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Abstract—Primary aldosteronism is characterized by autonomous production of aldosterone and arterial hypertension, and it occurs in 2 principal forms: aldosterone-producing adenoma (APA) and idiopathic hyperaldosteronism (IHA). APA can be cured through removal of the adenoma, whereas IHA leads to hypertension that must be treated with medication. The origin of the autonomous aldosterone production in IHA is poorly understood, but genetic factors may contribute to its cause. To test the hypothesis that variants of the aldosterone synthase gene may contribute to susceptibility to IHA, we compared genotypes at 3 polymorphic sites in the *CYP11B2* gene in patients with IHA (n=90) with those found in patients with APA (n=38), in patients with essential hypertension (n=72), and in normotensive individuals (n=102). We observed significant linkage disequilibrium among the 3 polymorphisms with 2 frequent haplotypes in all groups studied. One haplotype (C2R) was found to be increased in frequency in the IHA group (47%) compared with the other groups, which had a similar haplotype frequency (36%). The 3 polymorphisms studied have been implicated in either essential hypertension or excess aldosterone production in previous studies. Because of the strong linkage disequilibrium, the observed results could be due to the action of any 1 of the 3 alleles or to another allele in linkage disequilibrium with them. Our results suggest that variations in the *CYP11B2* gene may contribute to dysregulation of aldosterone synthesis and lead to susceptibility to IHA. **(*Hypertension*. 2000;35:694-698.)**

Key Words: aldosterone ■ hypertension, essential ■ hyperaldosteronism ■ genetics ■ polymorphism

Primary aldosteronism (PA) occurs in ≈1% to 2% of hypertensive patients, but it is usually considered in the differential diagnosis of hypertension, because some forms are curable with surgical intervention. A solitary adrenocortical aldosterone-producing adenoma (APA) is the most common cause of PA, occurring in ≈60% of cases. Idiopathic hyperaldosteronism (IHA), which can be associated with bilateral micronodular or macronodular adrenal hyperplasia, accounts for ≈40% of cases and is sometimes considered a variant of essential hypertension (EHT).[1] Very rarely, PA can be caused by the inheritance of a hybrid gene originating from a previous recombination between the *CYP11B1* and *CYP11B2* genes.[2]

The activity of the aldosterone synthase enzyme (CYP11B2) is necessary for normal aldosterone secretion. This enzyme has the steroid 11β-hydroxylase, 18-hydroxylase, and 18-oxidase activities that are required to catalyze the synthesis of aldosterone from 11-deoxycorticosterone.[3,4] The expression of CYP11B2 is normally limited to the zona glomerulosa of the human adrenal cortex, the normal site of aldosterone synthesis, where it is controlled principally by serum potassium and angiotensin II concentrations. The enzyme is encoded by the *CYP11B2* gene located on chromosome 8q24.[5,6] We hypothesized that polymorphic variants of the *CYP11B2* gene could contribute to susceptibility to PA and, in particular, to IHA.

In a sample of 128 patients with PA (38 APA and 90 IHA), in 72 patients with EHT, and in 102 normotensive volunteers, we studied 3 polymorphisms in the *CYP11B2* gene that may influence either its expression or the activity of the encoded enzyme. The first polymorphism was a frequent single–base pair substitution (C to T) at position −344 in the promoter of the gene, occurring in a sequence that binds to the steroidogenic factor-1 transcription factor.[7] The second polymorphism was a common gene conversion of *CYP11B2* in which most of intron 2 is replaced by that of the homologous *CYP11B1* gene,[7] and the third was a point mutation (R173K), previously reported to be at increased frequency in patients with low-renin EHT.[8] Genetic analysis of our samples revealed strong linkage disequilibrium between these markers, with 2 frequent haplotypes, of which 1, C2R (−344C, *CYP11B2* intron2 sequence, and 173R), had an increased frequency in the IHA group. No differences in haplotype frequencies were found among the normotensive individuals,

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From the Department of Medicine and Experimental Oncology (P.M., D.S., F.R., L.C., F.V.), Hypertension Unit, University of Torino, Torino, Italy; Department of Medical and Surgical Sciences (F.F., C.P.), Division of Endocrinology, University of Padova, Padova, Italy; and Fondation Jean Dausset CEPH (L.P.), Paris, France.

Correspondence to Dr Paolo Mulatero, University of Torino, Department of Medicine and Experimental Oncology, Hypertension Unit, San Vito Hospital, S San Vito 34, 10133 Torino, Italy. E-mail mulatero@tin.it

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TABLE 1. Clinical and Biochemical Parameters of Population Studied

Parameter	APA	IHA	Low-Renin Essential Hypertension	High-Renin Essential Hypertension	Control Subjects
Sample size	38	90	27	45	102
Age, y	51.1	50.3	49.6	51.2	52.1
Gender, M/F	20/18	47/43	14/13	22/23	51/51
Systolic BP, mm Hg	203.9±24.1	193.9±27.9	165±9.1	168±10	110±12.1
Diastolic BP, mm Hg	115.1±11.1	117.2±16.1	103±6.3	105±4.8	74±8.9
K^+, mmol/L	3.2±0.5	3.7±0.4	4.0±0.4	4.2±0.5	4.2±0.4
Supine PRA, ng · L^{-1} · s^{-1}	0.0361±0.02	0.044±0.03	0.08±0.017	0.57±0.33	0.278±0.22
Upright PRA, ng · L^{-1} · s^{-1}	0.047±0.03	0.078±0.05	...	...	...
Supine aldosterone, pmol/L	1104±352.3	685.2±244.1	239.9±124.8	418.8±169.2	277.4±88.8
Upright aldosterone, pmol/L	1306.5±449.4	1104±424.4	...	...	...

BP indicates blood pressure.

patients with APA, and patients with EHT. The association of the C2R haplotype with IHA is the first genetic polymorphism shown to be at an increased frequency in such patients.

Methods

Study Population

The patients recruited for this study were whites from northern Italy. The study was approved by a local ethics committee, and all subjects gave informed consent. We studied 90 patients with IHA, 38 patients with APA, 72 patients with EHT (of whom 27 were classified as having low-renin hypertension and 45 as having normal- to high-renin hypertension), and 102 normotensive volunteers. Clinical characteristics of the patients are given in Table 1. The criteria used for the differential diagnosis of the different forms of PA and EHT were as previously described.[9–11] Adrenal adenoma was confirmed at surgery and after histological examination. The presence of the dominantly inherited syndrome glucocorticoid remediable aldosteronism was excluded by either a long-polymerase chain reaction (PCR) test or Southern blot analysis, as previously described.[9]

Hormonal Measurements

Plasma aldosterone and plasma renin activity (PRA) were determined with radioimmunoassay kits purchased from Sorin Biomedical Diagnostics. The intra-assay and interassay coefficients of variation for aldosterone were 7.9% and 9.6%, respectively, with a normal range of 55.5 to 333 pmol/L supine and 139 to 832 pmol/L upright. The intra-assay and interassay coefficients of variations for PRA were 5.4% and 9.1%, respectively, with a normal range of 0.11 to 0.83 ng · L^{-1} · s^{-1} supine and 0.42 to 1.67 ng · L^{-1} · s^{-1} upright. Patients were left in a recumbent position for 1 hour before supine samples were collected and for 2 hours in an upright position before upright samples were collected.

PCR Amplification and Genotyping of *CYP11B2* Fragments

Genomic DNA was prepared from peripheral blood leukocytes with microspin columns (QIAamp Blood Kit; Qiagen). Analysis of the R173K substitution and the intron 2 gene conversion was performed by first amplifying this region with PCR with gene-specific primers 1S and 1A (Table 2). The 50-μL reaction was subjected to 35 cycles at 94°C for 1 minute, 65°C for 1 minute, and 72°C for 2 minutes with an additional 5 seconds each cycle, followed by a final extension at 72°C for 7 minutes. This reaction allows the amplification of a 2.8-kb fragment that includes exons 1 to 3 of *CYP11B2*.

The promoter region containing the −344C/T polymorphism was amplified with the oligonucleotide primers 2S and 2A (Table 2). This PCR was subjected to 35 cycles at 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension at 72°C for 10 minutes, producing a 160-bp fragment from the *CYP11B2* promoter region. The amplified DNA samples were subjected to electrophoresis in a 1% agarose gel and transferred to nylon membranes (GENE Screen Plus; DuPont-New England Nuclear) that were subsequently hybridized with oligonucleotides that had been radioactively labeled with γ-^{32}P-ATP and T4 polynucleotide kinase. The membranes were incubated with the appropriate radiolabeled allele-specific oligonucleotides (Table 2) in a solution containing 0.9 mol/L NaCl, 0.09 mol/L Na citrate, 1% SDS, and 1× Denhardt's solution (1% Ficoll, 1% polyvinyl pyrrolidone, and 1% BSA fraction V) for 4 hours at 42°C. The final stringent washes before autoradiography were conducted in 0.9 mol/L NaCl, 0.09 mol/L Na citrate, and 0.5% SDS at 56°C, 63°C, and 56°C for R173K, the intron 2 conversion, and −344C/T polymorphisms, respectively.

Statistical Analysis

Haplotype frequencies were estimated according to the method of maximum likelihood with the program EH,[12] with assumption of independence of the markers and separate allowance for linkage disequilibrium. Haplotype frequencies were estimated in each group individually and in the total data, and the statistical significance of comparisons was assessed by comparing twice the difference in

TABLE 2. List of Oligonucleotides Used for PCR Amplification and Allele-Specific Hybridization Experiments

Oligonucleotide	Sequence	Position	Use
1A	TTCCAGAGCAGGTTCCTGGG	Promoter	PCR
1S	TGGGGCTGGACCTTCCCGCAT	Intron 3	PCR
2A	CAGGGGGTACGTGGACATTT	Promoter	PCR
2S	CAGGGCTGAGAGGAGTAAAA	Promoter	PCR
3R	GGCCCTGAGGAAGAAGG	Exon 3	Hybridization
3K	GGCCCTGAAGAAGAAGG	Exon 3	Hybridization
4B1	GCAGAAAATCCCTCCCCCCTA	Intron 2	Hybridization
4B2	TTCTTGGAGAAAAGCCCTAC	Intron 2	Hybridization
5C	TCCAAGGCCCCCTCTCA	Promoter	Hybridization
5T	TCCAAGGCTCCCTCTCA	Promoter	Hybridization

All sequences are written 5′ to 3′, and polymorphic bases in the oligonucleotides are underlined.

TABLE 3. Frequencies of Most Common Genotypes in Patients With IHA and in Control Subjects

Geno-type	Control Subjects					Patients With EHT	
	IHA	APA	EHT	Normotensive	All	Low Renin	Normal/High Renin
TT11KK	0.14	0.29	0.25	0.28	0.27	0.22	0.27
TC12KR	0.40	0.31	0.37	0.46	0.41	0.41	0.36
CC22RR	0.21	0.18	0.15	0.11	0.14	0.15	0.16
TC12KR	0.10	0.05	0.04	0.04	0.04	0.00	0.00
TT12KK	0.06	0.15	0.12	0.08	0.11	0.04	0.18

EHT indicates essential hypertension; T, −344T; C, −334C; 1, CYP11B1 intron 2 sequence; 2, CYP11B2 intron 2 sequence; R, R173; K, K173; and low renin hypertension, hypertension with a PRA of <0.0833 ng · L^{-1} · s^{-1}.

logarithm of the likelihood (−2Ln L) with the χ^2 distribution with appropriate degrees of freedom.

Results

The numbers of control individuals and patients in each group, classified by genotype, are shown in Table 3. The estimated haplotype and allele frequencies in each of the groups are shown in Table 4. For the 3 polymorphic loci, 8 haplotypes are possible. However, the maximum likelihood estimation procedure suggests that there are 2 major haplotypes (C2R and T1K), 1 less frequent haplotype (T2K), and several rare haplotypes occurring in each of the groups (Table 4). Linkage disequilibrium was highly significant in the overall data ($\chi^2_4=861.4$, $P<0.001$) and in each subgroup ($\chi^2_4=232.6$, $P<0.001$, $\chi^2_4=125.8$, $P<0.001$, $\chi^2_4=201.0$, $P<0.001$, and $\chi^2_4=305.8$, $P<0.001$ for the IHA, APA, EHT, and control groups, respectively), as is expected for such tightly linked polymorphisms. The occurrence of the same haplotypes in each group is consistent with the fact that they were all drawn from the same population. No deviations of genotype frequencies from H-W equilibrium were observed for the alleles tested individually in each group.

There is significant heterogeneity between the haplotype frequencies in the IHA and control groups ($_{het}\chi^2_7=19.06$, $P<0.01$) but none between any of the other patient groups and the controls. The haplotype frequencies in these latter groups are remarkably uniform, whereas those in the IHA group are clearly different from them (Table 4). In particular, the frequency of the C2R haplotype is higher in the IHA group (47%) than in the other groups (36%), in large part at the expense of the T1K haplotype, which is decreased (38% versus 53%). A separate analysis of the intron 2 genotypes alone, which are not in complete linkage disequilibrium with the other 2 markers, revealed essentially identical results.

Because of the significant linkage disequilibrium in the region, the allele frequencies at each of the polymorphic sites are highly correlated, and similar differences in allele frequencies are seen for each marker (Table 4). We also reexamined our EHT group, classifying them into 27 patients characterized by low PRA (<0.0833 ng · L^{-1} · s^{-1}) and 45 patients with normal to high PRA. The haplotype and allele frequencies in the normal- to high-renin group were essentially identical to the control and APA values. The values for the low-renin group were intermediate between those of the controls and the IHA group (Table 4), although the difference from the control values was not statistically significant. The intron 2 polymorphism is not in complete linkage disequilibrium with the other polymorphisms.

TABLE 4. Haplotype and Allele Frequencies Observed in IHA and Control Samples at 3 Polymorphic Sites of *CYP11B2* Gene

Haplotype	Control Subjects				Patients with EHT		
	IHA	APA	EHT	Normotensive	All	Low Renin	Normal/High Renin
C1 K	0.01	0.00	0.01	0.00	0.00	0.04	0.00
T1 K	0.38	0.53	0.53	0.55	0.54	0.48	0.56
C2 K	0.00	0.00	0.00	0.00	0.00	0.00	0.00
T2 K	0.13	0.11	0.08	0.09	0.09	0.07	0.09
C1 R	0.00	0.00	0.00	0.00	0.00	0.00	0.00
T1 R	0.01	0.00	0.00	0.00	0.00	0.00	0.00
C2 R	0.47	0.37	0.36	0.36	0.36	0.41	0.33
T2 R	0.00	0.00	0.01	0.00	0.00	0.00	0.02
N	90	38	72	102	212	27	45
Allele	Allele Frequencies						
R	0.48	0.37	0.37	0.36	0.37	0.41	0.36
2	0.60	0.47	0.46	0.45	0.46	0.48	0.44
C	0.48	0.37	0.38	0.36	0.37	0.44	0.33

See Table 3 for abbreviations.

Discussion

PA is a frequent cause of endocrine hypertension. In some families, this disease is inherited as a dominant trait associated or not with the presence of a hybrid gene originating from a recombination between the *CYP11B1* and *CYP11B2* genes (familial hyperaldosteronism type I and II).[2,13] Although PA is generally believed to occur sporadically in most cases, a familial incidence of this disease may be masked by a low penetrance of susceptibility genes, leading to underestimation of its prevalence. This tendency would be aggravated if the genetic contribution to susceptibility only applied to a proportion of cases of PA, such as those caused by IHA. We recently showed that patients with IHA frequently have a family history of hypertension, whereas in patients with APAs, the familial history of hypertension is less evident.[9] Consequently, we decided to investigate genetic polymorphisms in genes responsible for aldosterone secretion in patients with IHA.

Aldosterone secretion is influenced by many factors, but all of these factors must eventually act through the aldosterone synthase enzyme, the only enzyme capable of synthesis of aldosterone, or through limitation of its substrates. Genetic polymorphisms in the aldosterone synthase gene that could render it insensitive to the normal transcriptional control or directly affect its activity are natural candidates to contribute to IHA. In contrast, APAs seem more likely to be caused by mutations in the genes that control growth and proliferation of adrenal cortical cells.

We studied the association of 3 different polymorphisms of *CYP11B2* with IHA, using as controls patients with APA or EHT and normotensive volunteers. Alleles of the −344T/C polymorphism were previously found to be associated with EHT[5] or with aldosterone levels[14] in French populations. This polymorphism is located in a site capable of binding to steroidogenic factor-1 transcription factor.[7] The R173K polymorphism has been associated with low-renin EHT in a Chilean population[8] but apparently does not influence the rate of production of aldosterone from the precursor deoxycorticosterone by CYP11B2 in vitro.[15] The third polymorphism is a common gene conversion in intron 2 of *CYP11B2*, in which most of the intron is replaced by that of *CYP11B1*.[7] It has been suggested that this intron may contain regulatory elements of the *CYP11B* genes.[16]

The 3 polymorphisms were in strong linkage disequilibrium, which is consistent with previous studies that found disequilibrium in this region in other populations.[7,17–19] The C2R haplotype was increased in frequency in the patients with IHA whom we studied. Due to the strong linkage disequilibrium in the region, it is not possible to determine whether this difference is due to 1 of the 3 markers studied or to another polymorphism that controls aldosterone synthesis that is in linkage disequilibrium with the 3 that were studied. Many genes could theoretically be involved, together with the *CYP11B2* gene, in the genetic predisposition to IHA, such as those involved in stimulation (angiotensin II, serotonin, endothelin, and so on) or inhibition (dopamine, adrenomedullin) of aldosterone synthesis. Mutations or regulatory polymorphisms in these genes could cause the altered aldosterone secretion seen in patients with IHA. For example, a recent study demonstrated the overexpression of *CYP11B2* mRNA in the lymphocytes of patients with IHA, suggesting an increase in stimulatory factors or variants in the promoter region causing the overproduction of aldosterone in this disease.[20] The present study provides the first demonstration of an association between a specific haplotype of a candidate gene and the risk of the development of IHA and indicates that *CYP11B2* gene polymorphisms may contribute to its pathogenesis.

In contrast, there was no association among the 3 *CYP11B2* polymorphisms that were studied and the occurrence of APAs in our study population. This observation suggests that the genes involved in adrenal tumorigenesis or hyperplasia are more important in the pathogenesis of PA due to APA than the genes involved in steroid biosynthesis. The mechanisms of adrenocortical tumorigenesis are still poorly understood.[21] The fact that the majority of these tumors are monoclonal in origin[22,23] suggests an accumulation of specific genetic aberrations, such as the activation of proto-oncogenes, overexpression of growth-promoting factors,[24] or inactivation of tumor suppressor genes,[25–28] leading to abnormal cell proliferation.[29] In this case, the excess aldosterone production would be secondary to proliferation of aldosterone-producing cells rather than dysregulation of its synthesis.

There also was no association between the *CYP11B2* polymorphisms and EHT in our study, although the number of patients studied was small. When the patients were reclassified into groups with either low PRA or normal to high PRA, we found that haplotype frequencies in the low-renin group were intermediate between the control values and those observed in the patients with IHA, although the difference in frequency was not statistically significant. It is possible that a subset of the low-renin hypertension group carrying the C2R haplotype may be susceptible to the development of IHA. Previous studies have found an association between the −344T allele and EHT in French and Scottish populations[5,18] and between the −344C allele and hypertension in a Japanese population. Furthermore, the R173 allele was associated with low-renin EHT in a Chilean population.[8] The association of different alleles with hypertension in different populations could be due to linkage disequilibrium of the alleles studied with a functional polymorphism that may have a different phase in each of the populations showing association with different alleles.

Associations of specific haplotypes with a disease may also arise from unsuspected population subdivision. For example, the presence of a genetically distinct subgroup that is susceptible to IHA could cause spurious associations of any genetic polymorphisms differing between the 2 groups and IHA, regardless of whether the variants had some causative role. However, our patients were all recruited from the same region in northern Italy, and we have no reason to suspect genetic heterogeneity. On the contrary, the uniformity of the haplotype frequencies in the 3 control groups suggests a genetically homogeneous population. In conclusion, we tentatively suggest that variants of the *CYP11B2* gene may be associated with an increased risk of the development of IHA.

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