Essential Hypertension and $\beta_2$-Adrenergic Receptor Gene
Linkage and Association Analysis


Abstract—A region on human chromosome 5 (5q31.1-qter) contains several genes that encode important blood pressure regulators and thus is a good candidate for analysis of linkage and association with hypertension. We recruited 638 individuals from 212 Polish pedigrees with clustering of essential hypertension. These subjects were genotyped for 11 microsatellite markers that span this region to test for linkage to essential hypertension and systolic and diastolic blood pressures. The segment of this region of $\approx 7$ cM delineated by D5S1480 and D5S500 markers was linked to blood pressures in multipoint analysis. In 2-point analysis, D5S1480—the marker in close proximity to $\beta_2$-adrenergic receptor gene—reached the maximal linkage to essential hypertension and adjusted systolic and diastolic blood pressures, implicating this gene as a positional candidate for further association studies. Arg16Gly, Gln27Glu, and Thr164Ile—3 functional single nucleotide polymorphisms within the $\beta_2$-adrenergic receptor gene—were tested for association with essential hypertension. None of these polymorphisms showed a significant association with essential hypertension, separately or in the haplotype. This study provided evidence of linkage of 5q31.1-5qter region to essential hypertension in the European population. Moreover, it implicated the chromosomal segment in close proximity to D5S1480 and D5S500. The detailed analysis of 3 single nucleotide polymorphisms does not support the role of the $\beta_2$-adrenergic receptor gene as a major causative gene for the detected linkage. (Hypertension. 2002;40:286-291.)

Key Words: chromosomes ■ adrenergic receptors ■ genes ■ linkage ■ blood pressure

Essential hypertension is a multifactorial complex trait with a strong hereditary component. Apart from genomewide scans and candidate gene approach (principal methods used in pursuit of genetic loci that may determine predisposition to essential hypertension), 1 a target chromosomal region approach combining the rationale of 2 major strategies has been postulated. 2 Selection of a small chromosomal region implicated by genome-wide searches and containing several candidate genes pathophysiologicaly related to the investigated phenotypes allows for denser saturation with microsatellite markers and may be followed by subsequent positional analysis. The distal segment of the long arm of chromosome 5 (5q31.1-qter) is an outstanding target chromosomal region for studies on essential hypertension, having been linked to both systolic 4 and postexercise diastolic blood pressure 4 in genome-wide scans performed in white populations. Furthermore, this region contains a cluster of genes coding for proteins known as important blood pressure regulators ($\beta_2$-adrenergic receptor gene [ADRB2], $\alpha_{1B}$-adrenergic receptor, dopamine D 1 receptor, annexin VI) and implicated as possible contributors to the pathogenesis of several cardiovascular disorders (platelet-derived growth factor receptor, glutathione peroxidase).

We performed a linkage analysis of this region using 3 related phenotypes: a diagnosis of essential hypertension (a qualitative trait) and 2 quantitative phenotypes—systolic and diastolic blood pressure. We searched for a linkage indicating positional locus for further association analyses. One of the loci in the implicated portion of the target region, ADRB2, was subsequently analyzed in association studies.

Subjects

The participants in this project (Silesian Hypertension Study) were recruited between 1999 to 2000 in Silesia, a region in the south of Poland with a high prevalence of cardiovascular morbidity and mortality. The study was designed to investigate for genetic predisposition to several cardiovascular phenotypes and was based on collecting probands with diagnosed essential hypertension along with their available parents and/or siblings. The project was approved by the local bioethical committee, and informed consent was obtained from each participant. We recruited 638 white individuals from 212 families with clustering of essential hypertension. Com-

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286
complete phenotypic information was obtained from 635 subjects representing 210 families. Six other individuals from 3 families were excluded because of the inconsistencies in Mendelian segregation.

Phenotyping
Phenotyping included clinical history obtained by standardized questionnaires, physical examination, and laboratory tests according to the recommendations of the World Health Organization.5 Hypertension was defined as systolic and/or diastolic blood pressure >140/90 mm Hg on 3 separate occasions and/or remaining on antihypertensive treatment.5 Subjects with secondary forms of hypertension were excluded from the study. Height and weight measurements were taken in standard conditions to calculate body mass index. Blood pressure was taken in a sitting position using mercury sphygmomanometer with a cuff size individually adjusted to the arm after 20 minutes of rest. Systolic blood pressure was taken at the return of arterial sounds (Korotkoff phase I), and disappearance of sounds (Korotkoff phase V) indicated diastolic blood pressure. The average of 3 consecutive recordings of both systolic and diastolic blood pressure was used to obtain the representative values. Blood pressure values from subjects remaining on antihypertensive therapy were nonparametrically adjusted for treatment effect according to the algorithm used previously in analyses of Framingham data.6 In brief, the adjustments were based on a nonparametric method in which the blood pressures of an individual receiving antihypertensive treatment were shifted upward by adding to them the mean of the residues of the age-regressed blood pressures of those with higher blood pressures and the individual itself. Both genders were analyzed separately. Observations from individuals not taking antihypertensive therapy remained unchanged.

Identification and Localization of Genetic Loci Within the Candidate Chromosomal Region
Eight microsatellite markers (DSS1480, DSS636, DSS820, DSS2093, DSS1471, DSS1456, DSS462, DSS211) spanning the 35-cM region on the distal portion of long arm of chromosome 5 (5q31.1-qter) were initially selected for molecular analysis. A set of 3 additional markers (DSS500, DSS642, DSS494) located proximally from DSS1480 and covering the distance of ~20 cM was chosen at a later stage to define the linkage region more accurately (Figure).

Location and distances between the markers were obtained from public databases, including the Center for Medical Genetics at Marshfield Medical Research Foundation (http://research.marshfieldclinic.org/genetics) and the Genetic Location Database (http://cedar.genetics.soton.ac.uk/pub/chrom5/map.htm). Candidate genes were localized within the region by use of integrated information from the Unified Database for Human Genome Mapping at the Weizmann Institute of Science (http://bioinformatics.weizmann.ac.il) and the database at University of California, Santa Cruz, Human Genome Project Working Draft (http://genome.ucsc.edu). Single nucleotide polymorphisms within the ADRB2 gene were identified using the Human Genome Variation Database (http://hgvbase.cgb.ki.se) and the Single Nucleotide Polymorphism (SNP) Database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/index.html) and were then prioritized based on their ability to affect the function of the receptor.

Genotyping
Genomic DNA was extracted from whole blood samples by use of the MasterPure™ DNA purification kit (Epicentre Technologies). Each microsatellite marker was amplified by polymerase chain reaction (PCR) using Tetrad DNA Engine (MJ Research). The sequences of the primers were obtained from Genome Database Bank (http://www.gdb.org) and sequenced (Applied Biosystems). The total volume of PCR master mixture was 20 μL and included 25 ng of genomic DNA, 200 μmol/L of each dNTP (Promega), 10 pmol of forward and reverse primer, 0.2 U of Taq DNA polymerase (HotStarTaq, Qiagen), 10× PCR buffer (with 1.5 mmol/L of MgCl₂, Qiagen), and polyoxyethylene ether (W-1 solution, Life Technologies). The 5’-end of the forward primers was labeled with 6-carboxyfluorescein (FAM) or its fluorescent analogs (HEX, NED). DNA polymerase activation in 95°C for 1 minute was followed by 34 cycles of 94°C (1 minute), annealing (1 minute), 72°C (1 minute), and the final extension in 72°C for 10 minutes. PCR products were pooled and resolved on 5% polyacrylamide gel by use of an ABI 377 Sequencer (Applied Biosystems). Genotyping was performed by use of Genescan and Genotyper software (Applied Biosystems), independently by 2 individuals, who were unaware of phenotypic data.

PCR amplification of the DNA fragment containing Arg16Gly polymorphism of the ADRB2 gene was performed in a volume of 20 μL, including 25 ng of genomic DNA, 200 μmol/L of each dNTP (Promega), 10 pmol of forward and reverse primer, 0.2 U of Taq DNA polymerase (HotStarTaq, Qiagen), and 10× PCR buffer (with 1.5 mmol/L of MgCl₂, Qiagen). DNA polymerase activation in 95°C for 15 minutes was followed by 34 cycles of denaturation (95°C, 1 minute) annealing (52°C, 1 minute), and extension (72°C, 1 minute), with a final extension (72°C, 10 minutes). The PCR product was digested with 2 U of BsrDI restriction enzyme (New England Biolabs) in 60°C for 6 hours and resolved on 3% 1000-agarose (Life Technologies) gel containing ethidium bromide, with subsequent visualization with a Fluoro-S Multi-imager (Biorad). The primers and the sizes of digestion products were the same as described previously.7

The amplification conditions for DNA fragment containing Gln27Glu polymorphism of the ADRB2 gene were similar to the Arg16Gly polymorphism, except for the primers sequence and annealing temperature (63°C). The PCR product was digested with 1.5 U of Hul restriction enzyme (Roche) in 37°C for 20 hours, resolved on 2.5% 1000-agarose (Life Technologies) gel containing ethidium bromide, and visualized by means of a Fluoro-S Multi-imager. The sequence of the primers and the sizes of digestion products were the same as described previously.7

The PCR conditions for the segment containing the Thr164Ile SNP of the ADRB2 gene were similar to that of the Arg16Gly polymorphism except for the sequence of primers and annealing temperature (55°C). The PCR product was digested with 2 U of MnlI restriction enzyme (New England Biolabs) in 37°C for 5 hours, resolved on 2% ultra pure-agarose (Life Technologies) gel containing ethidium bromide, and visualized by means of a Fluoro-S Multi-imager. The sequence of primers and the sizes of digestion products were the same as described previously.7 Direct sequencing
of the DNA fragment, including 3 functional SNPs within the ADRB2 gene, was performed in 15 randomly selected unrelated individuals to confirm the results of restriction fragment–length polymorphisms.

**Statistical Analysis**

Verification of genotypes for inconsistencies in Mendelian segregation was performed by means of PEDCHECK program. Multiple methods were used for linkage and association analysis of both qualitative and quantitative traits because this provides greater reliability of final result. Haseman-Elston regression analysis, based on reordering the siblings’ squared phenotype difference on their genetic similarity (defined as alleles shared identical by descent [IBD]) was used to test for 2-point linkage in case of microsatellite markers and the investigated phenotypes. Another IBD sib-pair test, SPLINK (Unix version 1.08), was applied to investigate for linkage of microsatellite markers to essential hypertension. Confirmatory 2-point linkage analysis based on estimation of alleles identical by state (IBS) at a microsatellite marker compared with the random distribution of alleles was performed for hypertension as a binary trait, by means of IBS \( \chi^2 \) test. The Haseman-Elston and IBS \( \chi^2 \) 2-point linkage tests were completed with SIB-PAIR program. For further confirmation of the results obtained in 2-point linkage analysis, multipoint nonparametric Z-score rank test was performed with quantitative phenotypes using MAPMAKER/SIBS.

The subsequent strategy, testing for association of essential hypertension with the ADRB2 gene as a positional candidate, was performed using family-based association tests. The transmission disequilibrium test (TDT) assessing the number of transmitted versus non-transmitted alleles from heterozygous parents to affected (hypertensive) probands (compared with expected 50%/50% transmission/nontransmission ratio) was used to test for association of essential hypertension with the SNPs of the ADRB2. Another IBD sib-pair test, \( \text{IBD} \) was used to test for 2-point linkage in case of microsatellite markers.

**Results**

**Clinical Characteristics**

There were 629 individuals (age, 45.8±15.7 years) from 207 families, with 313 (49%) men and 316 (51%) women included in the final analysis. Of these, 401 (63.7%) subjects were hypertensive, and 270 (67.3%) of the hypertensive subjects remained on treatment. The demographic and clinical data of all individuals divided into probands, parents, and siblings are shown in Table 1.

**Table 1. Demographic and Clinical Characteristics of the Individuals in Silesian Hypertension Study**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Probands (n=207)</th>
<th>Mothers (n=144)</th>
<th>Fathers (n=130)</th>
<th>Siblings (n=148)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, M/F</td>
<td>119/88</td>
<td>0/144</td>
<td>130/0</td>
<td>64/84</td>
</tr>
<tr>
<td>Age, y</td>
<td>36.2±15.6</td>
<td>54.2±10.8</td>
<td>55.8±10.0</td>
<td>42.4±14.8</td>
</tr>
<tr>
<td>Hypertensive subjects, n (%)</td>
<td>207 (100)</td>
<td>85 (59)</td>
<td>61 (47)</td>
<td>48 (32)</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>142.6±14.0</td>
<td>139.4±23.3</td>
<td>138.6±19.8</td>
<td>131.6±21.8</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>91.2±10.6</td>
<td>88.7±13.6</td>
<td>87.2±11.6</td>
<td>83.7±11.1</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.6±4.6</td>
<td>27.5±5.1</td>
<td>27.6±4.1</td>
<td>25.7±4.6</td>
</tr>
</tbody>
</table>

Values are mean±SD. SBP indicates systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index.

The transmission disequilibrium test (TDT) assessing the number of transmitted versus non-transmitted alleles from heterozygous parents to affected (hypertensive) probands (compared with expected 50%/50% transmission/nontransmission ratio) was used to test for association of essential hypertension with the SNPs of the ADRB2. The results of the TDTs were then verified by means of the empirical variance-family based association test (EV-FBAT) method, determining the value of an association test under the null hypothesis of linkage but no association by use of an empirical variance-covariance estimator. Unlike other family-based tests, this method is not affected by pedigree configurations and can be used in case of binary, quantitative, or time-to-onset traits, as well as multiallelic markers.

Clayton’s modified TDT was performed in case of haplotype combinations, using the program TRANSMIT. This test calculates a score vector that is equalized over all possible combinations of parental haplotypes and transmissions, in concordance with the observed data, and deals with the problem of partially unknown parental genotypes and haplotype phase uncertainty. Binary logistic regression analysis was performed in the parental generation to test for association between essential hypertension and each genetic variant of the ADRB2 in the presence of other covariates, including age, gender, and body mass index.
Association Studies of the Positional Candidate, ADRB2 Gene

Arg16Gly, Gln27Glu, and Thr164Ile polymorphisms were not associated with essential hypertension in the TDT (Table 3). This lack of association was confirmed by EV-FBAT test (Arg16Gly, \( P=0.67; \) Gln27Glu, \( P=0.55 \)). Testing for association of essential hypertension with Thr164Ile polymorphism using EV-FBAT could not be performed because of a rarity of Ile allele (only 17 individuals in the study were carriers of this allele).

Among 7 observed haplotypes (denoted A through G), B, D, and F represented the most common variants, comprising 97.4% of the total haplotypes (Table 4). The number of transmitted haplotypes from parents to hypertensive offspring was not significantly different from the expected number of transmissions (Table 4).

In the binary regression model, including age, gender, and body mass index as potential cofounders, none of the ADRB2 polymorphisms were associated with hypertension. The odds ratio for hypertension in subjects homozygous for a wild variant compared with heterozygous individuals and homozygous for a mutant allele was 0.85 (95% CI, 0.3 to 2.2; \( P=0.74 \)) and 1.46 (95% CI, 0.5 to 4.2; \( P=0.49 \)) for Arg16Gly, 1.33 (95% CI, 0.6 to 3.1; \( P=0.5 \)) and 1.54 (95% CI, 0.5 to 4.4; \( P=0.43 \)) for Gln27Glu, and 0.7 (95% CI, 0.1 to 4.9; \( P=0.72 \)) for Thr164Ile, respectively.

**Discussion**

In the present study, the maximal linkage was detected both in 2-point and multipoint analysis at the same chromosomal position corresponding to the D5S1480 microsatellite marker. In contrast, the linkage analysis of systolic blood pressure performed by Krushkal et al\(^2\) on the same chromosomal region implicated different markers located proximally to the telomere. This discrepancy is not surprising and may reflect several differences in ethnic (European versus American origin), demographic (age), and clinical (normotension versus hypertension) profile of the subjects between these studies.

To avoid a potential bias that may arise from a linkage analysis of a dichotomous trait based on arbitrary categorization (hypertension), we performed additional studies of systolic and diastolic blood pressures, detecting consistent linkage in the proximal segment of 5q31.1-qter chromosomal region for both qualitative and quantitative traits.

A qualitative-quantitative joint analysis has been postulated to increase the evidence for linkage, especially in case of multifactorial diseases,\(^15\) and it has been widely implemented in studies aiming to dissect genetic predisposition to atopic complex disorders.\(^16,17\)

Consistent linkage signal obtained in 2-point and multipoint analysis narrowed down searches for candidate genes to the chromosomal segment assigned by D5S1480 and D5S500. Among several candidates for further positional analyses, we selected the ADRB2, the gene located in close proximity to the marker of the highest linkage. The priority was given to this candidate also in light of the well-documented role of the ADRB2 in blood pressure regulation and its essential contribution to the development of several cardiovascular and metabolic phenotypes related to hypertension.\(^18\)

To test for the relationships between essential hypertension and the ADRB2, we performed association studies of 3

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**TABLE 2.** Results of 2-Point Linkage of Microsatellite Markers Spanning 5q31.1-5qter Region to Essential Hypertension and Systolic and Diastolic Blood Pressures

<table>
<thead>
<tr>
<th>Marker</th>
<th>HT</th>
<th>SBP</th>
<th>DBP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H-E</td>
<td>SPLINK</td>
<td>IBS</td>
</tr>
<tr>
<td>DSS1480</td>
<td>3.1</td>
<td>0.001</td>
<td>0.03</td>
</tr>
<tr>
<td>DSS636</td>
<td>1.2</td>
<td>0.12</td>
<td>0.58</td>
</tr>
<tr>
<td>DSS280</td>
<td>1.3</td>
<td>0.11</td>
<td>0.40</td>
</tr>
<tr>
<td>DSS2093</td>
<td>1.2</td>
<td>0.12</td>
<td>0.37</td>
</tr>
<tr>
<td>DSS1471</td>
<td>−0.9</td>
<td>0.81</td>
<td>0.57</td>
</tr>
<tr>
<td>DSS1456</td>
<td>0.9</td>
<td>0.17</td>
<td>0.57</td>
</tr>
<tr>
<td>DSS462</td>
<td>1.2</td>
<td>0.11</td>
<td>0.39</td>
</tr>
<tr>
<td>DSS211</td>
<td>1.3</td>
<td>0.09</td>
<td>0.14</td>
</tr>
</tbody>
</table>

HT indicates essential hypertension; H-E, Haseman-Elston regression analysis; \( t \), Haseman-Elston \( t \) statistics value; \( \chi^2 \), IBS \( \chi^2 \) statistics.

**TABLE 3.** Arg16Gly, Gln27Glu, and Thr164Ile Polymorphisms in TDT Test-Transmissions of Alleles From Heterozygous Parents to Offspring With Essential Hypertension

<table>
<thead>
<tr>
<th>SNP</th>
<th>Allele</th>
<th>Transmissions n (%)</th>
<th>TDT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg16Gly</td>
<td>Arg</td>
<td>54 (54.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gly</td>
<td>45 (45.5)</td>
<td>0.8</td>
</tr>
<tr>
<td>Gln27Glu</td>
<td>Gln</td>
<td>62 (49.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glu</td>
<td>64 (50.8)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Thr164Ile</td>
<td>Thr</td>
<td>4 (80)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ile</td>
<td>1 (20)</td>
<td>1.8</td>
</tr>
</tbody>
</table>

The numbers of informative parents were 99, 126, and 5 for Arg16Gly, Gln27Glu, and Thr164Ile polymorphisms, respectively.
Perspectives

Our study implicates a short 7-cM segment on the long arm of the chromosome 5 as harboring a gene or genes for human essential hypertension. Furthermore, detailed haplotype analysis of three functional SNPs excluded ADRB2 as a causative gene. Further studies will focus on the remaining positional candidate genes, thus bringing closer the dissection of complex cardiovascular traits.

Acknowledgments

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References


TABLE 4. Haplotype TDT Test for 3 Functional SNPs of the ADRB2

<table>
<thead>
<tr>
<th>H</th>
<th>Allele</th>
<th>Allele</th>
<th>Allele</th>
<th>Estimated Frequency</th>
<th>Observed Transmissions</th>
<th>Expected Transmissions</th>
<th>( \chi^2 )</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>Gly</td>
<td>Glu</td>
<td>Thr</td>
<td>42.2%</td>
<td>217</td>
<td>211</td>
<td>0.52</td>
<td>0.47</td>
</tr>
<tr>
<td>D</td>
<td>Arg</td>
<td>Gln</td>
<td>Thr</td>
<td>34.8%</td>
<td>178</td>
<td>174</td>
<td>0.59</td>
<td>0.44</td>
</tr>
<tr>
<td>F</td>
<td>Gly</td>
<td>Gln</td>
<td>Thr</td>
<td>20.4%</td>
<td>95</td>
<td>103</td>
<td>2.03</td>
<td>0.15</td>
</tr>
</tbody>
</table>

H indicates haplotype; and B, D, and F are the symbols corresponding to the most frequent haplotypes.


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