Hypertension and Genetic Variation in Endothelial-Specific Genes

Larsson, Erik; Wahlstrand, Bjorn; Hedblad, Bo; Hedner, Thomas; Kjeldsen, Sverre E.; Melander, Olle; Lindahl, Per

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Introduction

Hypertension is the major global risk factor for coronary heart disease and stroke. The pathogenesis is poorly understood and the primary cause is unknown in 90–95% of cases. Heritability has been estimated to be between 30 and 50% and ambitious efforts have been made to elucidate the genetic basis. While genome-wide association (GWA) studies usually detect common genetic variants with low-to-medium effect sizes, many contributing variants are not revealed, since they fail to reach significance after strong correction for multiple comparisons. The WTCCC study for hypertension, for example, failed to identify genome-wide significant associations. We hypothesized that genetic variation in genes expressed specifically in the endothelium may be important for hypertension development.

Results from the WTCCC study were combined with previously published gene expression data from mice to specifically investigate SNPs located within endothelial-specific genes, bypassing the requirement for genome-wide significance. Six SNPs from the WTCCC study were selected for independent replication in 5205 hypertensive patients and 5320 population-based controls, and successively in a cohort of 16537 individuals. A common variant (rs10860812) in the DRAM (damage-regulated autophagy modulator) locus showed association with hypertension (P = 0.008) in the replication study. The minor allele (A) had a protective effect (OR = 0.93; 95% CI 0.88–0.98 per A-allele), which replicates the association in the WTCCC GWA study. However, a second follow-up, in the larger cohort, failed to reveal an association with blood pressure. We further tested the endothelial-specific genes for co-localization with a panel of newly discovered SNPs from large meta-GWAS on hypertension or blood pressure. There was no significant overlap between those genes and hypertension or blood pressure loci. The result does not support the hypothesis that genetic variation in genes expressed in endothelium plays an important role for hypertension development. Moreover, the discordant association of rs10860812 with blood pressure in the case control study versus the larger Malmö Preventive Project–study highlights the importance of rigorous replication in multiple large independent studies.

Abstract

Genome-wide association (GWA) studies usually detect common genetic variants with low-to-medium effect sizes. Many contributing variants are not revealed, since they fail to reach significance after strong correction for multiple comparisons. The WTCCC study for hypertension, for example, failed to identify genome-wide significant associations. We hypothesized that genetic variation in genes expressed specifically in the endothelium may be important for hypertension development. Results from the WTCCC study were combined with previously published gene expression data from mice to specifically investigate SNPs located within endothelial-specific genes, bypassing the requirement for genome-wide significance. Six SNPs from the WTCCC study were selected for independent replication in 5205 hypertensive patients and 5320 population-based controls, and successively in a cohort of 16537 individuals. A common variant (rs10860812) in the DRAM (damage-regulated autophagy modulator) locus showed association with hypertension (P = 0.008) in the replication study. The minor allele (A) had a protective effect (OR = 0.93; 95% CI 0.88–0.98 per A-allele), which replicates the association in the WTCCC GWA study. However, a second follow-up, in the larger cohort, failed to reveal an association with blood pressure. We further tested the endothelial-specific genes for co-localization with a panel of newly discovered SNPs from large meta-GWAS on hypertension or blood pressure. There was no significant overlap between those genes and hypertension or blood pressure loci. The result does not support the hypothesis that genetic variation in genes expressed in endothelium plays an important role for hypertension development. Moreover, the discordant association of rs10860812 with blood pressure in the case control study versus the larger Malmö Preventive Project–study highlights the importance of rigorous replication in multiple large independent studies.


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These authors contributed equally to this work.
Although results have been contradictory, previous association studies indicate that genetic variation in endothelial genes such as endothelin-1 and endothelial nitric oxide synthase, both related to blood pressure (BP) regulation, may contribute to essential hypertension [9–11]. Based on the hypothesis that genetic variation in endothelium-specific genes may influence BP, we combined data from the WTCCC GWA study with previously published microarray gene expression data from mice [12], to select a subset of six SNPs, all located within endothelial marker genes, for independent replication in a case control study comprising more than 10,000 individuals. One SNP was further evaluated in a population-based study with 16537 participants (Figure 1).

Methods

Ethics Statement
The Nordic Diltiazem study (NORDIL) and the Malmo Diet and Cancer Cardiovascular Cohort study (MDC-CC) were approved by the local ethics committees at University of Gothenburg and Lund University, respectively. The Malmo Preventive Project (MPP) study was approved by the local ethics committee of Southern Sweden. All study participants had given written consent.

The Nordic Diltiazem Study
The Nordic Diltiazem study (NORDIL) is an intervention trial that between 1992 and 1999 included 10881 Swedish and Norwegian patients diagnosed with severe (grade 2) hypertension based on repeated diastolic $BP\geq 100 \, \text{mmHg}$ on different occasions, and prospectively compared cardiovascular outcome in patients randomized to diltiazem-based antihypertensive treatment as compared to patients randomized to diuretic and/or $\beta$-blocker based antihypertensive treatment. The design and main results have previously been described in detail [13]. The primary endpoint was fatal and non-fatal stroke, fatal and non-fatal myocardial infarction and other cardiovascular deaths, and there was no significant difference in cardiovascular outcome between the two treatment regimens. The Swedish subcohort of NORDIL participated in a genetic study and we obtained whole blood samples for DNA isolation from 5262 patients, of whom 5205 were successfully genotyped for the main SNP rs10860812 for the present investigation. BP was measured in the supine position after 10 minutes rest. These patients with diagnosed grade 2 hypertension formed our case group.

The Malmö Preventive Project
As a population control we used a Swedish cohort study, the population based Malmo Diet and Cancer Cardiovascular Cohort (MDC-CC) [14], which was designed to investigate the epidemiology of carotid artery disease. From a community-based prospective epidemiologic cohort of 28,449 persons enrolled between 1991 and 1996, 6,103 persons were randomly selected to participate in the MDC-CC. Whole blood samples for DNA extraction was obtained from 5445 subjects and genotypes for the main SNP rs10860812 were obtained from 5320 of these subjects. In the MDC-CC, BP was measured using a mercury-column sphygmomanometer after 10 minutes of rest in the supine position. Cardiovascular events (fatal and non-fatal stroke, fatal and non-fatal myocardial infarction and other cardiovascular deaths) were recorded during follow-up using national and local registers. Follow-up extended until December 31st 2005. Intima-media thickness (IMT) of the carotid artery was measured using 2D B-mode ultrasound as described previously [15]. The mean IMT of a 10 mm section of the common carotid artery (IMT$_{\text{mean}}$) and the maximum IMT of the carotid bulb (IMT$_{\text{max}}$) was recorded.

Characteristics of the grade 2 hypertension patients (NORDIL) and the population based control sample (MDC-CC) are shown in Table 1.

![Figure 1. Study design and workflow.](https://doi.org/10.1371/journal.pone.0062035.g001)
Genotyping

DNA was extracted from granulocyte or buffy coat suspensions, maintained at -80°C from the time of enrolment. Samples were thawed rapidly at 37°C and 200 μL aliquots were subjected to QiAamp mini-preps in 96-well format (Qiagen) according to the manufacturer’s instructions. SNPs rs893881, rs10060812, rs2269772, rs4684243, rs4981504 and rs6891143 were genotyped using 2.5 ng of DNA on the 7900HT instrument using TaqMan SNP Genotyping Assays (Applied Biosystems) in a total reaction volume of 6 μL in 384-well microtiter plates, according to the manufacturer’s instructions.

Animals

Adult C57Bl/6 mice were kept in groups at the Laboratory for Experimental Biomedicine at University of Gothenburg in a 12 h day/12 h night light cycle with food and water administered ad libitum in a temperature- and humidity-controlled room. All animal experiments were approved by the animal research ethics committee in Gothenburg, Sweden.

Isolation of Microvascular Fragments

Microvascular fragments were isolated from adult C57/Bl6 mouse brain and kidney. Brains or kidneys were dissected out, minced into pieces and digested with 5 mg Collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) dissolved in Hanks’ balanced salt solution (HBSS, Invitrogen AB, Lidingö, Sweden) including 1% BSA and 100U DNase at 37°C for 15 min with gentle agitation. The tissue was then gently pressed through a 100 μm cell strainer (Falcon, BD Biosciences, Stockholm, Sweden). Cells were washed out from the strainer in 2 ml of HBSS/1% BSA/100U DNase, pelleted at 200 g for 5 min, suspended in 1.5 ml HBSS/1% BSA/100U DNase, and again pelleted and resuspended. Rat anti-PECAM (BD Pharmingen, San Diego, CA, USA) antibody (Ab) -coated magnetic beads (Dynabeads M-450, sheep anti-Rat IgG, Dynal A.S., Oslo, Norway) were added, and after incubation at 4°C for 30 min with gentle agitation, microvascular fragments were isolated with a magnetic particle concentrator (MPC, Dynal) and washed three times with HBSS/1% BSA.

qPCR

mRNA expression levels were determined using SYBR Green quantitative qPCR (95°C, 55°C, 72°C, 40 cycles) on a 7900HT instrument (Applied Biosystems) using the following primers: Adcy4, 5'-CTTTGGTGTGCTTCTCTCGT-3' and 5'-ATGGCGTAA CACGGTGAAAGAT-3'; Gpr116, 5'-AAGACATGAGATCGCC AAAGG-3' and 5'-TTGGGGTCAATAGCTTCTCC-3'; Fgd5, 5'-GCTAGAGGCTGCTGTCTTT-3' and 5'-CCCTGCTGTA AAGTCACAGATA-3'; Arap3, 5'-GACTGAGCCCAATCTT CTTGG-3' and 5'-TCGCCCTGAAACTAATCGGA-3'; Itga3, 5'-TGTGAATATGTTGCGCTGAGA-3' and 5'-ATGCCG TCTGCAATAGTC-3'; Dram, 5'-GACACAGGAACACCTC CTCCA-3' and 5'-AAGCCGATGTGGCTGAGA-3'; Neb1, 5'-ATGTTTCCACTGCAGAGTC-3' and 5'-TGGATATGACC CTTGCGCATT-3'. Expression of Gapdh and Tie2 were measured using TaqMan assays under standard cycling conditions (Mm99999915_g1 and Mm00443242_m1, Applied Biosystems). Relative expression levels were determined using the standard curve method [17].

Bioinformatical Identification of Candidate Hypertension SNPs

Tab-delimited text files with hypertension association statistics for 469,557 SNPs were obtained from the WTCCC [2]. A list of 71 genes predicted to be specifically expressed in the microvascular endothelium were obtained from a recently published study [12]. Reciprocal human orthologs and genomic coordinates for these genes were determined using ENSEMBL and the UCSC browser [18,19]. For each gene region, here defined as the start of the first exon until the end of the last exon, the SNP with the lowest trend P-value in the WTCCC study was identified (Table 2).

Co-localization of SNPs Associated with Hypertension or Blood Pressure in Meta-GWAS with EC-specific Genes

549 SNPs with P<10^-5 based on data from 18 GWAS or meta-GWAS on hypertension or blood pressure were extracted from the PhenoR database [http://www.ncbi.nlm.nih.gov/gap/PhenoR] [2,17–32]. 670 putative associated genes were within 100 kb these SNPs, and these were evaluated for overrepresentation of EC-specific genes (71 gene list) using Fisher’s exact test.

Statistical Analysis

We assumed an additive model of inheritance and calculated allelic odds ratios (OR) and 95% confidence intervals (95% CI) for each SNP in relation to the main dependent variable (belonging to the grade 2 hypertension case group or belonging to the grade 2 hypertensive and received treatment either prior to or during the trial. doi:10.1371/journal.pone.0062035.t001

Table 1. Characteristics of study subjects.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NORDIL</th>
<th>MDC-CC</th>
<th>MPP base</th>
<th>MPP followup</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of individuals</td>
<td>5205</td>
<td>5320</td>
<td>16537</td>
<td>16537</td>
</tr>
<tr>
<td>Age (years ± SD)</td>
<td>60.2±6.6</td>
<td>57.4±5.9</td>
<td>45.6±6.9</td>
<td>69.1±5.5</td>
</tr>
<tr>
<td>Female sex (%)</td>
<td>50.2</td>
<td>57.7</td>
<td>35.8</td>
<td>35.8</td>
</tr>
<tr>
<td>Antihypertensive treatment (%)</td>
<td>100*</td>
<td>16.7</td>
<td>4.5</td>
<td>38.4</td>
</tr>
<tr>
<td>Smoker (%)</td>
<td>20.6</td>
<td>27.7</td>
<td>36.9</td>
<td>17.5</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg ± SD)</td>
<td>172.6±18.9</td>
<td>141.3±19.1</td>
<td>127.7±14.4</td>
<td>145.1±20.0</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg ± SD)</td>
<td>103.1±7.1</td>
<td>87.0±9.5</td>
<td>84.2±8.8</td>
<td>83.5±10.5</td>
</tr>
<tr>
<td>BMI (kg/m² ± SD)</td>
<td>28.1±4.4</td>
<td>25.9±4</td>
<td>24.3±3.4</td>
<td>27.2±4.1</td>
</tr>
<tr>
<td>Glucose (mmol/l ± SD)</td>
<td>5.32±1.56</td>
<td>5.19±1.42</td>
<td>4.90±0.75</td>
<td>5.85±1.4</td>
</tr>
<tr>
<td>Cholesterol (mmol/l ± SD)</td>
<td>6.31±1.17</td>
<td>6.17±1.09</td>
<td>5.63±1.0</td>
<td>5.59±1.1</td>
</tr>
</tbody>
</table>

BMI, body mass index; SD, standard deviation. *all NORDIL patients had grade 2 hypertension and received treatment either prior to or during the trial.
population controls) and to the secondary outcome variable (presence of cardiovascular events during follow-up in NORDIL or MDC-CC or no cardiovascular event during follow-up in NORDIL or MDC-CC) using crude and multivariate adjusted logistic regression. Continuous variables were related to rs10860812 using linear regression assuming an additive model of inheritance. Deviation from Hardy-Weinberg equilibrium was evaluated with a \( \chi^2 \) test using Levene's method (1949).

### Results

Putative Hypertension-associated SNPs in Endothelial-specific Genes

Since the endothelium has a functional role in BP regulation, we tested the hypothesis that genetic variation in genes expressed specifically in the endothelium may influence hypertension development. SNPs from the published WTCCC GWA analysis for human hypertension [2] were mapped onto a list of 71 endothelial genes obtained from a published study, where putative endothelial-specific genes were identified through analysis of a large mouse microarray compendium [12]. For each gene region,
the SNP with the strongest association to hypertension was identified (additive model). In consistency with our hypothesis, the number of moderately significant SNPs \( P<0.005 \) was significantly larger among the set of EC-specific genes compared to remaining genes (10% vs. 4%, \( P=0.03 \), Fisher’s exact test). Genes were ranked according to best \( P \)-value and SNPs in the top six candidates were selected for replication in an independent material. Association \( P \)-values in the WTCCC study for these SNPs were in the range of \( 1.8 \times 10^{-3} \) to \( 4.1 \times 10^{-4} \) (Table 2, italic). In addition, a SNP in the NEBL gene was included for validation due to its relatively strong association in the WTCCC (rs893081, \( P_{\text{add}}=1.5 \times 10^{-5} \)). NEBL is selectively expressed in endothelial cells and was highly ranked in the original analysis of EC-specific gene expression [12], but did not qualify for the 71-gene list due to additional strong expression in the heart.

To confirm expression of these genes in the vasculature, microvascular fragments were isolated from mouse brain and kidney using anti-CD31 (PECAM)-coated magnetic beads. Expression in vascular fragments vs. surrounding tissue was subsequently determined using real-time quantitative PCR. All genes were found to be significantly enriched in CD31+ fractions from both tissues \( (P<0.005) \) and all except one had >70-fold enrichment in at least one of the tissues (Figure 2). Gapdh was not differentially expressed, while Tie2, included as a positive control, was strongly enriched in CD31+ fractions from both tissues.

The DRAM Locus and Hypertension

An independent evaluation of the above selected SNPs was performed in 5205 hypertensive patients and 5320 population based controls (NORDIL vs. MDC-CC). Genotyping was successful (average genotyping success rate 98%) for six of the seven SNPs selected for validation, while one SNP (rs2021916, GPR116 locus) did not pass quality control. For all tested SNPs, allele frequencies were fairly similar in the WTCCC study and the MPP study. Association \( r^2 \) with each other was low (R2<0.15), suggesting that they may be in different LD blocks. The minor allele \( (A) \) was more frequent in the MPP study compared to the WTCCC study, where IMT was measured, the rs10860812 A-allele tended to be associated with lower \( (P=0.03, \text{Fisher’s exact test}) \). Genes with \( r^2 \) below 0.05 of the original analysis of EC-specific gene expression [12].

Secondary Analyses of DRAM Locus in the MDC-CC Study

Secondary analyses of rs10860812 (additive models) in relation to the continuous BP variable in the MDC-CC population sample did not reach significance, although results pointed toward a protective effect for the A-allele \( (\beta \text{-coefficient} \pm \text{SEM} \ (-0.425\pm0.574 \text{mmHg per A-allele, } P=0.26 \text{ for systolic BP and } -0.200\pm0.187 \text{mmHg per A-allele, } P=0.29 \text{ for diastolic BP}) \). The risk of incident cardiovascular disease during follow-up \( (n=894 \text{ cardiovascular events in MDC-CC+NORDIL}) \) was reduced in carriers of the rs10860812 A-allele \( (OR=0.90, 95\% \text{ CI 0.82–1.0 per A-allele; } P=0.04) \). Finally, in patients from the MDC-CC study, where IMT was measured, the rs10860812 A-allele tended to be associated with lower \( (\beta \text{-coefficient} \pm \text{SEM}) \) IMT \( =0.028\pm0.015 \text{ mm per A-allele; } P=0.06 \) and IMT \( =0.006\pm0.003 \text{ mm per A-allele; } P=0.07 \).

Secondary Analyses of DRAM Locus in the MPP Study

The rs10860812 variant was further evaluated (additive models) in relation to the continuous BP variable in the MPP study comprising 15637 individuals. There was no association between the rs10860812 variant and systolic or diastolic blood pressure neither at the MPP study baseline exam \( (\beta \text{-coefficient} \pm \text{SEM} \ (0.064\pm0.156 \text{mmHg per A-allele, } P=0.688 \text{ for systolic BP and } -0.068\pm0.097 \text{mmHg per A-allele, } P=0.486 \text{ for diastolic BP}) \) or at re-screening 15–25 years later \( (0.032\pm0.219 \text{ mmHg per A-allele, } P=0.883 \text{ for systolic BP and } -0.121\pm0.116 \text{ mmHg per A-allele, } P=0.297 \text{ for diastolic BP}) \). One possible confounding factor is that pharmacological treatment of hypertensive subjects in the study lowers the observed BP thus reducing the statistical power [20]. However, similar results were obtained when diastolic \(+10 \text{mmHg}) \) and systolic \(+15 \text{mmHg}) \) were corrected in

![Figure 2. Differential mRNA expression in CD31+ microvascular fragments.](image-url)
subjects on antihypertensive treatment (data not shown), as described in [6]. Moreover, the risk of incident cardiovascular disease during follow-up was not different in carriers of the rs10860812 A-allele, compared to the control population (data not shown).

Co-localization of EC-specific Genes and SNPs from Meta-GWAS

The lack of significant associations in our study suggests that variants in EC-specific genes may not be associated with hypertension or blood pressure more often than random genes. However, the publication of large meta-GWAS has expanded the repertoire of SNPs associated with those phenotypes, raising the possibility that newly discovered SNPs co-localize with EC-specific genes. To assess this possibility, we tested the 71 EC-specific genes against a recent catalogue of SNPs associated with hypertension or disease during follow-up was not different in carriers of the rs10860812 A-allele, compared to the control population (data not shown).

Table 3. Association between hypertension and SNPs in the study in WTCCC and NORDIL/MDC-CC.

<table>
<thead>
<tr>
<th>SNP</th>
<th>WTCCC hypertension study</th>
<th>NORDIL/MDC-CC replication study</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
<td>Chr.</td>
<td>Locus</td>
</tr>
<tr>
<td>rs4981504</td>
<td>14q11</td>
<td>ADCC4</td>
</tr>
<tr>
<td>rs4684243</td>
<td>3p25</td>
<td>FGDS5</td>
</tr>
<tr>
<td>rs6891143</td>
<td>5q31</td>
<td>CENTD3</td>
</tr>
<tr>
<td>rs2269772</td>
<td>17q21</td>
<td>ITGA3</td>
</tr>
<tr>
<td>rs10860812</td>
<td>12q23</td>
<td>DRAM</td>
</tr>
<tr>
<td>rs893881</td>
<td>10p12</td>
<td>NEBL</td>
</tr>
</tbody>
</table>

P-values were calculated assuming an additive model. Figures within parentheses are percentages.

doi:10.1371/journal.pone.0062035.t003

Discussion

Rare high-penetrance or common weakly penetrant genetic variants are not readily detected in GWA studies, opening up for alternative approaches that maintain the genome-wide perspective but reduce multiple testing. Here, we assess if genetic variants in endothelial-specific genes are associated with hypertension. The SNPs were selected for validation based on 1) endothelial-specific expression of the gene locus and 2) an association to hypertension that was observed in the WTCCC GWA study, although at a P-value below genome-wide significance. The selection process can thus be described as an intermediate between a candidate gene approach and a genome-wide approach.

Our case control study was substantially larger and our cases had a strictly defined and more severe clinical hypertension (grade 2) compared to the WTCCC, suggesting a greater power as the genetic contribution is likely to be larger in this case. Similar to the WTCCC, our control group was population based and thus included patients with hypertension. Our estimate of the genetic effect on risk therefore has relevance at the population level, but this may also have reduced the power in our study. To further validate effects on BP, SNPs were re-evaluated in relation to the continuous BP variable in the population based MPP study.

Our first replication suggested an association between a SNP (rs10860812) in the DRAM locus on chromosome 12q23 and hypertension. DRAM is a regulator of autophagy that plays a critical role in apoptosis [36]. Although open to question, the involvement of apoptosis in hypertension-related vascular remodeling has been suggested [37]. The minor allele (A) of this SNP was concluded to have a protective effect and this is consistent with results from the WTCCC study. Secondary analyses revealed an association with incident cardiovascular events and borderline significant associations with IMT\textsubscript{max} and IMT\textsubscript{mean}. However, evaluation of rs10860812 in relation to the continuous blood pressure variable in the MDC-CC population study and sequentially in the larger MPP study did not show association with BP. Moreover, there was no association of rs10860812 with cardiovascular disease in the MPP study.

Recent meta-analyses have to some part overcome the problems of the early hypertension GWAS, and 29 loci are now robustly associated with blood pressure and hypertension [3]. The EC-specific gene NRP3 is located near one of the 29 validated loci,
rs1173771, and encodes a receptor for natriuretic peptides that are implicated in the maintenance of blood pressure [30], supporting a causal role for the endothelium. A large proportion of blood pressure heritability remains unknown [3], and alternative methods such as the one we describe could help identify additional loci. However, our replication studies of moderately significant SNPs from the WTCCC study did not support a connection between genetic variation in the endothelium and hypertension. Similarly, the expanded investigation of 529 potential SNPs from the PheGenI catalogue failed to show significant overlap with EC-specific genes.

In conclusion, our study does not support that genetic variation in genes expressed in endothelium plays a major role in the development of hypertension. The result further underscores the importance of rigorous validation of genetic associations in large and independent populations.

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**Author Contributions**

Conceived and designed the experiments: EL BW BH SE OM PL. Performed the experiments: EL BH OM. Analyzed the data: EL OM PL. Contributed reagents/materials/analysis tools: EL TH SE BH OM PL. Wrote the paper: EL PL.

**References**