Gender-specific Association of the ANO1 Genetic Variations with Hypertension

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Development of hypertension is caused by complex contributions of genetic and environmental factors. In spite of the increased understanding of hypertension, genetic factors that contribute to hypertension largely remain elusive. ANO1 gene encoding a calcium-activated chloride channel has recently been reported to affect spontaneous hypertension in the animal model. In this report, we investigated possible association of the ANO1 gene with hypertension in human with ANO1 variants found in Korean population. Fourteen polymorphisms of ANO1 gene were analyzed to be associated with hypertension. Interestingly, the six polymorphisms that showed statistically significant association were all the male subjects. The highest significant SNP was rs7127129 (OR=1.14, CI: 1.02~1.28, additive P=0.023; OR=1.24, CI: 1.03~1.49, dominant P=0.025), and other five SNPs (rs2509153, rs11235473, rs10751200, rs10898827 and rs10899928) were also statistically associated with hypertension. Consequently, we found that the genetic variants of ANO1 present statistically significant associations with hypertension in human, especially, in male. To the best of our knowledge, this study is the first report describing association of genetic polymorphisms of ANO1 with hypertension in human.

Key Words: Hypertension, Calcium-activated chloride channel, ANO1, SNP, Association

INTRODUCTION

Hypertension is a status having consistent high blood pressure. Development of hypertension is caused by complex contributions of genetic and environmental factors. According to 'the Korea National Health and Nutrition Examination Survey (KNHANES)', prevalence of hypertension in Korean adult population has reached to 27.3%, which is almost similar with the prevalence of Americans (Horowitz et al., 2015) and is still growing. Seriousness of hypertension is its strong relationships with other metabolic diseases such as diabetes, obesity, dyslipidemia, and so on (Taylor et al., 2013; Kelly et al., 2014). In spite of its seriousness, genetic factors that are related with hypertension are still remains elusive. Hypertension studies with genetic information have been reported using KARE (Korean Association RESource) cohorts, such as Genome-wide association study (Cho et al., 2009), nonsynonymous SNPs association study (Hong et al., 2009 & Jin et al., 2012), and candidate genes study (Jin et al., 2010).

Blood pressure is determined by combination of cardiac output and resistances of peripheral blood vessels (Marc and Llorens-Cortes, 2011). Therefore, when the cardiac output is consistent, blood pressure is determined mainly by vascular contractions. Contraction of vascular smooth muscle cells are controlled by combinatorial functions of several ion channels, among which ANO1 gene (anoctamin 1), a calcium-activated chloride channel (CaCC), has been
suspected as a causal genetic factor for the development of hypertension (Large and Wang, 1996; Heinze et al., 2014). The ANO1 gene located on human chromosome 11q13.3, and induces smooth muscle contraction by transporting chloride ion. Calcium activates ANO1 (anion channel) in plasma membrane to efflux chloride ion (Cl\textsuperscript{-}) out of the cell by resulting in depolarization in the vascular smooth muscle cell. To compensate the chloride efflux, voltage-dependent Ca\textsuperscript{2+} channels lead influx of extracellular Ca\textsuperscript{2+} into the cytosol. Therefore, ANO1 overexpression could give rise to stronger arterial contraction and an increase of blood pressure. Actually, it has been reported that overexpression of ANO1 contributed to spontaneous hypertension in the spontaneously hypertensive male rats (SHRs) (Wang et al., 2015) which suggests ANO1 might be a novel genetic factor for hypertension.

We aimed to investigate whether genetic variations of ANO1 gene are associated with hypertension in humans. For this purpose, the 14 single nucleotide polymorphisms (SNPs) of ANO1 were collected from the Korean Association REsource (KARE) and were analyzed for their relationship with hypertension. Based on the results, we suggest that ANO1 could be a susceptible gene for hypertension in human males.

**MATERIALS AND METHODS**

**Subjects and clinical characteristics**

Subjects in the Korean population in the Korean Association REsource (KARE) study were described in more detail by other study (Cho et al., 2009). Briefly, 10,038 persons in the Ansung-Ansan prospective community cohorts were recruited. A two-community cohort study in South Korea was initiated beginning in 2001 as part of a major project for the Korean Health and Genome Study (KHGS) in Korea National Health and Genome Study (KNIH). Of the initial 10,038 subjects who were aged 40 to 69 years, 1196 were excluded due to poor genotyping data. In addition, to analyze accurate blood pressure traits, 330 subjects who were on drug treatments such as folk medicine that were likely to influence the blood pressure were also excluded. The remaining 8512 subjects were finally investigated in this study.

A case-control study was performed between hypertensive male cases (n = 910) and normotensive male controls (n = 2062). Hypertensive male cases were categorized with the systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg in addition to the subjects who were receiving hypertension medication. Normotensive male controls were defined as SBP < 120 mmHg and DBP < 80 mmHg. For quantitative blood pressure traits analysis, subjects who were undergoing antihypertensive treatment were excluded and the remaining 3747 males were investigated. Clinical characteristics of the subjects are summarized in Table 1. This study was approved by the Institutional Review Board of the Korean National Institute of Health (KNIH). Written informed consent was obtained from all subjects.

**Clinical characteristic measurement**

Blood sample were drawn for biochemical measurements [triglyceride, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL)]. Blood pressure measurements were taken three times in the supine position using a mercury sphygmomanometer (Baumanometer; W. A. Baum, Copiague, NY, USA) with an appropriate cuff size by trained nurses at clinics, and the average value data was used for this study. Before the first measurement, subjects rested for 5 min, and three measurements were taken at least 2 min apart.

**Genotyping and selection of SNPs**

The detailed genotyping, quality control processes and quantitative traits including SBP and DBP were described in the previous report (Cho et al., 2009). Briefly, most DNA samples were isolated from the peripheral blood of participants and genotyped using the Affymetrix Genome-Wide Human SNP array 5.0 (Affymetrix Inc., Santa Clara, CA, USA). The accuracy of the genotyping was calculated by Bayesian Robust Linear Modeling using the Mahalanobis Distance (BRLMM) algorithm (Rabbee and Speed, 2006). Samples that had genotyping accuracies were lower than 98%, high missing genotype call rates (≥4%), high heterozygosity (≥30%), or gender biases were excluded.
The 14 SNPs that we analyzed were selected from the KARE data, based on their positions within the *ANO1* gene boundary (5 kb upstream and downstream of the first and last exons, respectively) (Table 2). The positions of the SNPs were validated in the NCBI human genome build 36. For the *in silico* functional analysis, we used HaploReg v3 (http://www.broadinstitute.org/mammals/haploreg/haploreg_v3.php), which is a tool for exploring annotations of the noncoding variants. The clinical information and genotype data that we used were graciously provided by the Center for Genome Science, KNIH, Korea Center for Disease Control (KCDC).

### Statistical analysis

Most statistical analyses were performed using PLINK version 1.07 (http://pngu.mgh.harvard.edu/~purcell/plink) and PASW Statistics version 18.0 (SPSS Inc., Chicago, IL, USA). The 14 selected SNPs were also analyzed in hypertension case-control studies using logistic regression analysis, controlling for cohort, age and body mass index (BMI) as covariates. Linear regression was used to analyze for the clinical characteristics as quantitative traits in the final 3747 men, controlling for cohort, age and body mass index (BMI) as covariates. The association tests were based on an additive, dominant, and recessive genetic model, and *P*-values were not adjusted for multiple tests. Statistical significance was determined at a two-tailed value of *P* < 0.05. For the regional association plot, we had used the SNP Annotation and Proxy Search (SNAP) database (http://www.broadinstitute.org/mpg/snap/) using the CHBJPT (Chinese and Japanese) population panel originated from HapMap database for the recombination rate.

## RESULTS

### Association analyses between SNPs in *ANO1* gene and hypertension in male

Clinical characteristics of the study subjects were listed in Table 1. Mean age of the hypertensive males (*n*=910), mean systolic blood pressure (SBP), and mean diastolic blood pressure (DBP) was 55.06 years, 137.56 ± 16.27, and 88.12 ± 10.37 (Table 1), respectively. Means and variances of BMI, SBP, DBP, total cholesterol, LDL, and triglyceride were statistically different between normotensive and hyper-

### Table 1. Basic characteristics of the male subjects in the KARE study cohort

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Quantitative trait analysis</th>
<th>Case-control analysis*</th>
<th>P value***</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normotensive</td>
<td>Hypertensive</td>
</tr>
<tr>
<td>Number of subjects</td>
<td>3747</td>
<td>2062</td>
<td>910</td>
</tr>
<tr>
<td>Age (M years ± SD)</td>
<td>51.27 ± 8.68</td>
<td>49.66 ± 8.17</td>
<td>55.06 ± 8.72</td>
</tr>
<tr>
<td>Body mass index (BMI) (M kg/m² ± SD)</td>
<td>24.14 ± 2.9</td>
<td>23.82 ± 2.76</td>
<td>24.99 ± 3.01</td>
</tr>
<tr>
<td>Systolic blood pressure (SBP) (M mmHg ± SD)</td>
<td>116.5 ± 16.17</td>
<td>105.57 ± 8.64</td>
<td>137.56 ± 16.27</td>
</tr>
<tr>
<td>Diastolic blood pressure (DBP) (M mmHg ± SD)</td>
<td>75.76 ± 10.99</td>
<td>68.94 ± 7.62</td>
<td>88.12 ± 10.37</td>
</tr>
<tr>
<td>Total cholesterol (M mg/dl ± SD)</td>
<td>191.33 ± 36.24</td>
<td>190.44 ± 34.63</td>
<td>193.89 ± 38.25</td>
</tr>
<tr>
<td>High density lipoprotein cholesterol (M mg/dl ± SD)</td>
<td>43.79 ± 9.99</td>
<td>43.5 ± 9.68</td>
<td>43.35 ± 10.29</td>
</tr>
<tr>
<td>Low density lipoprotein cholesterol (M mg/dl ± SD)</td>
<td>114.26 ± 33.40</td>
<td>115.76 ± 31.86</td>
<td>112.73 ± 35.48</td>
</tr>
<tr>
<td>Triglyceride (M mg/dl ± SD)</td>
<td>176.66 ± 119.48</td>
<td>163.73 ± 110.17</td>
<td>202.42 ± 127.02</td>
</tr>
</tbody>
</table>

Abbreviations: M, mean value; SD, standard deviation. *Individuals who are not using hypertensive medications. **Controls (normotensive), SBP < 120 mmHg and DBP < 80 mmHg; Cases (hypertensive), SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg and/or antihypertensive medication. ***Significant differences in characteristics between the normotensive and hypertensive subjects were determined by the two-tailed Student’s t-test.
Tensive groups by Student's *t*-test (Table 1). Minor allele, minor allele frequency, and function of *ANO1* gene and its 14 SNPs were presented (Table 2). Regional association plots for the 14 SNPs in the *ANO1* were presented (Fig. 1).

The six SNPs of *ANO1* were associated with hypertension status. One of the six SNPs, rs7127129, had the highest significance with the hypertension (OR=1.14, CI: 1.02–1.28, additive *P*=0.023, Table 2). The significance of the rs7127129 in hypertension was statistically meaningful in additive and dominant genetic models (OR=1.24, CI: 1.03–1.49, dominant *P*=0.025, Table 2). The five other SNPs (rs2509153, rs11235473, rs10751200, rs10898827 and rs10899928) were also associated with the hypertension (Table 2). Among the six SNPs, rs10751200, rs10898827, and rs10899928 showed resistance to the hypertension (OR<1), and were composed in one LD block (*r*^2^>0.99). Otherwise, rs11235473 and rs7127129 were susceptible to hypertension (OR>1), and had high correlation with each other (*r*^2^>0.99).

### Table 2. The association analysis results of SNPs in the *ANO1* gene with the hypertension in the KARE males

<table>
<thead>
<tr>
<th>No.</th>
<th>SNP</th>
<th>Minor allele</th>
<th>MAF</th>
<th>Function</th>
<th>Hypertension (controls 2062: cases 910)</th>
<th>OR (95% CI)</th>
<th>Add P</th>
<th>OR (95% CI)</th>
<th>Dom P</th>
<th>OR (95% CI)</th>
<th>Rec P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>rs2509153</td>
<td>T</td>
<td>0.288</td>
<td>intron</td>
<td>1.08 (0.92–1.28)</td>
<td>0.017</td>
<td>0.334</td>
<td>1.36 (1.01–1.83)</td>
<td>0.045</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>rs2515274</td>
<td>T</td>
<td>0.145</td>
<td>intron</td>
<td>0.98 (0.83–1.15)</td>
<td>0.781</td>
<td>0.800</td>
<td>0.95 (0.52–1.73)</td>
<td>0.858</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>rs2509166</td>
<td>G</td>
<td>0.494</td>
<td>intron</td>
<td>1.06 (0.88–1.28)</td>
<td>0.253</td>
<td>0.534</td>
<td>1.13 (0.94–1.36)</td>
<td>0.205</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>rs2515267</td>
<td>T</td>
<td>0.412</td>
<td>intron</td>
<td>0.96 (0.79–1.16)</td>
<td>0.658</td>
<td>0.499</td>
<td>0.97 (0.78–1.21)</td>
<td>0.790</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>rs2509175</td>
<td>T</td>
<td>0.095</td>
<td>intron</td>
<td>0.96 (0.79–1.16)</td>
<td>0.657</td>
<td>0.42</td>
<td>0.96 (0.63–1.43)</td>
<td>0.280</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>rs948173</td>
<td>G</td>
<td>0.450</td>
<td>intron</td>
<td>0.99 (0.88–1.11)</td>
<td>0.845</td>
<td>0.769</td>
<td>1.00 (0.81–1.23)</td>
<td>0.999</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>rs1940236</td>
<td>A</td>
<td>0.232</td>
<td>intron</td>
<td>0.90 (0.78–1.04)</td>
<td>0.138</td>
<td>0.194</td>
<td>0.81 (0.55–1.19)</td>
<td>0.275</td>
<td></td>
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</tr>
<tr>
<td>8</td>
<td>rs10160639</td>
<td>A</td>
<td>0.195</td>
<td>intron</td>
<td>1.04 (0.88–1.24)</td>
<td>0.287</td>
<td>0.646</td>
<td>1.50 (1.00–2.24)</td>
<td>0.051</td>
<td></td>
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<tr>
<td>9</td>
<td>rs10793024</td>
<td>T</td>
<td>0.154</td>
<td>intron</td>
<td>0.92 (0.78–1.08)</td>
<td>0.309</td>
<td>0.171</td>
<td>1.17 (0.70–1.96)</td>
<td>0.546</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>rs11235473</td>
<td>T</td>
<td>0.468</td>
<td>intron</td>
<td>1.14 (1.01–1.28)</td>
<td>0.029</td>
<td>0.171</td>
<td>1.17 (0.96–1.42)</td>
<td>0.115</td>
<td></td>
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</tr>
<tr>
<td>11</td>
<td>rs10751200</td>
<td>G</td>
<td>0.226</td>
<td>intron</td>
<td>0.89 (0.75–1.05)</td>
<td>0.059</td>
<td>0.166</td>
<td>0.66 (0.44–0.99)</td>
<td>0.046</td>
<td></td>
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</tr>
<tr>
<td>12</td>
<td>rs10898827</td>
<td>A</td>
<td>0.225</td>
<td>intron</td>
<td>0.89 (0.75–1.05)</td>
<td>0.065</td>
<td>0.184</td>
<td>0.66 (0.44–0.99)</td>
<td>0.045</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>rs10898828</td>
<td>G</td>
<td>0.226</td>
<td>intron</td>
<td>0.89 (0.75–1.05)</td>
<td>0.057</td>
<td>0.161</td>
<td>0.66 (0.44–0.99)</td>
<td>0.046</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>rs7127129</td>
<td>G</td>
<td>0.475</td>
<td>intron</td>
<td>1.14 (1.02–1.28)</td>
<td>0.023</td>
<td>1.16</td>
<td>1.16 (0.95–1.40)</td>
<td>0.140</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: SNP, single nucleotide polymorphism; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval; Add P, additive genetic model *P* value; Dom P, dominant genetic model *P* value; Rec P, recessive genetic model *P* value. Controls were the subjects with SBP < 120 mmHg and DBP < 80 mmHg, and hypertension cases were the subjects with SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg and/or antihypertensive medication. Statistically significant values (*P* < 0.05) are indicated in bold and underline.
Association analyses between the six SNPs in ANO1 gene and the clinical characteristics

The six SNPs having association with the hypertension were further analyzed for the analysis with quantitative traits. The male subjects' clinical characteristics were collected such as SBP, DBP, total cholesterol, HDL, LDL, and triglyceride (Table 1). Linear regression analysis was used to analyze associations between the SNPs and the clinical characteristics by controlling for age, BMI, and cohort as covariates. The two SNPs (rs2509153 and rs7127129) were significantly associated with the clinical characteristics. The rs2509153 was associated with both total cholesterol ($\beta=2.57$, dominant $P=0.024$) and LDL ($\beta=2.97$, dominant $P=$...
4.8×10^{-3}). On the other hand, the rs7127129 was associated with HDL (β=-0.85, recessive \( P=0.027 \)). The six SNPs had no association with the characteristics indicating blood pressures such as SBP, and DBP.

### Association analyses between the SNPs in \textit{ANO1} gene and the hypertension in female

Additionally, association between the genetic variations of \textit{ANO1} gene and hypertension in female was examined. The SNPs had no significant association with the hypertension except the rs2515274 (OR=1.19, CI: 1.01~1.40, additive \( P=0.033 \), Table 4). These results indicate there is gender-specific association between the SNPs in \textit{ANO1} gene and hypertension.

**DISCUSSION**

In this study, we investigated the association of genetic variations of \textit{ANO1} gene with hypertension in the Korean population. The six SNPs of \textit{ANO1} were associated with
hypertension in a gender-specific manner (Table 2, Fig. 1). Among the six SNPs, the two SNPs were also associated with total cholesterol, LDL, or HDL (Table 3). The six SNPs were analyzed in silico using HaploReg v3 which presented the motif changes in all of the six SNPs (Table S1). On the other hand, the 14 SNPs of ANO1 had no relationship with hypertension in the Korean females except one SNP (Table 4). Unlike the hypertension results, we were not able to find relationships between the genetic variations of ANO1 gene and SBP and/or DBP. Whereas, ANO1 had been shown to have a relationship with blood pressure in the experiments with SHRs (Wang et al., 2015). The discordance might be caused by limited number of subjects (79 male subjects) having blood pressure above 160 mmHg and heterogeneous reasons for the hypertension. Therefore, it will be necessary to collect increased number of subjects having high blood pressure and select hypertensive subject caused by arterial resistance in the future study.

Our statistical results showed a gender-specific association of the SNPs in the ANO1 gene with hypertension in the male (Tables 2 and 4). Several gender-specific associations of the SNPs with hypertension have been reported: association of AGT polymorphisms with hypertension in female (Dhanachandra Singh et al., 2014), association of ESR1 polymorphisms with hypertension in male (Kelly et al., 2013), and association of KNG1 polymorphisms with essential hypertension in male (Zhao et al., 2009). In a recent study, significant gender-specific differences in concentration of serum metabolites and the metabolism-related genes have been revealed (Mittelstrass et al., 2011). Nevertheless, the accurate mechanisms of these gender-specific associations of genetic polymorphisms still remain to be clarified.

We further analyzed the influence of smoking to the high prevalence of hypertension in the males. Smoking status is very different between Korean males and females. Korean females have low smoking habit (164 females, 3.52%), whereas Korean males show very high smoking habit (2,064 males, 49.34%) from KARE data. Case control analyses were conducted toward the male hypertension group having smoking habit or the non-smoking male hypertension group, separately. The results indicated that only the male hypertension group having smoking habit had association with the SNPs of ANO1 gene: among the 14 SNPs of ANO1 gene, three SNPs were associated with hypertension (Table S2). These results suggest that smoking could be a reason for the gender-specific differences.

Despite a couple of reports implicating the relationship between calcium-activated chloride channel and hypertension, there has been no report for the association between ANO1 gene and hypertension in human. Even in the genetic association databases (HuGE Navigator: http://hugenavigator.net), we were not able to find any significant association of ANO1 polymorphisms with metabolic disease. Therefore, this is the first report describing genetic polymorphisms in the ANO1 gene associated with hypertension in human.

In summary, we investigated whether the ANO1 gene associated with hypertension in Korean population. And, we found that the ANO1 polymorphisms were statistically associated with hypertension in the Korean male subjects. Therefore, this study suggests that the ANO1 gene could be a causal genetic factor for hypertension.

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Conflict of interest
The authors declare that they have no competing interests.

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