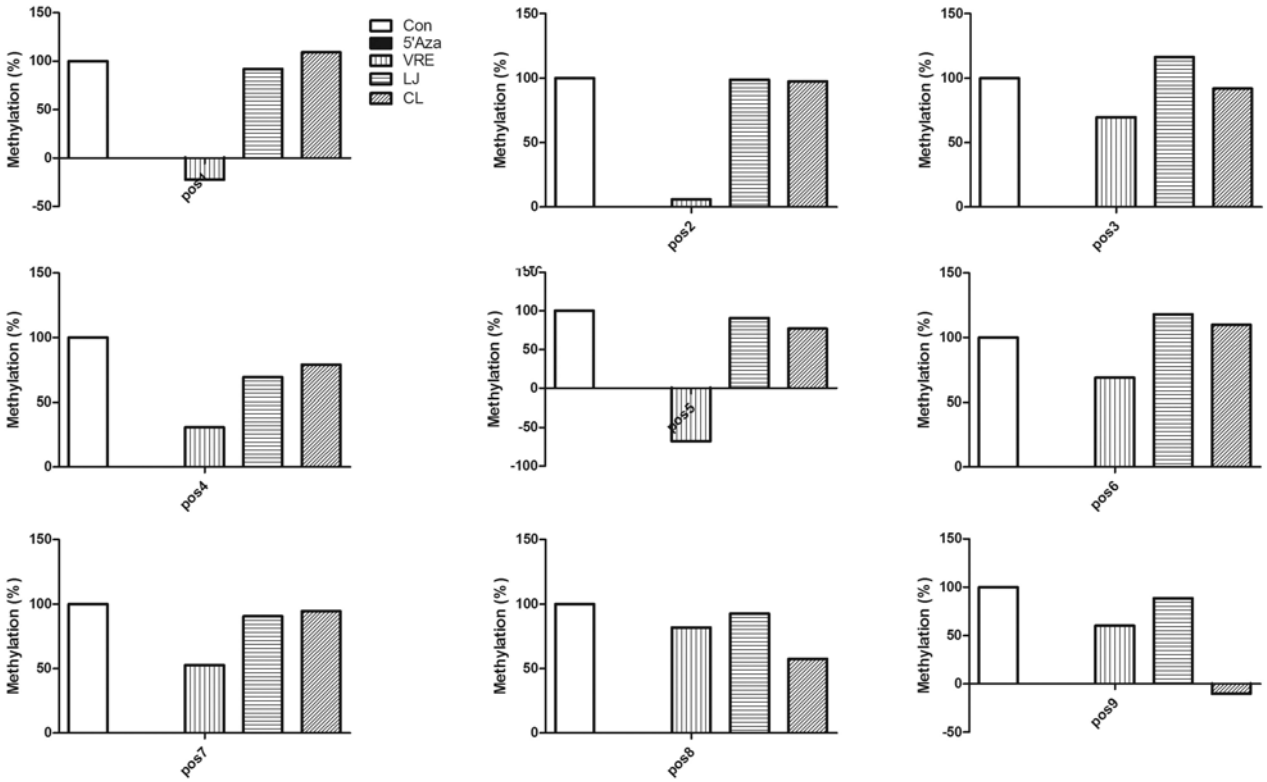

Figure 3. Quantitative DNA methylation analysis. The percentage of DNA methylation levels at promoter CpG islands were analyzed in bisulfite-modified genomic DNA extracted from non-treated and herb-treated SW620 cells. The percentage of methylated cytosines in the samples as obtained from pyrosequencing is plotted on the y-axis. The percentage of methylated cytosines was normalized such that the positive control (5-aza-2-deoxycytidine-treated cells) becomes 0% and control (non-treated cells) becomes 100% for all datasets. Abbreviations: 5-aza: 5-aza-2-deoxycytidine, VRE: *Vitex rotundifolia*, LJ: *Lonicera japonica*, and CL: *Curcuma longa*. Pos: selected position number at promoter CpG islands.

exceptions, displayed methylated DNA (data not shown). The exceptions were VRE-, LJ-, and CL-treated SW620 cells, where unmethylated DNA from the *ZIC1* gene was detected (Figure 1).

Validation of *ZIC1* gene expression via real-time RT-PCR

We normalized the mRNA expression levels of the *ZIC1* gene by dividing by the expression of β-actin as determined in the real-time RT-PCR analysis. Real-time RT-PCR revealed that *ZIC1* expression was restored in SW620 CRC cells after treatment with 5-aza-2-deoxycytidine as a positive control and following treatment with VRE, LJ, and CL (Figure 2).

Validation of DNA methylation of the *ZIC1* gene via pyrosequencing

We used pyrosequencing to analyze the methylation status of the *ZIC1* gene. For *ZIC1*, the percentage (%) of methylation at a specific promoter was compared between the non-treated and the herb-treated SW620 cells (Figure 3). The pyrosequencing analysis was repeated. The *ZIC1* gene was frequently hypermethylated in the non-treated samples. The pyrosequencing data showed a distinct methylation pattern and was found to be hypomethylated in VRE-treated SW620 cells compared to LJ- or CL-treated SW620 cells (Figure 3).

Discussion

Epigenetic regulation is important in the regulation of gene expression. Epigenetic modifications are heritable and are transmitted from a mother cell to the daughter cells. The gene structure consisting of exons, introns, and a promoter is complicated by the presence of often-unknown alternative gene promoters, and other regulatory sequences such as suppressor and enhancer elements that have been identified in international efforts such as ENCODE project[22] or post-GWAS initiatives

(2010). In addition, epigenetic changes, including promoter DNA methylation, can induce the inactivation of tumor suppressor genes[23,24]. A growing number of tumor suppressor genes including *ZIC1*, UCHL1, CDKN2A/p16, APC, KRAS, p53, and TBX5 are frequently silenced through promoter hypermethylation in CRC[20,25-30]. The molecular mechanisms by which *ZIC1* functions as a tumor suppressor remain unknown. *ZIC1* inhibits cell proliferation through p-Erk1/2 and p-Akt inactivation in colon cancer cells[20]. Erk1/2 and Akt, once activated by phosphorylation, can function as key effectors of pI3K and MAPK pathways to promote cell survival and proliferation[31-35]. In addition, *ZIC1* is induced during apoptosis in colon cancer cells. Caspase-3 and the Bcl2 family play central roles in apoptosis[36,37]. The expression of cleaved-Caspase-3 and Bad is induced through re-expression of *ZIC1*, while Bcl-xl and p-Bad are suppressed[20].

Natural products have long been used in traditional medicine to treat various diseases. Recently, the raw materials of such products have been used to develop novel drugs[38,39]. In this study, we assayed the DNA methylation status of genomic DNA and demethylation of the *ZIC1* gene in SW620 CRC cells following treatment with the spray-dried extracts of 30 medicinal plant products to determine their efficacy as therapeutic agents for the treatment of CRC. Changes in DNA methylation patterns, which include localized hypermethylation at promoter CpG island regions of genes and global demethylation, are some of the most common molecular alterations in tumors[40,41]. Global demethylation has been observed in human colon cancer tissues[42]. In addition, global DNA demethylation is also correlated with chromosomal instability in human colorectal cancer tissues[43]. Among the 30 tested extracts, 21 (*Curcuma longa* (CL), *Astragalus complanatus, Vitex rotundifolia* (VRE), *Chrysanthemum, Verbena officinalis, Oryza sativa, Cynanchum atratum, Notopterygium incisum, Artemisia anomala, Tussilago farfara, Cannabis sativa, Ophiopogon japonicas, Ephedra sinica, Morinda officinalis, Trichosanthes kirilowii, Angelica daburica, Sophora subprostata, Sinapis alba, Santalum album, Magnolia liliflora*, and *Lonicera japonica* (LJ) showed the highest levels of global DNA hypermethylation (Table 1). Among these, 3 herbal medicines (CL, VRE, and LJ) were selected for further study. They did not induce cytotoxicity at the test concentrations (data not shown). MS-PCR results revealed that the *ZIC1* gene was unmethylated in CL-, VRE-, and LJ-treated SW620 cells (Figure 1). In addition, real-time RT-PCR results showed that *ZIC1* expression was restored after treatment of SW620 cells with 5-aza-2-deoxycytidine (positive control), CL and VRE (Figure 2). However, *ZIC1* expression was not restored in high dose (10 and 50 μg) CL and VRE. Pyrosequencing results showed that the *ZIC1* gene was demethylated in VRE- and CL-treated SW620 cells compared to non-treated SW620 cells (Figure 3). On the other hands, *ZIC1* expression was not restored in LJ treated sample (Figures 2 and 3). Such notable oversensitivity with false-positive methylation (LJ treated sample) in MS-PCR data can be the result of inadequate completeness of bisulphate treatment (inherent PCR specificity limitations) or the use of high numbers of PCR cycles.

VRE is a medical plant that is widely used in Korea and China for the treatment of asthma, chronic bronchitis, myalgia, migraine, pain, cold, headache, sore eyes, inflammation, and gastrointestinal infections such as bacterial dysentery and diarrhea[39,44,45]. Shin *et al*[46] found that VRE inhibited the allergic reactions of rat peritoneal mast cells caused by anti-dinitrophenyl IgE. It was shown that CL has been used for centuries in indigenous medicine for the treatment of a variety of medical conditions. This compound possesses anti-oxidant, anti-inflammatory, and anti-cancer properties[47,48]. It should be noted that several reports have shown that the major component of CL, curcumin, down-regulates NF-kB and has anti-cancer effects[49,50]. In addition, CL is a powerful scavenger of the superoxide anion, hydroxyl radicals and nitrogen dioxide, and protects DNA against singlet-oxygen-induced strand breaks[51]. In addition, it was reported that LJ has anti-inflammatory and hepatoprotective effects associated with suppression of NF-kB activation through reduction of IkB degradation in lipopolysaccharide-challenged rats[52]. In addition, LJ exhibits neuroprotective effects associated with the suppression of hydrogen peroxide-induced apoptosis via MAPKs and PI3K/Akt in neuroblastoma cells[53]. A number of compounds isolated from LJ, including biflavonoids, quercetin, luteolin, and dicaffeoylquinic acid, have various pharmacological properties such as antimicrobial, antiviral, antioxidative, and anti-inflammatory effects[54,55]; therefore, there is considerable interest in the various health-promoting benefits of this drug.

In conclusion, the results of this study indicate that *Vitex rotundifolia* has significant hypermethylation effects on global DNA and restores *ZIC1* expression. More studies will be necessary to elucidate the mechanism(s) of action to aid in the discovery of new therapeutic agents for the treatment of CRC.

Materials & Methods

Preparation of herbs

Water-extracted dried herbal medicines were purchased from Sun Ten Pharmaceutical (Taipei, Taiwan). For

the initial screening procedure, water-extracted herbal medicines were used, as these products were produced in a Good Manufacturing Practice (GMP) facility using a strict quality control system. Stock solutions were prepared by dissolving each herbal medicine in distilled water (DW) overnight at room temperature to a final concentration of 10 mg/mL. Each sample was centrifuged for 10 min at 3000 rpm, and the supernatant was collected and sterilized by passage through a 0.22 μm syringe filter. Each sterilized supernatant was used for the experiments. A sample specimen of each herbal preparation was deposited at the Herbarium of College of Oriental Medicine, KyungHee University, Korea. Dr. Minkyu Shin, the director of the herbarium, identified the plants and assigned the herbarium sheet number (No. PMP0081).

Cell culture

Human colorectal cancer cell line (SW620) was obtained from the Korean Cell Line Bank (KCLBTM, Korea). SW620 cells were grown and maintained in 100% humidity and 5% CO_2 at 37°C in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin-streptomycin (Invitrogen Life Technologies, Rockville, MD, USA). SW620 cells were plated in RPMI medium in a 100-mm-diameter dish at a density of 2×10^6 cells/well. The medium was changed every 2 days until the cells became 80-90% confluent, and they were then used for the experiments.

Global DNA methylation

Genomic DNA was extracted from 2×10^6 cells using the DNeasy® blood and tissue kit (Qiagen, Valenica, CA, USA) according to the manufacturer's instructions. The isolated DNA was then quantified using a model ND-1000 apparatus (NanoDrop Technologies). Global methylation levels for the cell line were determined by the MethylFlash Methylated DNA Quantification Kit (Epigentek, Farmingdale, NY, USA), which is an enzyme-linked immunosorbent assay (ELISA)-based colorimetric procedure. The assay was performed according to the manufacturer's instructions using 100 ng of genomic DNA. A microplate reader (Molecular Devices, Sunnyvale, CA, USA) was used to take measurements at a wavelength of 450 nm. Relative quantification was determined by normalizing the readings to the positive control provided with the kit. Data are reported as mean ± standard deviation (SD). The slope of the standard curve was determined using a linear regression, and the percentage of 5-hydroxymethylcytosine (5-hmC) in total DNA was calculated using the following formula:

$$\text{5-hmC } \% = \frac{\text{Sample OD} - \text{negative control OD}}{\text{Slope} \times \text{input DNA amount}} \times 100$$

Methylation-specific PCR (MS-PCR)

Approximately 1 μg of DNA was treated with bisulfate using the EZ DNA Methylation-Gold Kit according to the manufacturer's protocol (Zymo Research, Irvine, CA, USA). Briefly, 1 μL of bisulfate-treated DNA was amplified using primers specific for either methylated or unmethylated DNA under the following conditions: 95°C for 10 min; 40 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and a final extension for 10 min at 72°C. The sequences of methylation-specific (MS) primers and unmethylated-specific (US) primers for *ZIC1* have previously been reported[20] and were as follows: *ZIC1* (MS) (forward 5′-GGA TTT TTT GTT TCG TAA TC-3′, reverse 5′-CCC GTT AAC CAC GTT AAA CG-3′), *ZIC1* (US) (forward 5′-GGG ATT TTT TGT TTT GTA ATT-3′, reverse 5′-CCC ATT AAC CAC ATT AAA CA-C-3′). Twenty microliters of each PCR product was loaded onto 2% agarose gels and visualized by ethidium bromide staining.

Real-time RT-PCR

SW620 cells were initially cultured in a 100-mm-diameter dish (1×10^6/mL) for 24 h, and then treated with water-extracted herbs (0.1, 10, or 50 μg/mL) or 5-aza-2-deoxycitidine (20 μM). The cells were then incubated at 37°C for 3 days. RNA was isolated using the RNeasy® mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, after which the RNA was quantified using a model ND-1000 apparatus (NanoDrop Technologies, Wilmington, DE, USA). The integrity of the RNA was confirmed by denaturing agarose gel electrophoresis. Single-stranded cDNA was prepared using First Strand cDNA Synthesis Kit (Roche Diagnostics Korea Applied Science, Seoul, Korea). The integrity of the cDNA was confirmed by amplifying β-actin. The PCR parameters used were 10 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C; and a 7 min final extension at 72°C. The sequences of the human primers were *ZIC1* (forward 5′-AAA CTG GTT AAC CAC ATC CGC-3′, reverse 5′-CTC AAA CTC GCA CTT GAA GG-3′); and β-actin (forward 5′-TTG-CCG-ACA-GGA-TGC-AGA-AG-3′, reverse 5′-AGG-TGG-ACA-GCG-AGG-CCA-GG-3′).

Pyrosequencing

Approximately 1 μg of DNA was treated with bisulfate using the EZ DNA Methylation-Gold Kit according to the manufacturer's protocol (Zymo Research,

Irvine, CA, USA). PCR primers were designed to assay the methylation status of CpGs within 0.5 kb from the transcription start site. PCR primer sequences and sequencing primer sequences were as follows: *ZIC1* PCR (forward 5′-AGG GGT TAG TGG AGA AGT AAG GAG GG-3′, reverse 5′-AAC ATA AAA CAT CTC AAC CCC CTA A-3′) and *ZIC1* sequencing (forward 5′-GGG TTAGTGGAGAAGTAAG-3′). Either one-step or two-step PCR reactions were performed using 2 μL of bisulfite-converted genomic DNA and either one or two sets of different bisulfite PCR primers in a standard PCR reaction mix. One of the primers (reverse primer) in the final PCR reaction was biotinylated to create an ssDNA template for the pyrosequencing reaction. Where indicated, we used a previously described amplification protocol[21] based on the universal primer approach. Briefly, the biotinylated reverse primer was substituted with a reverse primer and a biotinylated universal primer at a ratio of 1 : 9 in the PCR reaction. The integrity of the PCR product was verified on 1.5% agarose gels with ethidium bromide staining. The PCR product was immobilized on streptavidin-Sepharose beads (Amersham), washed, and denatured, and the biotinylated strands were released into an annealing buffer containing the sequencing primer. Pyrosequencing was performed using the PSQ HS96 Gold SNP Reagents on a PSQ 96HS machine (Qiagen).

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