CYP11B2 gene haplotypes independently and in concurrence with aldosterone and aldosterone to renin ratio increase the risk of hypertension

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Abstract

Objectives: Aldosterone synthase produces aldosterone, which regulates electrolytes and thereby blood pressure. Polymorphisms in aldosterone-synthase gene (CYP11B2) may associate with heterogeneous aldosterone production and hypertension. Hence, we investigated −344T/C, Iw/Ic polymorphisms of CYP11B2, plasma renin activity (PRA) and aldosterone concentration (PAC).

Design and methods: Consecutive ethnically-matched 450 hypertensive patients and 360 controls were screened by PCR-RFLP for genotypes and haplotypes; PRA and PAC were measured.

Results: The Iw/Ic polymorphism distribution differed significantly between the two groups (LRT χ² =15.8, df=2, P=0.000). The mutant allele-Ic and genotype-Ic/Ic were overrepresented in patients (35% versus 27% and 13% versus 7%). Overrepresentation of T-Ic haplotype in patients was identified as risk haplotype (P=0.000). Patients had significantly higher PAC and aldosterone-to-renin ratio (ARR; P=0.000), which was Ic-allele dependent.

Conclusions: The haplotype T-Ic associated with hypertension susceptibility. Correlation between Ic-allele and raised ARR likely serve in hypertension management.

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Keywords: CYP11B2 gene; Hypertension; Polymorphisms; Aldosterone levels

Introduction

Aldosterone, the principal human corticosteroid, mainly expressed in the zona glomerulosa of the adrenal gland, plays a vital role in body fluid and electrolyte regulation [1]. Aldosterone synthase (CYP11B2) is the key rate-limiting enzyme in aldosterone synthesis [2]. The aldosterone synthase gene (CYP11B2) polymorphisms have been associated with altered aldosterone levels and hypertension [3–5]. Among the several, the −344T/C polymorphism was shown to influence aldosterone secretion and putative binding site for the transcriptional regulatory factor (SF-1), though its physiological significance and association with hypertension remain controversial [6–11]. An association has been reported among intron 2 conversion (Iw/Ic) polymorphism, hypertension and an elevated aldosterone to renin ratio (ARR), and also between the T-Ic haplotype and elevated ARR [12]. These two polymorphisms are known to be in linkage disequilibrium [13,14]. It is believed that inappropriate aldosterone activity and raised ARR in hypertension are likely related to CYP11B2 polymorphisms [15,16].

Because of its significance, the genetic variability in this gene has generated lot of interest among various investigators. As a consequence, various ethnic groups have been investigated for relevance of this gene to sodium homeostasis.
and thereby blood pressure [14–20]. Investigation of yet another population with different ethnicity than the previously studied populations may add to the existing knowledge and support the worldwide efforts in identifying markers of global relevance.

In the present study, therefore, a well-characterized cohort with case-control design was investigated for the distribution of genotypes and haplotypes of the −344T/C and Iw/Ic polymorphisms; further, the aldosterone, renin levels were measured and ARR was determined, and correlation analyses were performed.

Material and methods

Study subjects and clinical evaluation

This study was approved by institutional ethics committee; informed consent was obtained from each subject. Out of total 2400 North Indian ethnically matched participants only 810 subjects were included due to various restrictive factors. The selection criteria, BP measurements, clinical and biochemical characteristics of the study subjects are described in detail in our previous report [21]; briefly, the subjects underwent physical and clinical tests to avoid any interference in the selection criteria. A detailed questionnaire regarding health, family history, life style, and migration status was fulfilled individually. The recruitment criteria of controls included: aged ≥25 years, systolic blood pressure (SBP) <140 mm Hg and diastolic blood pressure (DBP) <90 mm Hg, absence of antihypertensive medication and any other disease; whereas the recruitment of the patients was based on; aged ≥25–60 years and newly diagnosed, SBP ≥140 mm Hg and DBP ≥90 mm Hg (JNC VII). The point at which the first of two or more Korotkoff sounds are heard is defined as SBP and the disappearance of Korotkoff sound as DBP. Three measurements of BP, in supine position, using a calibrated mercury sphygmomanometer with appropriate adult cuff size were recorded by two trained observers with at least 10 min interval. Blood samples were collected after overnight fasting. Peripheral blood leukocytes were used for DNA extraction and plasma for biochemical analyses. The samples were preserved at appropriate subzero temperatures.

Hormonal assays

Patients were rested for at least 30 min prior to drawing blood in supine position. Plasma renin activity (PRA, ng/mL/h) and aldosterone concentration (PAC, pmol/L) were measured in duplicate by means of radioimmunoassay (M/s Immunotech, France) on a gamma counter (Ria Calc WIZARD 1470, USA). Aldosterone to Renin ratio (ARR) as an index of inappropriate aldosterone activity was derived by dividing PAC with PRA. The results were analyzed through a logit–log curve fit. Total cholesterol, triglycerides, glucose and uric acid were estimated on an Autoanalyzer (Elecsys 2010, Roche, Germany). The intra- and interassay coefficients of variation were <10% for all the measurements.

Genotyping and haplotyping

Genomic DNA was isolated from peripheral blood leukocytes by using a standard protocol [22]. The −344T/C, Iw/Ic polymorphisms were screened by modification of standard PCR-RFLP [23,24]. The reaction mixture, 20 μL, contained 50 ng of DNA, 10 pmol of each primer, 1× buffer, 0.33 U of Taq DNA polymerase and 0.2 mmol/L of dNTPs. For detection of alleles at −344T/C locus, 10 μL of the PCR product (537 bp) was digested with 2.5 U of Hae III for 4 h at 37 °C. The −344T allele was detected on 2% agarose gel as fragment of 273 bp, and −344C allele as fragment of 202 bp. The no-conversion allele (Iw) was detected on 1.5% agarose gel as fragment of 406 bp and the intron conversion allele (Ic) as fragment of 420 bp. An independent observer confirmed all the genotypes; discrepancies, if any, were resolved by repeated PCR-RFLP. We also looked for tagging efficiency of the selected SNPs taking SNP data from the HapMap study. Haplotypes were scored from the two polymorphisms and associations were identified.

Statistical analysis

SPSS 12.0 (SPSS Inc., Chicago, Illinois, USA) and EPIINFO version 6 softwares were used for statistical analysis. Allele frequencies were calculated by allele counting. Multiple logistic regression model and a backward elimination procedure were used to investigate whether genotypes of the −344T/C and Iw/Ic polymorphisms were independent predictors for the probability of having hypertension, while adjusting for age, gender and BMI. Baseline characteristics and demographic features were compared with the unpaired t-test for continuous data and the χ² test for categorical data. The measure of Linkage disequilibrium (LD) was calculated using Haploview version 3.2 software. The frequency of the haplotypes was estimated using the expectation maximization (EM) algorithm estimates [25], and compared by simple contingency 2×2 table between hypertensives and controls. For determining the haplotypes and permutation analysis, SNPAlalyze ver. 6.0 was used (DYNACOM Co., Ltd., Yokohama, Japan). P values for pairwise differences were corrected for multiple comparisons by Bonferroni correction test. A P value of ≤0.05 was considered statistically significant.

Results

Clinical characteristics

The demographic and clinical characteristics of study population were determined (Supplementary Table 1). SBP, DBP and pulse rate differed significantly (P<0.0001); whereas, BMI (Body Mass Index; BMI is calculated as weight in kilograms divided by the square of height in meters) and lipid profile did not differ significantly.

Genotype and haplotype analyses

The overall percentage of successful genotyping in the studied population was at least 99.2%. The tagging efficiency...
for the −344T/C and K173R polymorphisms were looked using HapMap data. Both the SNPs were self-tagged. The −344T/C polymorphism was in complete LD with the K173R polymorphism \( (D' = 1.0, P = 0.000) \), therefore the latter was not analyzed further. The genotype frequency of both, −344T/C and Iw/Ic, polymorphisms were consistent with Hardy–Weinberg Equilibrium \( (P > 0.05) \). The genotype distribution is presented in Table 1. The Iw/Ic polymorphism differed significantly between the two groups \( (\chi^2 = 15.8, df = 2, P = 0.000) \). The Ic/Ic genotype was over-represented in patients as compared to controls (13% versus 7%), as a consequence, the Ic allele was more abundant in patients (35% versus 27%). The logistic regression analysis revealed that the risk of hypertension for the homozygote Ic/Ic and heterozygote Iw/Ic was significantly higher when compared with the most common genotype, Iw/Iw \( (OR = 2.5, 95\% CI = 1.5–4.1, OR = 1.7, 95\% CI = 1.1–1.9, \text{ respectively}) \); moreover the risk for the two genotypes remained significantly higher even after adjustment for age, gender, and BMI \( (P = 0.000, P = 0.008, \text { respectively}) \). Further, multiple logistic regression analysis revealed that the risk of hypertension increased significantly with Ic allele \( (OR = 1.5, 95\% CI = 1.2–1.9, P = 0.000) \). This polymorphism was found to be independent predictor of hypertension. When the genotypes of the Iw/Ic polymorphism were analyzed as recessive, dominant and additive models, the significance and the risks were conserved \( (P = 0.004, P = 0.001, P = 0.000, \text { respectively}) \). The distribution of the genotypes and alleles for the −344T/C polymorphism did not reach significance between the two groups.

The −344T/C and Iw/Ic polymorphisms were in complete LD in both the groups as well as the overall sample \( (\text{Table } 2) \). The maximum likelihood procedure revealed significant effects for global haplotypes after permutation test \( (P = 0.01) \). The controls were significantly prevalent with haplotype T-Iw \( (P = 0.02) \), whereas, the patients were over-represented with haplotype T-Ic \( (P = 0.000) \).

**Association analyses**

We investigated whether the Iw/Ic polymorphism influenced BP levels. Univariate analysis revealed that the Iw/Ic genotype had a significant influence on DBP \( (F \text{ ratio} = 4.1, P = 0.014) \) but not on SBP \( (F \text{ ratio} = 0.52, P = 0.59) \). As shown in Table 3, after excluding the extreme outliers \( (n = 40) \) for normalization, Shapiro–Wilk W test revealed normal distribution for the PRA and PAC. The patients showed significantly higher PAC than the controls \( (245 ± 78 \text{ pmol/L versus } 69.4 ± 22 \text{ pmol/L, respectively; } P = 0.000) \), whereas PRA was lower in patients than controls \( (0.75 ± 0.22 \text{ ng A-I/mL/h versus } 1.2 ± 0.37 \text{ ng A-I/mL/h; } P = 0.000) \). Further, PAC and ARR were stratified and compared on the basis of the Iw/Iw and Iw/Ic + Ic/Ic genotypes in the two groups. PAC and ARR were significantly higher in patients for the Iw/Ic + Ic/Ic genotypes compared to the Iw/Iw genotype; the PAC was 300±80 versus 190±49 pmol/L and ARR was 400 versus

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**Table 1**

Genotype and allele distributions of the CYP11B2 gene polymorphisms in patients and controls.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients</th>
<th>Controls</th>
<th>Crude OR (CI %95)</th>
<th>Adjusted OR (CI %95)</th>
<th>( \chi^2 )</th>
<th>( P^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>−344T/C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>178 (39%)</td>
<td>131 (36%)</td>
<td>1.0</td>
<td>1.0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TC</td>
<td>202 (45%)</td>
<td>169 (47%)</td>
<td>1.1 (0.8–1.5)</td>
<td>1.0 (1.0–1.1)</td>
<td>2.1</td>
<td>0.14</td>
</tr>
<tr>
<td>CC</td>
<td>70 (16%)</td>
<td>60 (16.6%)</td>
<td>0.8 (0.56–1.3)</td>
<td>0.6 (0.4–1.0)</td>
<td>3.12</td>
<td>0.07</td>
</tr>
<tr>
<td>T</td>
<td>558 (62%)</td>
<td>431 (60%)</td>
<td>0.9 (0.7–1.1)</td>
<td>–</td>
<td>0.77</td>
<td>0.38</td>
</tr>
<tr>
<td>C</td>
<td>342 (38%)</td>
<td>289 (40%)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Recessive Model</td>
<td>0.9 (0.62–1.3)</td>
<td>1.0 (0.8–1.7)</td>
<td>0.47</td>
<td>0.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dominant Model</td>
<td>0.9 (0.6–1.2)</td>
<td>0.7 (0.4–1.3)</td>
<td>0.62</td>
<td>0.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additive Model</td>
<td>0.8 (0.5–1.1)</td>
<td>0.7 (0.5–1.1)</td>
<td>2.1</td>
<td>0.31</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| **Iw/Ic** | | | | | | |
| Iw/Iw     | 188 (42%) | 193 (53%) | 1.0 | 1.0 | – | – |
| Iw/Ic     | 202 (45 %) | 143 (40%) | 1.7 (1.1–1.9) | 1.5 (1.0–1.4) | 7.13 | 0.008 |
| Ic/Ic     | 60 (13%)  | 24 (7%)  | 2.5 (1.5–4.1) | 2.7 (1.6–4.9) | 14.2 | 0.000 |
| Iw        | 578 (65%) | 529 (73%) | 1.5 (1.2–1.9) | – | 15.8 | 0.000 |
| Ic        | 322 (35%) | 191 (27%) | – | – | – | – |
| Recessive Model | 2.7 (1.6–4.5) | 2.1 (1.3–3.6) | 8.42 | 0.004 |
| Dominant Model | 1.8 (1.3–2.5) | 1.6 (1.2–2.1) | 11.2 | 0.001 |
| Additive Model | 2.4 (1.7–3.4) | 2.2 (1.6–2.9) | 28.3 | 0.000 |

\( n, \) number of subjects; \( \text{Values are } n (%); \) OR, odd ratio; CI, confidence interval.

\( ^* \) \( P \) values were corrected using multiple comparison Bonferroni correction.

\( ^\dagger \) ORs are estimated from multiple logistic regression adjusted for the confounding factors including age, gender and BMI \((\text{Body Mass Index}, \text{BMI, is calculated as weight in kilograms divided by the square of height in meters}).\) Recessive model compares a combination of heterozygous and the homozygous for the most frequent allele to the variant allele homozygous genotype. Dominant model compares a combination of heterozygous and homozygous for the least frequent allele to the homozygous for the most frequent allele. Additive model compares a combination of the two genotypes with weight 2 and 1 respectively to the homozygous for the most frequent allele.
Table 2
Distribution of two-polymorphism haplotypes of the CYP11B2 gene in patients and controls.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Overall</th>
<th>Patients</th>
<th>Controls</th>
<th>( \chi^2 ) *</th>
<th>( P^\dagger )</th>
<th>Permutation^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iw/Ic</td>
<td>0.34</td>
<td>0.33</td>
<td>0.36</td>
<td>1.68</td>
<td>0.19</td>
<td>0.22</td>
</tr>
<tr>
<td>Iw/Ic</td>
<td>0.34</td>
<td>0.31</td>
<td>0.37</td>
<td>6.21</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>Ic/Ic</td>
<td>0.27</td>
<td>0.31</td>
<td>0.23</td>
<td>14.6</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>C/Ic</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.18</td>
<td>0.67</td>
<td>0.72</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td></td>
<td></td>
<td>16.0</td>
<td>0.001</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Iw, intron conversion polymorphism. \(^*\chi^2\) and \(^\dagger P\) were calculated using 2 by 2 contingency table comparing each haplotype with all the haplotypes combined together between patients and controls.

\(^2P\) values are based on 1000 permutations. LD, linkage disequilibrium.

253.3 \((P=0.000, \text{each})\) and a similar trend was present even in controls.

Discussion

The well-defined physiologic function of aldosterone is to enhance sodium reabsorption in the kidney and at other secretory epithelial sites at the expense of potassium and hydrogen ions [26]. It has been suggested that variations at the CYP11B2 gene influence aldosterone secretion thereby associating with essential hypertension [26–28]. Our investigation of two common polymorphisms, −344T/C and Iw/Ic, provided interesting insights. The prevalence of the Ic/Ic genotype and Ic allele in the patients suggested of an association with hypertension. Literature presents conflicting reports on association of these two polymorphisms with hypertension [8,13,15]. We further looked for the contribution of these polymorphisms as haplotypes as it accounts for greater power and are more informative. The prevalence of T-Ic haplotype in hypertensive patients likely serves as risk-predisposing haplotype, whereas T-Iw haplotype being more prevalent in controls serves as protective. A report on ethnicities also yielded similar results such that the common haplotypes generated were T-Ic (38%), T-Iw (16%) and C-Iw (45%) [25]. Sub-haplotype analysis strengthened the notion that in our study population the Iw/Ic polymorphism could be the risk determinant.

Table 3
Stratification of PAC on the basis of Iw/Ic CYP11B2 gene polymorphism in patients and controls.

<table>
<thead>
<tr>
<th>Iw/Ic</th>
<th>Patients (n=303)</th>
<th>Controls (n=205)</th>
<th>( P^\dagger )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PAC ARR</td>
<td>PAC ARR</td>
<td></td>
</tr>
<tr>
<td>IwIw</td>
<td>190±49</td>
<td>253.3</td>
<td>0.000*</td>
</tr>
<tr>
<td>IwIc+Ic</td>
<td>300±80</td>
<td>400</td>
<td>71.5±23</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD. \(^*P\) values were calculated by one-way ANOVA.

If genetic variations in CYP11B2 gene contribute to hypertension, this will be more obvious in individuals with relative heterogeneous aldosterone levels. To test this, we measured PRA and PAC and determined the ARR which may reflect a relative excess of aldosterone secretion. Higher PAC and ARR in our patients, possibly, suggested of low renin hypertension. It is reported that on an average, one third of the hypertensive population has low renin and higher aldosterone and/or ARR [29]. We found an excess of Ic allele (Iw/Ic+Ic/Ic) in patients with raised PAC/ARR and diastolic hypertension phenotype. Our findings are in agreement with a previous report wherein a significant excess of the Ic allele associated with a raised ARR in hypertension [12]. These findings support the contention that genetic variations in CYP11B2 gene contribute to an intermediate phenotype with differential aldosterone concentration and ARR thereby contributing to hypertension.

It may not also be ruled out that the Iw/Ic polymorphism is in close LD with functional sites in the gene or a quantitative trait locus elsewhere in the regulatory region, thus associating with increased transcription factor availability, which in turn results in altered expression of the gene. For example, it is known that these two polymorphisms are associated with raised levels of the 11-deoxycortisone, 11-deoxycorticosterone (DOC), and 11-deoxy cortisol. These steroids are converted to corticosterone and cortisol by means of 11β-hydroxylase encoded by the gene 11β-hydroxylase (CYP11B1), which is highly homologous to the present gene under investigation i.e. CYP11B2; the two genes are located adjacent to each other [30] and the impaired activity of CYP11B1 was consistently associated with essential hypertension [31–33]. This strengthens the perception that the Iw/Ic CYP11B2 polymorphism is likely in close LD with functional quantitative trait loci in CYP11B1 that influence the gene expression. In contrast, the −344T/C polymorphism in our study did not associate with intermediate phenotypes and essential hypertension. The −344T/C variant as a binding site has no known function and hence directly may not implicate in transcriptional activity in vivo but likely serves as a marker for as-yet-undefined polymorphism.

Although, we did not carry out further functional tests to verify these significant associations, the results underlined the hypothesis that genetic variability at the CYP11B2 gene could
be a characteristic of patients with essential hypertension. We believe that performing further family-based studies with sufficient information on dietary electrolyte intake and aldosterone excretion rate in different ethnicities will strengthen the findings. Furthermore, linkage of the Iw/Ic polymorphism with other functional sites within the CYP11B2 gene or relevant variants with a quantitative trait in the adjacent CYP11B1 gene or elsewhere could elucidate the present findings.

We do visualize few limitations. The precise molecular mechanisms or environmental factors that intervene the unfavorable effects of the studied variants and haplotypes remain to be explored. The incomplete gene coverage possibly cannot present the whole gene function. Further, hormonal and physiological assessments with regulated salt intake are required to find out the physiological effects of this genetic variability. In addition, we did not measure urine sodium and potassium excretion over a 24-hour period, as these factors are crucial environmental factors that influence aldosterone levels. Variability. In addition, we did not measure urine sodium and potassium excretion over a 24-hour period, as these factors are crucial environmental factors that influence aldosterone levels.

In conclusion, the Iw/Ic polymorphism of CYP11B2 associated with essential hypertension. Although, the −344T/C polymorphism was not associated with hypertension, the haplotype T-Ic was more frequent in hypertensive patients. The correlation between the over-represented Ic allele and raised PAC/ARR in patients signifies the role of the Iw/Ic CYP11B2 polymorphism and also the potential of the interaction in hypertension development.

**Conflict of interest statement**

None declared.

**Acknowledgments**

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clinbiochem.2009.09.015.

**References**