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Genomics & Informatics
Vol. 9(3) 102-113, September 2011

http://dx.doi.org/10.5808/GI.2011.9.3.102
pISSN 1598-866X eISSN 2234-0742

Differentially Expressed Genes by Inhibition of C-terminal Src Kinase by siRNA in Human Vascular Smooth Muscle Cells and Their Association with Blood Pressure

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Abstract

C-terminal SRC kinase (CSK) is a ubiquitously expressed, cytosolic enzyme that phosphorylates and inactivates several SRC family protein tyrosine kinases. Recent genomewide association studies have implicated CSK in the regulation of blood pressure. The current study aim is to determine the blood pressure association of the genes regulated by CSK down-regulation. The CSK mRNA expression was downregulated in vascular smooth muscle cells using small interfering RNA (siRNA). CSK mRNA levels fell by 90% in cells that were treated with CSK siRNA; the RNA from these cells was examined by microarray using the Illumina HumanRef-8 v3 platform, which comprises 24,526 reference mRNA probes. On treatment with CSK siRNA, 19 genes were downregulated by more than 2-fold and 13 genes were upregulated by more than 2-fold. Three (CANX, SLC30A7, and HMOX1) of them revealed more than 3 fold differential expression. Interestingly, the HMOX1 SNPs were associated with diastolic blood pressure in the 7551 Koreans using Korea Association REsource data, and the result was supported by the other reports that HMOX1 linked to blood vessel maintenance. Among the remaining 29 differentially expressed genes, seven (SSBP1, CDH2, YWHAE, ME2, PFTK1, G3BP2, and TUFT1) revealed association with both systolic and diastolic blood pressures. The CDH2 gene was linked to blood pressures. Conclusively, we identified 32 differentially expressed genes which were regulated by CSK reduction, and two (HOMX1 and CDH2) of them might influence the blood pressure regulation through CSK pathway.

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Accepted 20 June 2011

Keywords: CSK, blood pressure, microarray, expression, association analysis

Introduction

Blood pressure is regulated by many genetic and environmental factors. To identify genes that mediate this regulation, genomewide association studies (GWASs) have been performed using large samples from various ethnicities. The Wellcome Trust Case Control Consortium (WTCCC) (WTCCC, 2007), Amish study (Wang *et al.*, 2009), KORA (Org *et al.*, 2009), KARE (Cho *et al.*, 2009), the Global BPgen consortium (Newton-Cheh *et al.*, 2009), and the CHARGE consortium (Levyt *et al.*, 2009) have conducted GWASs on hypertension and blood pressure, identifying 14 independent loci that govern blood pressure that reached genomewide significance: 6 enzymes, 2 solutes channels, 2 transcription factors, 1 growth factor, 1 cell signaling protein, 1 structural protein, and 1 hypothetical gene (reviewed in Ehret, 2010).

The CSK (C-terminal SRC tyrosine kinase) locus has repeatedly been linked to blood pressure in Korean and European studies (Fig. 1) (Hong *et al.*, 2010a; Levy *et al.*, 2009; Newton-Cheh *et al.*, 2009). CSK is a ubiquitously expressed protein tyrosine kinase that is related structurally to SRC kinases (Nada *et al.*, 1991). The Src family of kinases (SFKs) comprises 9 members (c-src,

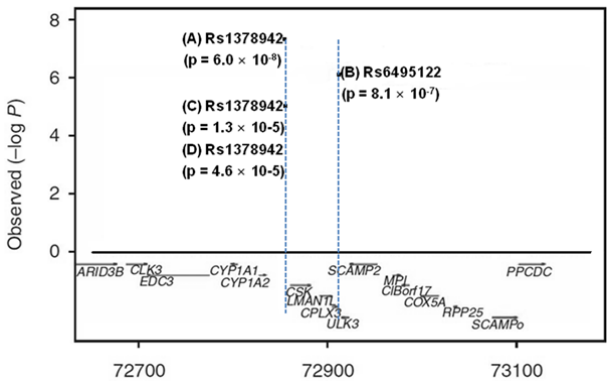

Fig. 1. The highest signals in 15q24 from 3 association studies on blood pressure. A. Signal in Newton-Cheh, *et al.*, 2009. B. Signal in Levy, *et al.*, 2009. C. Signal in Hong, *et al.*, 2010a. D. Signal in Takeuchi, *et al.*, 2010.

c-yes, fyn, c-fgr, yrk, lck, hck, lyn, and blk) that have similar C-terminal domains that are phosphorylated by CSK (Cheng *et al*., 1996). SFKs regulate signal transduction through diverse cell surface receptors in many environments (reviewed in Parsons and Parsons, 2004).

CSK-mediated phosphorylation of SFKs at their regulatory tyrosine inhibits their kinase activity. Many reports have documented the importance of CSK in cellular development and the growth control (Chow and Veillette, 1995; Imamoto and Soriano, 1993; Nada *et al*., 1993). Studies in CSK-deficient mice have shown that CSK regulates embryonic development, particularly the central nervous system (Imamoto and Soriano, 1993). The CSK-deficient mice have also shown that CSK is required both for angiogenic sprouting and vascular remodeling (Duan *et al*., 2004). In addition, the critical role of c-SRC signaling pathway was reported in angiotensin II-mediated vascular smooth muscle cell proliferation (Sayeski and Ali, 2003).

To understand the CSK function in blood pressure regulation, we reduced CSK mRNA expression in the human vascular smooth muscle cells (VSMCs) using CSK siRNA, examined the expression profile of differentially expressed genes (DEGs) by microarray, and analyzed the genetic association of DEGs with blood pressure traits. DEGs in the VSMCs downregulated by CSK could be candidate genes responsible for the blood pressure regulation through CSK signaling. Among the DEGs, of course there may be genes irrelevant to the blood pressure regulation, but other cellular controls through CSK. Therefore, we performed the association study of DEGs with the blood pressure to determine which DEGs are involved in the blood pressure regulation in this study.

Methods

Cell culture and reagents

Human vascular smooth muscle cell lines (CRL-1999™-T/G HA-VSMC) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cell lines were cultured in Kaighn's-modified Ham's F-12K medium (ATCC, Manassas, VA) with 0.05 mg/ml ascorbic acid, 0.01 mg/ml insulin, 0.01 mg/ml transferrin, 10 ng/ml sodium selenite, 0.03 mg/ml endothelial cell growth supplement, 10 mM HEPES, 10 mM TES (Sigma, St. Louis, MO), 100× antibiotic-antimycotic (Gibco, Grand Island, NY), and 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA) in a humidified incubator at 37°C and 5% CO_2.

siRNA design and transfection

An siRNA oligonucleotide that targeted CSK was designed based on the published mRNA sequence of CSK (nm number) and synthesized by Genolution Co. Ltd. (Seoul, Korea). Scrambled nontargeting siRNA was also synthesized by Genolution Co. Ltd. (Seoul, Korea). The CSK siRNA sequence is 5'-CTGGCCATCCGGTACAGAA-3'. Twenty-four hours before the transfection, VSMCs were diluted in fresh medium without antibiotics and transferred to 6-well plates. Cells were grown to 80% confluence and transfected with 150 nmol/L (final concentration) of siRNA using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA) per the manufacturer's instructions.

RNA isolation and real-time PCR

RNA was extracted using RNAiso Plus (Takara, Shiga, Japan). We generated cDNA from 400 ng of total RNA using the PrimeScript™ RT reagent kit (TaKaRa, Shiga, Japan) used per the manufacturer's manual. *CSK* expression was analyzed by real-time PCR. One-tenth of the cDNA reaction was added to a final volume of 25 μl for each real-time PCR reaction using SYBR Green I (TaKaRa, Shiga, Japan). The primers for CSK and GAPDH were (5'-ACCTCAGACGCAGATGGACT/AGCATCACGTCTCCGAACTC-3') and (5'-GCTCTCTGCTCCTCCTGTTC/CAATACGACCAAATCCGTTG-3') respectively (forward/reverse sequence).

Reactions were run on an ABI Step One real-time PCR system (Applied Biosystems, Foster City, CA) with the following program: 35 cycles of 95°C for 40s, 60°C for 40s, and 72°C for 40s. All reactions were performed in triplicate. The significance of differences in relative levels of expression was determined ($p < 0.05$) by two-way analysis of variance. The size of the RT-PCR products was analyzed by agarose gel electrophoresis (data not shown).

Labeling and purification of probe for microarray

Total RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Ambion, Austin, USA) to generate biotinylated cRNA per the manufacturer's instructions. Briefly, 550 ng of total RNA was reverse-transcribed using T7 oligo (dT) primer. Second-strand cDNA was synthesized, in vitro-transcribed, and labeled with biotin-NTP. After purification, the cRNA was quantified on an ND-1000 spectrophotometer (NanoDrop, Wilmington, USA).

Hybridization and data export

Seven hundred fifty nanograms of labeled cRNA was hybridized to each human-8 expression bead array for 16-18 h at 58°C, per the manufacturer's instructions (Illumina, Inc., San Diego, USA). Array signals were detected using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) per the bead array manual. Arrays were scanned on an Illumina Bead Array Reader per the manufacturer's instructions. Array data were exported, processed, and analyzed using Illumina GenomeStudio v2009.2 (Gene Expression Module v1.5.4).

Raw data preparation and statistical analysis

The quality of hybridization and overall chip performance was monitored by visual inspection of internal quality control checks and raw scanned data. Raw data were extracted using the software that was provided by the manufacturer (Illumina GenomeStudio v2009.2 (Gene Expression Module v1.5.4)). Array data were filtered using a detection p-value < 0.05 (similar to signal-to-noise) for all samples. Selected gene signal values were log-transformed and normalized by a quantile method. The comparative analysis between test group and control group was evaluated by LPE test using adjusted FDR (false discovery rate) p-values. FDR was controlled by adjusting p-values using the Benjamini-Hochberg algorithm.

The gene ontology analysis was performed using PANTHER (http://www.pantherdb.org/panther/ontologies.jsp), using text files containing the Gene ID list and accession number of the Illumina probe ID. Gene Set Enrichment Analysis (GSEA) was performed if an a priori-defined set of genes showed differential patterns with regard to both biological process and molecular function. One-tail Fisher exact test was used to measure the gene enrichment in annotation terms. All data analysis and visualization of differentially expressed genes were conducted using R 2.4.1 (www.r-project.org). The biological ontology-based analysis was performed using the Panther database (http://www.pantherdb.org).

Association study samples

The KARE subjects and their genotypes have been reported in the original study (Cho *et al.*, 2009). Briefly, 7551 individuals from the KyungGi-Do province, near Seoul, Korea, were recruited for the association analysis between blood pressure and SNP genotypes.

In the original study, blood pressure was measured 3 times with the patient in the supine position. Before the first measurement, the participants rested for 5 minutes, and 3 measurements were taken at least 30 seconds apart. The average of the 3 measurements was used for this study. This study was approved by the International Review Board of the Korea National Institute of Health.

Genotypes and statistical analysis

KARE DNA samples from the original study were isolated from the peripheral blood of participants and genotyped using the Affymetrix Genomewide Human SNP array 5.0 (Affymetrix, Inc., Santa Clara, CA, USA). The quality controls have been described elsewhere (Cho *et al.*, 2009). Briefly, the accuracy of the genotyping was calculated by Bayesian robust linear modeling using the Mahalanobis distance (BRLMM) genotyping algorithm (Rabbee and Speed, 2006).

Samples that had accuracies below 98% and a high missing genotype call rate ($\geq$ 4%), high heterozygosity ($>$ 30%), or inconsistency in sex were excluded from subsequent analyses. Individuals who had a tumor or were undergoing antihypertensive therapy were excluded. Related individuals whose estimated identity-by-state (IBS) values were high ($>$ 0.80) (WTCCC, 2007) were also excluded. The 1956 SNPs that were examined in this study were extracted from the KARE genotype data if they lay within $\pm$5 kb of the genes and did not deviate from Hardy-Weinberg equilibrium (HWE) ($p > 1\times10^{-6}$).

The KARE dataset, comprising 351,677 SNPs for 7751 individuals, was merged with that of International HapMap Phase II JPT (Japanese)+HCB (Chinese) panel 2. The genotypes of the KARE individuals were imputed using IMPUTE (Howie *et al.*, 2009) as described (Hong *et al.*, 2010b)

Most statistical analyses were performed using PLINK version 1.07 (http://pngu.mgh.harvard.edu/~purcell/plink/) (Purcell *et al.*, 2007) and SAS (version 9.1; SAS institute Inc., Cary, NC, USA). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were tested for their association with SNPs by linear regression analysis with an additive model (1-d.f.) after adjustments for area, age, sex, and BMI (Hong *et al.*, 2010a; Hong *et al.*, 2010c).

Results

CSK inhibition by siRNA

VSMCs were treated with CSK siRNA for 24 hr. By quantitative real-time PCR (qRT-PCR), CSK mRNA levels fell by 90% compared with controls that were treated

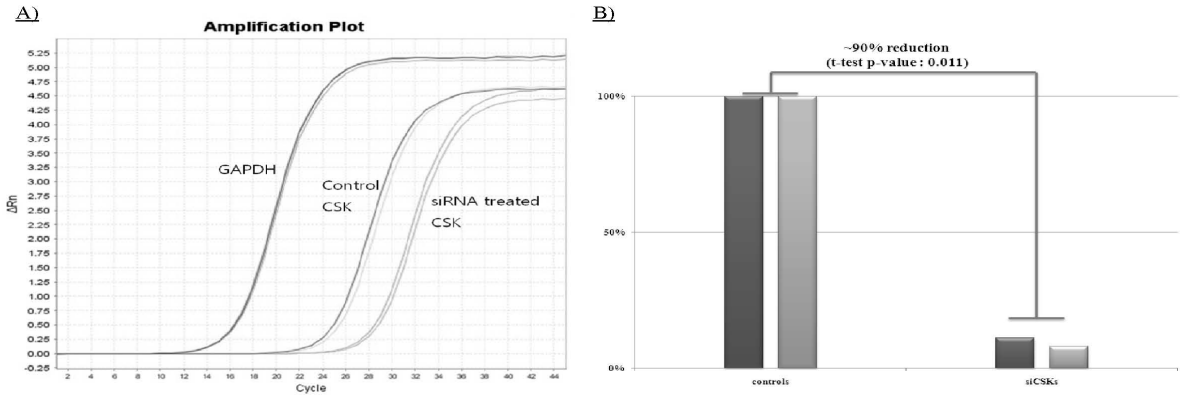

Fig. 2. Real-time PCR for *CSK* and *GAPDH* expression. A) Real-time PCR amplication curve in scramble siRNA-treated (Controls) and CSK siRNA-treated samples (siCSK). B) Comparison of *CSK* and *GAPDH* expression between siCSK and controls.

with scramble siRNA (Fig. 2).

Four RNA samples-from 2 CSK siRNA-treated (siCSK) and 2 scramble siRNA-treated (control) groups were examined by microarray. *CSK* mRNA levels declined in siCSK-treated cells by approximately 80% compared with the controls, mirroring the reductions in VSMCs by qRT-PCR.

Expression microarray profiling

After false discovery rate (FDR) correction, the expression of 631 genes changed significantly with an adjusted p-value $<$ 0.05 (288 genes were upregulated and 343 were downregulated), 32 of which changed their expression by more than 2-fold (19 downregulated and 13 upregulated) (Table 1). Notably, a chaperone protein (CANX: calnexin) and a zinc ion transporter (SLC30A7: solute carrier family 30 member 7) decreased their expression by more than 3-fold (-3.85 $\pm$ 0.17 and -3.11 $\pm$ 0.08, respectively); conversely, an oxidoreductase (HMOX1: heme oxygenase (decycling) 1) increased its expression by greater than 3-fold (3.15$\pm$0.44).

Gene ontology analysis of DEGs

We analyzed the ontology of DEGs with regard to biological process (Fig. 3). A gene set enrichment analysis (GSEA) of biological processes was also performed (the results are marked with * over the process in Fig. 3A, B, and C). Two biological processes-signal transduction and development-were significantly enriched in upregulated DEGs (Fig. 3B), and 6 categories-signal transduction, phosphate metabolism, immunity and defense, homeostasis, development, and cell cycle-were represented in downregulated DEGs (Fig. 3C).

In the GSEA, one molecular function (signaling molecule) was significantly enriched in upregulated DEGs (Fig. 3E), and 4 functions (signaling molecule, receptor, kinase, and ion channel) were preferential in downregulated DEGs (Fig. 3F).

Genetic association analysis

We analyzed the genetic association of the 32 DEGs (differentially expressed by more than 2-fold) in Table 1 with blood pressure traits in 7551 Koreans. We obtained 1956 SNPs in the DEGs from Korean Association REsource (KARE) data (Table 2) and examined their association with systolic blood pressure (SBP) and diastolic blood pressure (DBP), controlling for cohort, age, sex, and BMI as covariates. The clinical characteristics of the subjects are described in Table 3.

Three hundred ninety-one SNPs from 16 genes were linked to SBP or DBP at p-value $<$ 0.05. Among them, two SNPs (rs2071746 and rs2071748) of HMOX1 gene, the most increased its expression by CSK down regulation, were associated with diastolic blood pressure (Table 4). Seven of less regulated genes (3 $>$ |fold change| $>$ 2) genes (SSBP1, CDH2, YWHAE, ME2, PFTK1, G3BP2, and TUFT1) were correlated with both SBP and DBP, and the minor allele frequencies of their associated SNPs were higher than 0.05 (Table 5).

Discussion

By microarray, we examined DEGs in siCSK-treated cells. The gene-set enrichment analysis revealed that the signaling molecules were greatly changed in the expression by the CSK reduction. Among the DEGs, thirty-two genes in Table 1 were differentially expressed by more than 2-fold, and calnexin (CANX), a molecular chaperone, experienced the most extensive down-

Table 1. Differential expressed genes (± 2-fold) on downregulation of CSK

Illumina chip ID	Gene symbol	Gene description	Fold change	Signal intensity				Molecular function
			Mean ± S.D.	Con 1	Con2	CSK1	CSK2	
ILMN_2401057	CANX	*Calnexin*	-3.85 ± 0.17	2,669.7	2,271.4	644.9	590.6	Chaperone
ILMN_1789999	SLC30A7	*Solute carrier family 30 member 7 (Zinc transcporter 7)*	-3.11 ± 0.08	7,472.6	6,797.4	2,218.5	2,201.7	Zinc Ion Transporter
ILMN_1658917	SLC1A1	*Solute Carrier Family 1 Member 1 (Excitatory amino acid carrier 1)*	-2.98 ± 0.07	3,175.9	2,936.9	947.7	1,038.3	Ion Transporter
ILMN_1794875	AGPAT9	*1-acylglycerol-3-phosphate O-acyltransferase 9*	-2.78 ± 0.10	2,143.6	1,875.3	699.9	696.2	Acyltransferase
ILMN_1809478	SSBP1	*Single-stranded DNA binding protein 1*	-2.42 ± 0.04	11,892.8	11,315.3	4,552.2	4,583.2	Nucleic acid binding
ILMN_1762764	SH3BGRL2	*SH3 domain binding glutamic acid-rich protein like 2*	-2.40 ± 0.11	1,111.0	1,127.5	429.2	479.7	Molecular function unclassified
ILMN_1652357	PDHX	*Pyruvate dehydrogenase complex, component X*	-2.30 ± 0.16	3,644.9	3,472.5	1,567.6	1,412.6	Molecular function unclassified
ILMN_2173004	RAB8B	*RAB8B, member RAS oncogene family*	-2.24 ± 0.04	2,251.6	2,112.7	935.6	948.5	G-protein
ILMN_1779228	CDH2	*Cadherin 2, type 1, N-cadherin (neuronal)*	-2.23 ± 0.19	18,865.0	16,507.2	7,998.3	7,270.1	Cadherin
ILMN_1734950	LOXL1	*Lysyl oxidase-like 1*	-2.19 ± 0.12	2,744.9	2,650.0	1,096.0	1,282.3	Receptor
ILMN_2252136	YWHAE	*Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide*	-2.12 ± 0.09	2,583.0	2,479.5	1,081.2	1,221.0	Chaperone
ILMN_1794501	HAS3	*Hyaluronan synthase 3*	-2.11 ± 0.07	712.5	685.0	332.4	312.5	Glycosyltransferase
ILMN_1720048	CCL2	*Chemokine (C-C motif) ligand 2*	-2.10 ± 0.09	2,401.5	2,208.5	998.0	1,113.3	Chemokine
ILMN_1714622	TNRC6A	*Trinucleotide repeat containing 6A*	-2.10 ± 0.32	2,061.0	1,548.4	733.0	931.4	Molecular function unclassified
ILMN_1673363	CD97	*CD97 molecule*	-2.05 ± 0.38	3,710.8	4,438.1	1,600.4	2,280.3	Receptor
ILMN_2048636	ME2	*Malic enzyme 2, NAD(+)-dependent, mitochondrial*	-2.04 ± 0.18	2,108.0	1,694.9	873.7	914.1	Acyltransferase
ILMN_1703074	CPD	*Carboxypeptidase D*	-2.04 ± 0.09	3,590.9	3,147.9	1,536.8	1,640.9	Protease
ILMN_2136177	CNOT6	*CCR4-NOT transcription complex, subunit 6*	-2.02 ± 0.11	1,932.0	1,750.1	898.2	858.0	Exoribonuclease
ILMN_1667030	HSBP1	*Heat shock factor binding protein 1*	-2.00 ± 0.05	8,729.5	8,938.4	4,133.9	4,312.5	Molecular function unclassified
ILMN_2174127	DCBLD2	*Discoidin, CUB and LCCL domain containing 2*	2.05 ± 0.19	23,803.1	21,492.5	42,166.3	49,121.0	Molecular function unclassified
ILMN_1781374	TUFT1	*Tuftelin 1*	2.11 ± 0.12	1,153.2	1,110.9	2,122.0	2,511.5	Molecular function unclassified
ILMN_2347068	MKNK2	*MAP kinase interacting serine/ threonine kinase 2*	2.13 ± 0.22	1,346.2	1,343.2	2,415.3	3,082.6	Kinase
ILMN_2381753	G3BP2	*GTPase activating protein (SH3 domain) binding*	2.14 ± 0.16	3,501.9	2,896.9	6,281.0	6,839.7	Signaling molecule
ILMN_2380418	BICD2	*Bicaudal D homolog 2 (Drosophila)*	2.19 ± 0.12	2,168.6	1,849.0	4,119.2	4,290.0	Molecular function unclassified
ILMN_1811264	CCDC32	*Coiled-coil domain containing 32*	2.19 ± 0.06	1,112.0	1,116.4	2,352.7	2,366.3	Molecular function unclassified
ILMN_1790533	PHACTR2	*Phosphatase and actin regulator 2*	2.26 ± 0.47	906.0	882.3	1521.1	2,522.4	
ILMN_1719219	ZNF616	*Zinc finger protein 616*	2.29 ± 0.06	314.4	309.6	697.9	682.2	Transcription factor
ILMN_1805271	ZNF721	*Zinc finger protein 721*	2.66 ± 0.08	571.6	561.6	1,359.7	1,536.3	Transcription factor
ILMN_2140974	TPM4	*Tropomyosin 4*	2.70 ± 0.18	1,495.2	1,213.2	3,363.2	3,593.0	Cytoskeletal protein
ILMN_2171295	PFTK1	*PFTAIRE protein kinase 1*	2.75 ± 0.15	546.7	472.5	1,252.0	1,433.5	Kinase
ILMN_1770228	KRT34	*Keratin 34*	2.77 ± 0.18	1,901.5	1,760.4	5,175.5	4,532.8	Cytoskeletal protein
ILMN_1800512	HMOX1	*Heme oxygenase (decycling) 1*	3.15 ± 0.44	1,937.3	1,508.6	6,123.3	4,361.8	Oxidoreductase

regulation (-3.85 ± 0.17-fold). CANX is a calcium-binding, endoplasmic reticulum (ER)-associated protein that interacts transiently with newly synthesized N-linked glycoproteins, facilitating protein folding and assembly (Kleizen and Braakman, 2004). Zinc transporter 7 (ZnT7 or SLC30A7) was downregulated by more than 3-fold

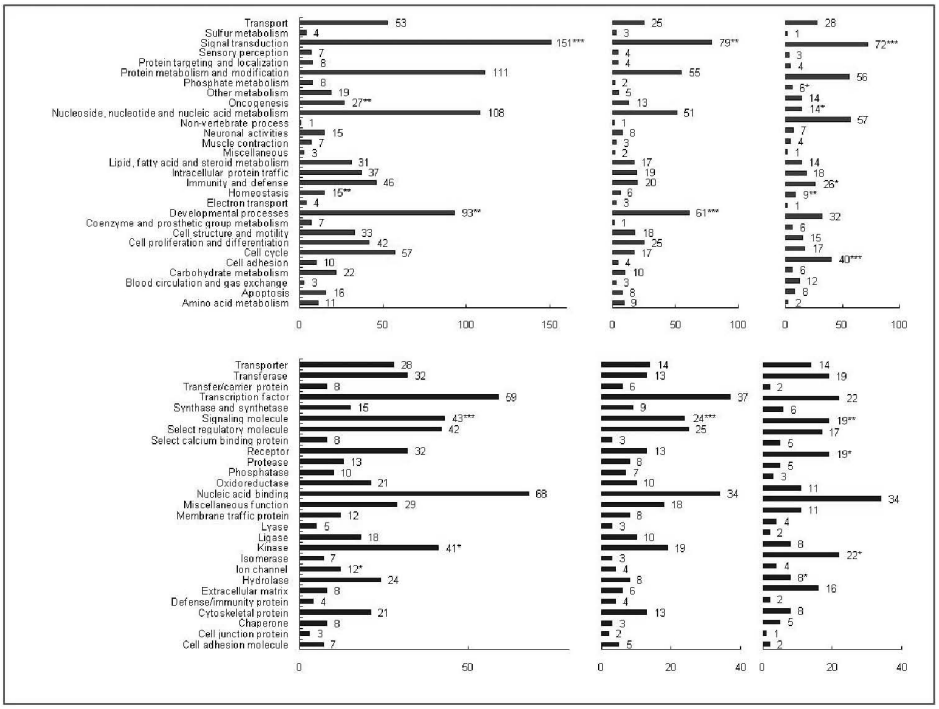

Fig. 3. Gene ontology analysis of biological processes (A, B, C) and molecular function (D, E, F). All differentially expressed genes (DEGs) are described in panels A and D; upregulated DEGs are listed in panels B and E, and downregulated DEGs are shown in panels C and F. Gene set enrichment analysis p-values are denoted as follows: $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

(-3.11 ± 0.08-fold). ZnT7, a novel member of the zinc transporter (ZnT) family, localizes to the Golgi apparatus and cytoplasmic vesicles, suggesting that ZnT7 mediates zinc transport from the cytoplasm into the Golgi (Kirschke and Huang, 2003). In spite of the great differential expression, the association studies of both CANX and ZnT7/SLC30A7 with blood pressure in Koreans were not significant. Possibly these genes may be involved in the cellular controls through CSK signaling, but irrelevant to the blood pressure regulation.

On the other hand, heme oxygenase 1 (HMOX1), the most significantly upregulated (3.15 ± 0.44-fold), was associated with diastolic blood pressure, indicating its involvement in the blood pressure regulation through CSK. Heme oxygenase, an essential enzyme in heme catabolism, cleaves heme to form biliverdin, which is subsequently converted to bilirubin and carbon monoxide, a putative neurotransmitter (Yoshida *et al.*, 1988). Human HMOX1 polymorphisms were linked to the risk of coronary artery disease in type II diabetes patients (Chen *et al.*, 2002). Also, HMOX1 overexpression in VSMCs protects them from free radical attack, implicating HMOX1 in the maintenance of blood vessel (Zhang *et al.*, 2002).

Seven gene loci such as SSBP1, CDH2, YWHAE, ME2, TUFT1, C3BP2 and PFTK1 showed the association tendency with both SBP and DBP. Based on a literature search, CDH2 among these genes appears to related to the regulation of blood pressure. CDH2, also known as neuronal cadherin (N-cadherin), is a classical member of the cadherin superfamily (Reid and Hemperly, 1990). It is a calcium-dependent cell-cell adhesion glycoprotein that comprises 5 extracellular cadherin repeats, a transmembrane region, and a highly conserved cytoplasmic tail (Garcia-Castro *et al.*, 2000). Deletion of N-cadherin from mouse endothelium results in vascular defects, leading to midgestational embryonic lethality, implying that it regulates blood pressure (Luo and Radice, 2005).

The SSBP1 is a single-stranded DNA binding protein, and is a housekeeping gene involved in mitochondrial biogenesis (Tiranti *et al.*, 1995). It is also a subunit of a single-stranded DNA (ssDNA)-binding complex involved in the maintenance of genome stability (Huang *et al.*, 2009). The YWHAE gene belongs to the 14-3-3 family of proteins which mediate signal transduction by binding to phosphoserine-containing proteins (Ikeda *et al.*, 2008). The ME2 gene encodes Mitochondrial NAD(+)-dependent malic enzyme (EC 1.1.1.39), and linked to the conversion of amino acid carbon to pyruvate (Loeber *et al.*, 1991). The TUFT1 has been suggested to play an important role during the development and mineralization of enamel (Mao *et al.*, 2001). The G3BP2 gene is a member of GTPase-activating protein SH3 domain binding protein family and unwound DNA/DNA, RNA/DNA, and RNA/RNA substrates (Costa *et al.*, 1999). The PFTK1 is a member of the CDC2-related protein kinase family, and involved in the control of the

Table 2. SNP distribution in significantly differentially expressed genes (± 2 fold) and the number of significant SNPs

Gene symbol	Chr	Gene+-5Kb		Number of tested SNPs			Mean distance (bp)	Number of SNPs			
		From	to	Total	Genotyped	Imputed	between SNPs	Total	Any BP ($p < 0.05$)	Both BPs ($p < 0.05$)	MAF > 0.05%
CANX	5	179053536	179085167	5	0	5	1895	5	0	0	0
SLC30A7	1	101129266	101222582	35	6	29	2564	35	0	0	0
SLC1A1	9	4475427	4582469	97	36	61	1071	97	0	0	0
AGPAT9	4	84671677	84751050	38	13	25	2100	38	1	1	0
SSBP1	**7**	**141079645**	**141101726**	**14**	**1**	**13**	**1308**	**14**	**14**	**4**	**4**
SH3BGRL2	6	80392719	80475088	23	5	18	2773	23	0	0	0
PDHX	11	34889253	34979251	115	29	86	753	115	0	0	0
RAB8B	15	61263781	61352026	35	10	25	2442	35	1	0	0
CDH2	**18**	**23779933**	**24016189**	**224**	**40**	**184**	**1023**	**224**	**88**	**23**	**23**
LOXL1	15	72000842	72036531	26	6	20	1156	26	0	0	0
YWHAE	**17**	**1189584**	**1255306**	**33**	**6**	**27**	**1810**	**33**	**9**	**1**	**1**
HAS3	16	67693944	67714071	2	0	2	7040	2	0	0	0
CCL2	17	29601409	29613333	9	4	5	896	9	0	0	0
TNRC6A	16	24643550	24750048	67	19	48	1573	67	10	1	0
CD97	19	14348213	14385535	2	2	0	7643	2	2	0	0
ME2	**18**	**46654430**	**46735160**	**45**	**12**	**33**	**1778**	**45**	**2**	**1**	**1**
CPD	17	25725110	25824825	32	3	29	3200	32	0	0	0
CNOT6	5	179849023	179942959	74	16	58	1278	74	1	0	0
HSBP1	16	82394094	82409095	5	3	2	3585	5	0	0	0
DCBLD2	3	99992504	100108223	75	9	66	1558	75	6	0	0
TUFT1	**1**	**149774405**	**149827683**	**37**	**11**	**26**	**1358**	**37**	**24**	**19**	**19**
MKNK2	19	1983470	2007243	3	2	1	4762	3	0	0	0
G3BP2	**4**	**76781977**	**76822691**	**25**	**7**	**18**	**1464**	**25**	**8**	**6**	**4**
BICD2	9	94508466	94571904	46	9	37	1331	46	0	0	0
CCDC32	15	38627634	38647511	34	10	24	576	34	5	0	0
PHACTR2	6	143966010	144199015	173	46	127	1325	173	13	0	0
ZNF616	19	57304465	57340003	3	3	0	8595	3	0	0	0
ZNF721	4	418779	488442	26	5	21	2698	26	0	0	0
TPM4	19	16043135	16079813	13	5	8	915	13	0	0	0
PFTK1	**7**	**90058459**	**90682416**	**618**	**122**	**496**	**1007**	**618**	**205**	**8**	**2**
KRT34	17	36782447	36797162	7	2	5	1573	7	0	0	0
HMOX1	22	34102060	34125207	15	3	12	1164	15	2	0	0

Table 3. Clinical characteristics of the KARE and replication study subjects

Variables	Count/mean ± standard deviation
Number of individuals	7,751
Gender [men (%)/women (%)]	3,747 (50)/3,804 (50)
Age	51.44±8.79
BMI	24.4±3.2
SBP	115.65±17.25
DBP	74.21±11.27

eukaryotic cell cycle, whose activity is controlled by an associated cyclin (Shu *et al*., 2007). Even though these genes were found their association with blood pressure in this study, we could not find previous reports about these six genes related to the vascular function as well as blood pressure.

Our study limitation is that none of the 391 associated SNPs passed the multiple correction criteria (Bonferroni p-value $< 2.6 \times 10^{-5}$). Therefore, the further replication study will be necessary and awaiting for the functional analysis validating these findings.

Conclusively, we identified 32 differentially expressed genes which were regulated by CSK reduction, and two (HOMX1 and CDH2) of them might be influence the blood pressure regulation through CSK pathway.

Table 4. Association results of three most regulated genes (CANX, SLC30A7, and HMOX1)

Chr	SNP RSID	Data Source	BP		Minor allele	MAF	Systolic blood pressure			Diastolic blood pressure		
							Best	Se	p-value	Best	Se	p-value
	CANX	*Calnexin*										
5	rs4701197	I	179128235	INTRONIC	T	0.29	0.19	0.29	0.52	0.02	0.19	0.93
5	rs11744662	I	179128706	INTRONIC	G	0.45	0.01	0.26	0.96	-0.09	0.18	0.63
5	rs12374446	I	179131143	INTRONIC	T	0.45	0.01	0.26	0.96	-0.09	0.18	0.62
5	rs7735702	I	179132612	INTRONIC	A	0.15	-0.23	0.36	0.51	-0.17	0.24	0.49
5	rs6893300	I	179135815	DOWNSTREAM	A	0.15	-0.23	0.36	0.51	-0.17	0.24	0.49
	SLC30A7	*Solute carrier family 30 member 7 (Zinc transcporter 7)*										
1	rs3737580	I	101360215	5PRIME_UTR	T	0.17	0.14	0.35	0.68	0.05	0.24	0.82
1	rs1074739	I	101362371	UPSTREAM	A	0.08	-0.55	0.47	0.24	-0.36	0.32	0.26
1	rs12569251	I	101372040	INTRONIC	G	0.07	-0.52	0.51	0.31	-0.21	0.34	0.54
1	rs12040671	I	101373328	INTRONIC	A	0.43	-0.35	0.28	0.21	-0.10	0.19	0.61
1	rs17408326	I	101375854	INTRONIC	C	0.04	0.13	0.63	0.84	-0.21	0.43	0.62
1	rs10493940	I	101377845	INTRONIC	G	0.08	-0.38	0.46	0.41	-0.20	0.31	0.52
1	rs10493939	I	101378026	INTRONIC	G	0.07	-0.52	0.51	0.31	-0.21	0.35	0.54
1	rs12738779	I	101378072	INTRONIC	G	0.04	0.13	0.63	0.84	-0.21	0.43	0.63
1	rs17408457	I	101385693	INTRONIC	G	0.09	-0.32	0.44	0.47	-0.19	0.30	0.53
1	rs12569174	G	101386179	INTRONIC	C	0.09	-0.32	0.44	0.47	-0.18	0.30	0.54
1	rs17123521	G	101386897	INTRONIC	C	0.31	0.28	0.27	0.30	0.11	0.18	0.54
1	rs11583674	I	101396560	INTRONIC	G	0.14	-0.15	0.38	0.69	-0.21	0.26	0.41
1	rs11578366	I	101396852	INTRONIC	A	0.13	-0.15	0.38	0.69	-0.19	0.25	0.46
1	rs12121879	I	101403815	INTRONIC	A	0.44	-0.32	0.28	0.25	-0.07	0.19	0.73
1	rs6698798	I	101407028	INTRONIC	T	0.12	-0.40	0.39	0.31	0.05	0.26	0.86
1	rs11581062	I	101407519	INTRONIC	G	0.13	-0.15	0.38	0.68	-0.19	0.25	0.46
1	rs3850453	I	101408933	INTRONIC	C	0.13	-0.15	0.38	0.68	-0.19	0.25	0.46
1	rs6577219	I	101415707	INTRONIC	G	0.13	-0.15	0.38	0.68	-0.18	0.25	0.47
1	rs11582211	I	101416871	INTRONIC	A	0.12	-0.14	0.39	0.73	-0.18	0.27	0.50
1	rs6577221	I	101420406	INTRONIC	A	0.09	-0.31	0.44	0.48	-0.17	0.30	0.57
1	rs3903905	I	101420957	INTRONIC	T	0.13	-0.18	0.38	0.62	-0.20	0.25	0.43
1	rs17448827	I	101421407	INTRONIC	A	0.09	-0.31	0.44	0.49	-0.15	0.30	0.62
1	rs6693339	G	101424431	INTRONIC	G	0.08	-0.22	0.45	0.63	-0.10	0.31	0.75
1	rs6693456	I	101424561	INTRONIC	G	0.13	-0.16	0.37	0.66	-0.18	0.25	0.49
1	rs11588568	G	101426960	INTRONIC	T	0.04	0.08	0.62	0.90	-0.25	0.42	0.55
1	rs17610202	G	101429724	INTRONIC	G	0.07	-0.57	0.51	0.26	-0.30	0.34	0.38
1	rs17525507	I	101430504	INTRONIC	A	0.07	-0.61	0.51	0.23	-0.32	0.35	0.36
1	rs7537601	I	101435878	INTRONIC	A	0.04	0.11	0.62	0.86	-0.24	0.42	0.57
1	rs12730160	I	101438791	INTRONIC	A	0.13	-0.18	0.37	0.63	-0.22	0.25	0.39
1	rs3087816	I	101441775	3PRIME_UTR	C	0.07	-0.59	0.51	0.24	-0.30	0.35	0.38
1	rs17123572	I	101442211	3PRIME_UTR	C	0.04	0.11	0.62	0.86	-0.25	0.42	0.55
1	rs17449022	I	101442367	3PRIME_UTR	G	0.09	-0.31	0.44	0.49	-0.18	0.30	0.54
1	rs10493941	I	101445821	3PRIME_UTR	T	0.04	0.11	0.62	0.86	-0.25	0.42	0.55
1	rs12726628	G	101446276	3PRIME_UTR	G	0.04	-0.27	0.64	0.67	-0.54	0.43	0.21
1	rs6700743	I	101447384	DOWNSTREAM	T	0.13	-0.18	0.37	0.63	-0.22	0.25	0.39
	HMOX1	*heme oxygenase (decycling) 1*										
22	rs2071746	I	35776672	INTRONIC	T	0.48	-0.29	0.26	0.27	**-0.52**	**0.18**	**3.8E-03**
22	rs2071747	I	35777185	NON_SYNONYMOUS_CODING	C	0.04	0.86	0.64	0.18	0.04	0.44	0.92
22	rs2071748	I	35777618	INTRONIC	A	0.45	-0.37	0.26	0.16	**-0.44**	**0.18**	**0.01**
22	rs9306300	G	35778278	INTRONIC	G	0.04	0.68	0.62	0.28	0.05	0.42	0.90
22	rs5995097	I	35778961	INTRONIC	C	0.04	0.96	0.68	0.16	0.10	0.46	0.83
22	rs8139532	I	35779568	INTRONIC	A	0.03	-0.43	0.72	0.55	-0.36	0.49	0.46
22	rs8140669	G	35779844	INTRONIC	A	0.04	0.76	0.63	0.22	0.13	0.42	0.76
22	rs9607267	I	35781207	INTRONIC	C	0.45	-0.06	0.26	0.83	-0.11	0.18	0.54

Table 4. Continued

Chr	SNP RSID	Data Source	BP		Minor allele	MAF	Systolic blood pressure			Diastolic blood pressure		
							Best	Se	P-value	Best	Se	P-value
22	rs6518952	I	35782513	INTRONIC	T	0.04	-0.66	0.67	0.32	-0.47	0.45	0.30
22	rs2071749	G	35783413	DOWNSTREAM	A	0.29	-0.25	0.28	0.37	-0.03	0.19	0.89
22	rs11912889	I	35783617	DOWNSTREAM	A	0.04	-0.66	0.67	0.32	-0.47	0.45	0.30
22	rs5755720	I	35786873	DOWNSTREAM	G	0.43	0.02	0.26	0.93	-0.07	0.18	0.70
22	rs5995098	I	35787167	DOWNSTREAM	G	0.43	0.02	0.26	0.93	-0.07	0.18	0.70
22	rs2285112	I	35789263	INTRONIC	G	0.47	-0.10	0.26	0.70	-0.15	0.18	0.38
22	rs743811	I	35792974	DOWNSTREAM	T	0.33	0.04	0.27	0.87	-0.03	0.18	0.88

Table 5. Significant SNPs that are associated with both systolic and diastolic blood pressure

Chr	SNP RSID	Data Source	Base Pair	Location	Minor allele	MAF	Systolic blood pressure			Diastolic blood pressure		
							Best	Se	P-value	Best	Se	P-value
	SSBP1	*single-stranded DNA binding protein 1*										
7	rs1008318	I	141433634	UPSTREAM	G	0.32	-0.65	0.28	0.020	-0.42	0.19	0.027
7	rs11761832	I	141439390	UPSTREAM	A	0.32	-0.64	0.28	0.021	-0.42	0.19	0.027
7	rs7784221	I	141439404	UPSTREAM	C	0.32	-0.64	0.28	0.021	-0.42	0.19	0.027
7	rs12537498	I	141446915	INTRONIC	A	0.32	-0.64	0.28	0.022	-0.41	0.19	0.030
	CDH2	*cadherin 2, type 1, N-cadherin (neuronal)*										
18	rs665781	I	25579313	INTRONIC	T	0.36	-0.62	0.26	0.020	-0.41	0.18	0.022
18	rs584936	I	25581773	INTRONIC	A	0.36	-0.61	0.26	0.021	-0.41	0.18	0.024
18	rs597591	G	25582289	INTRONIC	C	0.35	-0.61	0.27	0.021	-0.41	0.18	0.024
18	rs614966	I	25583892	INTRONIC	A	0.36	-0.61	0.26	0.022	-0.40	0.18	0.025
18	rs1122356	I	25585263	INTRONIC	G	0.36	-0.58	0.26	0.029	-0.38	0.18	0.033
18	rs623234	I	25589391	INTRONIC	T	0.36	-0.60	0.27	0.024	-0.39	0.18	0.033
18	rs490820	I	25596894	INTRONIC	T	0.36	-0.68	0.27	0.012	-0.39	0.18	0.033
18	rs673008	I	25599702	INTRONIC	A	0.37	-0.67	0.27	0.012	-0.37	0.18	0.043
18	rs656642	I	25603085	INTRONIC	G	0.37	-0.67	0.27	0.012	-0.37	0.18	0.043
18	rs643555	I	25603666	INTRONIC	T	0.37	-0.67	0.27	0.012	-0.37	0.18	0.043
18	rs576467	I	25603719	INTRONIC	T	0.37	-0.67	0.27	0.012	-0.37	0.18	0.043
18	rs568575	I	25612462	INTRONIC	C	0.37	-0.69	0.27	0.010	-0.38	0.18	0.037
18	rs539075	I	25613439	INTRONIC	G	0.37	-0.69	0.27	0.010	-0.38	0.18	0.037
18	rs533602	I	25614034	INTRONIC	C	0.37	-0.71	0.27	0.008	-0.39	0.18	0.033
18	rs8087457	I	25617625	INTRONIC	C	0.35	-0.76	0.28	0.006	-0.45	0.19	0.017
18	rs1234682	I	25624066	INTRONIC	G	0.40	-0.64	0.26	0.015	-0.35	0.18	0.049
18	rs694943	I	25626303	INTRONIC	A	0.40	-0.65	0.26	0.013	-0.36	0.18	0.045
18	rs500643	G	25629299	INTRONIC	T	0.40	-0.63	0.26	0.015	-0.35	0.18	0.046
18	rs8087860	I	25673138	INTRONIC	C	0.38	-0.66	0.29	0.021	-0.44	0.19	0.024
18	rs1220035	I	25692866	INTRONIC	G	0.38	-0.77	0.27	0.004	-0.41	0.18	0.025
18	rs1148377	I	25693919	INTRONIC	C	0.37	-0.74	0.27	0.006	-0.38	0.18	0.041
18	rs1148378	I	25694909	INTRONIC	G	0.37	-0.74	0.27	0.006	-0.38	0.18	0.041
18	rs1148379	I	25695088	INTRONIC	C	0.37	-0.74	0.27	0.006	-0.38	0.18	0.041
	YWHAE	*tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide*										
17	rs7210877	I	1267472	INTRONIC	G	0.45	-0.71	0.27	0.008	-0.36	0.18	0.046
	ME2	*malic enzyme 2, NAD(+)-dependent, mitochondrial*										
18	rs661327	I	48479444	DOWNSTREAM	C	0.10	-0.88	0.42	0.037	-0.62	0.29	0.031
	PFTK1	*PFTAIRE protein kinase 1*										
7	rs705349	G	90673308	INTRONIC	A	0.50	-0.67	0.25	0.008	-0.46	0.17	0.007
7	rs705352	I	90698364	INTRONIC	T	0.50	0.61	0.26	0.017	0.43	0.17	0.014

Table 5. Continued

Chr	SNP RSID	Data Source	Base Pair	Location	Minor allele	MAF	Systolic blood pressure			Diastolic blood pressure		
							Best	Se	p-value	Best	Se	p-value
	G3BP2	*GTPase activating protein (SH3 domain) binding*										
4	rs17000733	G	76580894	INTRONIC	A	0.08	0.97	0.46	0.034	0.75	0.31	0.015
4	rs6531816	G	76581775	INTRONIC	C	0.08	-1.16	0.46	0.011	-0.77	0.31	0.013
4	rs17000740	I	76590608	INTRONIC	A	0.08	0.91	0.46	0.048	0.73	0.31	0.019
4	rs3775071	I	76593147	INTRONIC	A	0.08	0.91	0.46	0.048	0.73	0.31	0.019
	TUFT1	*tuftelin 1*										
1	rs3811411	G	151511268	DOWNSTREAM	G	0.50	0.66	0.25	0.008	0.42	0.17	0.014
1	rs3790507	I	151513247	DOWNSTREAM	T	0.36	-0.79	0.27	0.003	-0.49	0.18	0.006
1	rs11204844	G	151515458	DOWNSTREAM	G	0.27	-0.79	0.29	0.006	-0.47	0.20	0.016
1	rs4970957	I	151517388	INTRONIC	A	0.50	-0.67	0.25	0.007	-0.41	0.17	0.016
1	rs6587597	I	151520731	INTRONIC	A	0.36	-0.83	0.27	0.002	-0.52	0.18	0.004
1	rs17640579	I	151521933	INTRONIC	G	0.27	-0.82	0.29	0.005	-0.49	0.20	0.013
1	rs17640598	I	151524275	INTRONIC	G	0.27	-0.81	0.29	0.005	-0.49	0.20	0.012
1	rs11204848	I	151529918	INTRONIC	C	0.36	-0.96	0.27	0.000	-0.55	0.18	0.002
1	rs4132646	I	151534142	INTRONIC	A	0.36	-0.94	0.27	0.001	-0.55	0.18	0.003
1	rs3748610	I	151542099	INTRONIC	T	0.41	0.51	0.26	0.048	0.38	0.18	0.033
1	rs1539490	G	151542626	INTRONIC	C	0.41	0.53	0.26	0.041	0.40	0.18	0.025
1	rs3790505	I	151545057	INTRONIC	A	0.28	-0.60	0.28	0.036	-0.39	0.19	0.044
1	rs12751350	G	151550673	INTRONIC	A	0.41	0.52	0.26	0.045	0.37	0.18	0.037
1	rs3748608	I	151551320	INTRONIC	A	0.41	0.53	0.26	0.042	0.38	0.18	0.031
1	rs10494267	I	151553918	INTRONIC	T	0.41	0.54	0.26	0.037	0.39	0.18	0.027
1	rs1891592	I	151554503	3PRIME_UTR	C	0.41	0.54	0.26	0.038	0.39	0.18	0.027
1	rs11204853	G	151557131	DOWNSTREAM	C	0.29	-0.61	0.28	0.031	-0.38	0.19	0.043
1	rs4970919	I	151557258	DOWNSTREAM	G	0.41	0.55	0.26	0.036	0.39	0.18	0.027
1	rs1935886	I	151560153	DOWNSTREAM	C	0.41	0.57	0.26	0.030	0.41	0.18	0.020

Acknowledgments

This work was supported by the Basic Science Research Program through a National Research Foundation of Korea (NRF) grant, funded by the Korean government (MEST) (2010- 0012080).

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