

Evidence for Involvement of the Type 1 Angiotensin II Receptor Locus in Essential Hypertension

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Abstract—Components of the renin-angiotensin system play an important role in the normal regulation of blood pressure. We carried out a comprehensive genetic linkage study of the genes involved in the renin-angiotensin cascade in Finnish hypertensive twins and their affected siblings. We found no evidence for linkage between essential hypertension and the genes coding for renin, angiotensinogen, angiotensin-converting enzyme, or kallikrein 1 in the 329 hypertensive individuals of 142 families studied. In contrast, two intragenic markers for the type 1 angiotensin II receptor (AT₁) showed some evidence for linkage in the total sample. A closer examination of this gene locus was carried out using subgroups of nonobese sibpairs with early onset of hypertension and uniform geographical origin. These stratifications yielded suggestive evidence for linkage of hypertension to the genetic area containing the AT₁ gene, with a maximal multipoint logarithm of the odds (LOD) score of 2.9. A genetic association study carried out in an independent series of 50 hypertensive cases and 122 normotensive controls showed an increase in the frequency of the A1166→C allele of the AT₁ gene in the hypertensive individuals. In a novel variant of model-free multipoint linkage analysis allowing linkage disequilibrium in the calculations, an LOD score of 5.13 was obtained. Sequence analyses of the entire coding region and 848 bp of promoter region in the DNA sample on 8 index samples did not reveal previously unpublished sequence variations. The data provide evidence that a common genetic variant of the AT₁ gene locus influences the risk of essential hypertension in the Finnish population. (*Hypertension*. 1999;33:844-849.)

Key Words: hypertension, essential ■ receptor, angiotensin II ■ siblings ■ linkage ■ Finnish population

Essential hypertension is considered to be a multifactorial trait resulting from a combination of environmental factors and several predisposing genes whose products likely interact with each other. The possible role of the genes coding for components of the renin-angiotensin cascade in the pathogenesis of hypertension has been studied extensively. Ample evidence exists for the role of the angiotensinogen gene (AGT) in induction of elevated blood pressure (BP),¹⁻³ but studies on other components of this metabolic pathway have resulted in negative or controversial data.⁴⁻¹⁵

Linkage studies of essential hypertension pose several problems, including delayed onset of phenotypic expression, varying penetrance, and lack of unequivocal diagnostic criteria. Because the parameters are impossible to accurately specify, model-free methods such as affected sibpair linkage analyses are often applied, although these will also suffer from lack of power in the case of a very common multifactorial disorder such as essential hypertension. Under these conditions, efforts to identify causative gene loci may be facilitated by accepting only severely affected and relatively

young subjects in genetic linkage or association studies sampled from a genetically homogenous population, such as the Finns.

We conducted the present study to determine whether any of the genes coding for major components of the renin-angiotensin pathway are involved in the etiology of elevated diastolic BP in a population-based sample of middle-aged hypertensive twins and their siblings in the Finnish population. The studied loci include the genes coding for renin, AGT, type 1 angiotensin II receptor (AT₁), angiotensin-converting enzyme (ACE), and kallikrein 1 (KLK1). Our results suggest involvement of the AT₁ locus on chromosome 3 in the pathogenesis of essential hypertension.

Methods

This study was approved by the ethics committee of the National Public Health Institute of Finland. The subjects gave informed consent. All samples were taken according to the Helsinki declaration.

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TABLE 1. Demographic Characteristics of the Study Samples

Sample	n (F/M)	Age, y	Age of Onset, yr	Body Mass Index, kg/m ²
Linkage study sample	329 (209/120)	59.3±8.1	43.3±8.9	27.8±3.9
Association study, cases	50 (32/18)	53.0±6.2	38.6±9.7	24.5±1.8
Association study, controls	122 (72/50)	57.2±4.0	...	26.5±3.2

Linkage Study Subjects

Subjects were ascertained through the older part of the Finnish Twin Cohort, which consists of 9581 dizygotic like-sexed and 4307 monozygotic twin pairs born before 1958.¹⁶ From questionnaire surveys carried out on the entire cohort in 1975, 1981, and 1990, 476 dizygotic pairs and 264 monozygotic pairs potentially concordant for hypertension were identified. A detailed health questionnaire was sent to collect information on each individual's history of hypertensive, cardiovascular, and renal diseases; current health status; and occurrence and characteristics of cardiovascular diseases in first-degree relatives. In families with large sibships, the questionnaire was also sent to the siblings of the twins. Further confirmation and details on the subjects' hypertensive status were obtained through medical records using both the inpatient hospital discharge register and the medication reimbursement register of the Finnish Social Insurance Institute. On the basis of this information, a total of 329 hypertensive individuals (120 men and 209 women) from 142 families were selected for the present study (Table 1). The total number of possible families was 119 sibships with 2 affected, 10 with 3 affected, 7 with 4 affected, 4 with 5 affected, and 2 with 6 affected. In 6 families, the sibship consisted of 1 twin from a monozygotic twin pair concordant for hypertension and 1 or more of their siblings. In 9 families, only 1 twin from a dizygotic pair was included in the affected sibship. All the selected subjects met the criteria of established diagnosis of essential hypertension at an age younger than 60 years, a history of recorded diastolic BP of at least 95 mm Hg, current use of antihypertensive medication, and absence of renal failure. No parents of the hypertensive individuals were available for genotyping.

Association Study Subjects

A case-control study was carried out in an independent study sample of 50 cases and 122 controls. The cases for the study were ascertained through the Finnish Twin Cohort,¹⁶ using the same selection criteria as in the linkage material in subgroup 8 (Table 2). Subjects were accepted as affected only if they were taking antihypertensive medication, had been diagnosed as hypertensive before the age of 50 years, and had a body mass index <27 kg/m² (Table 1). Twenty-five of the subjects represented individuals who had a

hypertensive monozygotic cotwin. The rest of the cases represented dizygotic twins, whose cotwin's affected status was unknown. None of the cases belonged to the families used in the linkage study. Both parents of each case were born in Southwestern Finland. One hundred twenty-two control individuals were ascertained either through the Finnish Twin Cohort (19 individuals representing 19 normotensive monozygotic twin pairs) or from a previous cross-sectional survey on risk factors of coronary heart disease in Finland (103 unrelated individuals).¹⁷ The control subjects reported no history of elevated BP; reported systolic and diastolic BP measurements <146 and 86 mm Hg, respectively; and were at least 55 years of age. Since the incidence of hypertension strongly correlates with age and body mass index, we chose the controls to be older and more obese than the cases. The parents of the controls ascertained through the Twin Cohort were born in Southwestern Finland, whereas the controls ascertained through the cross-sectional survey were born in Southwestern Finland.

Genotyping and Sequencing

The subjects were genotyped using 19 polymorphic markers (Figure 1). See www.ktl.fi/molbio/wwwpub/ht/index.html for genotyping details. Marker order and distances were based on sex-averaged genetic maps from the Genetic Location DataBase.¹⁸ In the case of the AGT and KLK1 loci, the orders of the flanking markers were determined by radiation hybrid mapping using an 8000r RH-map (Research Genetics). A total of 848 bp of the AT₁ promoter¹⁹ and the coding region of the gene²⁰ were sequenced as explained in the information available at www.ktl.fi/molbio/wwwpub/ht/index.html.

Linkage and Association Analyses

To test for linkage of the markers to disease, affected sibpair identity-by-descent methods were used. The 2-point affected sibpair analyses were performed using the likelihood-based statistic in the program SIBPAIR.²¹ Multipoint sibpair analysis was initially performed with the MAPMAKER/SIBS program.²² Marker allele frequencies were estimated from the family data by taking 1 randomly selected individual from each sibship. The differences in marker allele frequency distribution between cases and controls were tested by the likelihood ratio test of the DISLAMB program in the case of

TABLE 2. Two-Point Affected Sibpair Analyses Between AT₁-ac Marker and Hypertension in Subgroups of the Original Linkage Study Sample

Subgroup (number of families)	Alleles Shared	Alleles Not Shared	P
1. All patients (142)	87	69	0.03
2. Female-female pairs (90)	46	32	0.02
3. Male-male pairs (53)	30	29	0.42
4. BP >104 mm Hg for both sibs (52)	20	15	0.15
5. Body mass index <27 kg/m ² for at least 1 sib (84)	41	26	0.02
6. Age of onset <50 years for both sibs (63)	37	18	0.001
7. Pairs with low risk* (45)	29	11	0.0005
8. Southwestern pairs with low risk*† (21)	19	4	0.0001
9. Northeastern pairs with low risk*† (20)	10	5	0.04

*Selection criteria were as follows: age of onset <50 years for both sibs and body mass index <27 kg/m² for at least 1 sib.

†In the case of 4 pairs, the parents originated from both areas.

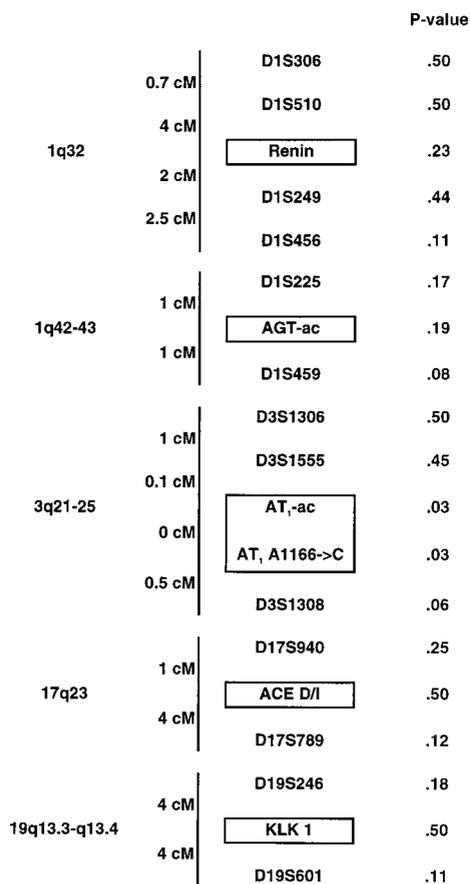


Figure 1. Two-point affected sibpair linkage analyses between hypertension and markers at and flanking candidate loci.

multiallelic markers²³ and by a χ^2 test in the case of diallelic markers. Finally, a model-free logarithm of the odds (LOD) score analysis in which haplotype frequencies were treated as a nuisance parameter was performed using ILINK of the LINKAGE package.²⁴ This analysis does not assume complete penetrance. In these analyses, 2 modes of inheritance were considered in which affected relatives were expected to share the transmission of either 1 or 2 copies of the disease allele. The disease locus was modeled as a diallelic locus, with both parents arbitrarily assigned disease locus genotype 1/2, and affected children, genotype 2/2 (in the recessive analyses). Alternatively, in the dominant analyses, 1 parent was assumed to be 1/1 and the other 1/2, with affected children having genotype 1/2 (since neither parent was genotyped for the marker, it causes no loss of generality to randomly assign the fathers to be heterozygous). The association sample was included in this analysis as well by making dummy pedigrees with disease locus genotypes as above,²⁵ except that the control individuals were assigned trait locus genotype 1/1 to contrast them maximally with the cases. Unaffected sibs were given unknown genotype at the trait locus. Since parents were not available, they were left unknown at the marker locus, but their disease locus genotypes were assigned as for the sibpair pedigrees. Thus, these analyses make use of all the data jointly to extract the maximum linkage and association information possible. For multipoint analysis, a similar approach was used to extract the maximum phase information from 3 diallelic intragenic markers. A more detailed description of the method, as well as the software needed, is available from J.T. (joseph.terwilliger@columbia.edu).

Results

Linkage Analyses in the Total Material

In the first phase of the study, all subjects were genotyped for 19 markers representing the 5 genomic areas as indicated in

Figure 1. The results of the analyses in the primary study group of 329 individuals from 142 families are summarized in Figure 1. We obtained no evidence for linkage between hypertension and markers at or near the loci coding for renin, AGT, ACE, or KLK1 with either 2-point (Figure 1) or multipoint (data not shown) linkage analyses. However, we did find some evidence for the involvement of the AT₁ locus already in this primary screen with the intragenic diallelic marker A1166→C ($P=0.03$, Figure 1) and the ac-repeat marker AT₁-ac ($P=0.03$, Figure 1).

Linkage Analyses in Sample Subgroups

Linkage analyses were performed not only on the total sample and on men and women separately but also on predefined sample subgroups that potentially could exhibit greater genetic homogeneity as regards the etiology of hypertension. First, all the individuals being treated for diabetes mellitus ($n=30$) were excluded from the analyses. From the remaining patients in 124 families, we identified those sets of sibpairs that had an early age of onset, with a relatively severe elevation of BP and normal or low body weight (subgroups 4 through 7, Table 2). In this analysis, we obtained conspicuous evidence for linkage between the AT₁ locus and hypertension in several of the subgroups (Table 2), whereas no evidence for linkage to hypertension emerged for the markers from the remaining 4 candidate gene loci (data not shown). In the case of the AT₁ gene locus, the strongest evidence of linkage was obtained when the sibpairs were required to have both an early onset of the disease and normal body weight (Table 2, subgroup 7). Here the group is called the low-risk group. Because of the long-present, clearly demonstrated difference in hypertension risk factors between individuals who live in Southwestern and Northeastern Finland¹⁷ and the suggested difference in the genetic origin between the eastern and western Finns,²⁶ the study sample was further stratified according to geographical origin. In the 21 sibpairs representing the low-risk Southwestern group, an LOD score of 3.0 ($P=0.0001$) was obtained with the intragenic dinucleotide repeat marker AT₁-ac (subgroup 8, Table 2, Figure 2A), and a maximal multipoint LOD score of 2.9 was demonstrated in the analysis of 6 markers within and flanking the AT₁ gene (Figure 2A). The analogous analysis in the low-risk Northeastern group of sibpairs failed to reveal any evidence for linkage in the multipoint analyses.

Association Analyses

On demonstration of the strongest linkage of the AT₁ gene markers to a particular type of hypertension, we considered it prudent to search for possible association of the various AT₁ alleles with essential hypertension using an independent group of patients chosen by similar criteria. DNA samples were analyzed from 50 hypertensive patients whose parents were all born in Southwestern Finland. For each individual, genotypes for 6 markers at or tightly linked to AT₁ were determined and compared with the corresponding genotypes of 122 control subjects. We found that the A1166→C variant was significantly more frequent among the hypertensive cases than among the controls (28% versus 16%, $P=0.01$, Table 3). This difference was more significant in a joint association analysis. In this approach, 1 randomly selected

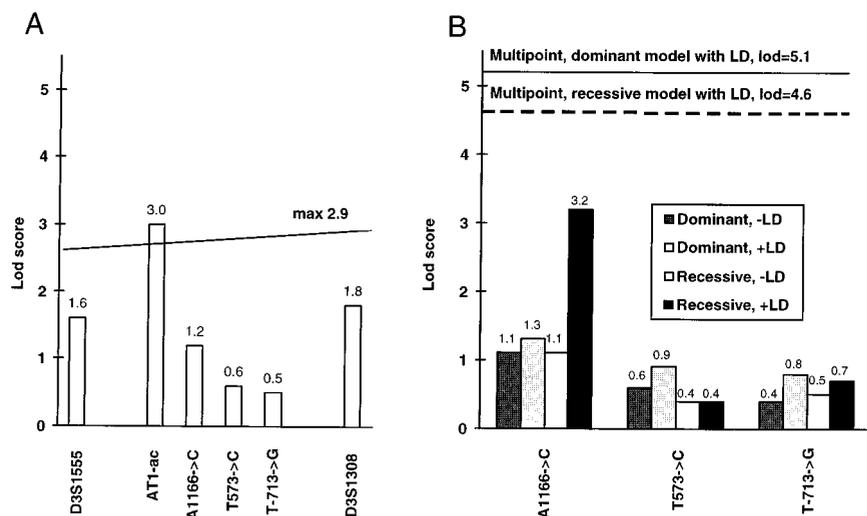


Figure 2. A, Two-point linkage analyses between hypertension and markers at and flanking the AT₁ gene area in the subgroup of nonobese sibpairs with early onset of hypertension and uniform geographical origin (subset 8, Table 2). The line represents maximal (max) LOD score obtained in the multipoint sibpair linkage analyses of the 6 makers using the MAPMAKER/SIBS program. B, LOD scores obtained under different models in linkage analyses of the subgroup of nonobese sibpairs with early onset of hypertension and uniform geographical origin (subset 8, Table 2). Data of 3 intragenic diallelic markers were analyzed, first under simple dominant and recessive models and consequently under the same models allowing linkage disequilibrium (LD). Bars represent 2-point LOD scores between hypertension and each marker. The solid and dotted lines represent maximal multipoint LOD scores under dominant and recessive models, respectively.

index case from each of the 21 Southwestern low-risk families (subgroup 8) was added in the association case material. This resulted in the variant AT₁ allele frequencies of 31% and 16% ($P=0.0007$, Table 3) in cases and controls, respectively. Also, the T-713→G polymorphism was marginally associated with the trait ($P=0.01$, Table 3).

Linkage Analyses Using Linkage Disequilibrium

To assess the overall involvement of the AT₁ locus in the etiology of hypertension, we performed a test of linkage allowing for the presence of linkage disequilibrium using the intragenic diallelic markers A1166→C, T573→C, and T-713→G. When allowing for linkage disequilibrium in the analysis, an

LOD score of >3 was found in a 2-point dominant analysis with the marker A1166→C; and in the multipoint analysis, extracting information of all 3 intragenic diallelic markers, an LOD score of 4.57 was obtained with the recessive model and 5.13 with the dominant model (Figure 2B).

Sequencing

We sequenced the entire coding region (1080 bp) and 848 bp of the promoter area of the AT₁ gene from 7 index cases and 1 control representing different AT₁ A1166→C genotypes. A C→T variation at -861 of the promoter region was seen in all individuals sequenced. This fragment was sequenced from an additional 20 hypertensive cases and 20 controls, and all

TABLE 3. Tests of Association Between Hypertension and Markers Within and Flanking the AT₁ Locus

Marker	Allele	Controls (n=122)	Cases (n=50)	P	Joint Analysis* (n=71)	P
D3S1555	1	71	32		41	
	2	62	28		42	
	3	56	24		34	
	Other	39	12	0.50	19	0.50
AT ₁ -ac	1	130	67		86	
	2	37	13		22	
	3	22	14		20	
	Other	33	6	0.15	12	0.49
AT ₁ 1166	A	192	72		97	
	C	36	28	0.01	43	0.0007
AT ₁ 573	T	141	54		72	
	C	86	44	0.24	64	0.09
AT ₁ -713	T	215	84		117	
	G	19	16	0.03	23	0.01
D3S1308	1	46	11		17	
	2	98	44		63	
	3	66	30		42	
	4	20	15	0.45	20	0.26

*Includes 1 randomly selected proband from each family in subgroup 8.

were T-T homozygous for this variation; hence, it most probably represents a population difference between Finns and the published sequence.¹⁹ No other previously unknown sequence variants were observed in the coding region or the promoter region.

Discussion

In the present study we adapted multiple strategies to identify genetic loci predisposing to hypertension. The Finnish population represents an optimal group for molecular studies of complex diseases by virtue of its genetic isolation and the maintenance of excellent records by the healthcare system. Several candidate genes of the renin-angiotensin cascade were screened, and data on linkage analyses were supplemented with data from association studies using independent samples of patients and controls to increase the reliability of the positive findings recorded. The advantage of using like-sex dizygotic twins in affected sibpair linkage analysis is that they provide perfect control for age and sex, which are significant confounding factors in genetic studies of hypertension. Restriction of the selection criteria for individuals with low risk factors of the disease strengthened the initially observed linkage of hypertension to AT₁ in our study. The advantage of performing linkage analyses for complex diseases in strictly defined subsets of patients has been well-demonstrated in previous studies on non-insulin-dependent diabetes mellitus²⁷ and bronchial asthma.²⁸ Furthermore, a distinct difference in the occurrence of cardiovascular morbidity and cardiovascular risk factors, including elevated BP, has been demonstrated between individuals living in the northeastern and southwestern parts of Finland, with clustering of unfavorable risk factors in the east.¹⁷ This difference follows a postulated cultural border, which divides the southwestern area from the rest of the population. On these grounds we reasoned that any environmental burden of deleterious risk factors on the development of hypertension should be less heavy in Southwestern Finland, and accordingly, genetic component(s) contributing to BP elevation would be attenuated to a lesser extent than would be the case in Northeastern Finland. Moreover, a genetic difference between these two populations has been implicated.²⁶ Accordingly, the most significant linkage was obtained in the sibpair sample originating in Southwestern Finland. Interestingly, no linkage was found in the subgroups consisting of men or those with a diastolic BP >104 mm Hg. Further studies in other patient cohorts are needed to reveal whether AT₁ has some age-, sex-, or population-specific effect on BP.

Multiple testing may result in a type I error if not controlled for. Therefore, we replicated our findings in an independent association sample and furthermore, in a joint association analysis representing 71 unrelated hypertensive cases; these data were consistent with the original findings. Finally, the joint linkage analysis of the data conditional on linkage disequilibrium yielded even more statistically significant results, including a maximal multipoint LOD score of 5.13. Allowing linkage disequilibrium in the model increases the power of the analyses by increasing the amount of phase information available in the study. Affected sibpair analysis without parents is normally phase-unknown, but when link-

age disequilibrium exists, allowing for it has the effect of altering the parental phase probabilities, thus increasing the effective number of meioses in the sample.

Only 1 of the analyzed genetic variations, AT₁ A1166→C, yielded significant evidence of linkage disequilibrium with the trait phenotype in the single-marker χ^2 tests we applied. It is possible that the A1166→C substitution represents the causative mutation itself; or more likely, it is situated in the immediate vicinity of the causative mutation, thus showing linkage disequilibrium with it. It is of interest that the same A1166→C variant showed a significant increase in allelic frequency in approximately 200 hypertensive patients compared with normotensive individuals, even though no evidence for linkage was noticed in 267 sibpairs in the French population.⁸ In contrast to the present study and a study by Wang et al¹¹ that demonstrate the increased frequency of the 1166→C variant among hypertensive individuals, BP values were significantly lower in individuals carrying the CC genotype in 1 study.¹⁰ Takayanagi et al¹⁹ suggested the presence of a negatively regulating element or elements within 848 bp upstream of the first exon of the AT₁ gene. During our screening for variations in the coding and promoter areas of the AT₁ gene in a subsample of 8 hypertensive individuals, we were unable to demonstrate DNA alterations leading to changes in the protein structure. A previously reported sequence variation (T-713→G)²⁹ within the suggested inhibitory region of the promoter area was marginally associated with hypertension, and its role remains unknown.

Interestingly, for the diallelic markers within this gene that were analyzed jointly and showed evidence of allelic association with the disease allele, there was no predominant haplotype, and the pattern of disequilibrium observed was not consistent with a single ancestral founder effect model for linkage disequilibrium. When 3 markers and disease were analyzed jointly, the maximum likelihood estimates of all haplotype frequencies were nonzero, indicating that the proportion of each haplotype varies between cases and controls rather than a single ancestral haplotype being enriched.

In conclusion, whether examination is made using a linkage or association approach or a combination of the 2 approaches, our study in the genetically unique Finnish population provides evidence for the assumption that common genetic variation at the AT₁ gene locus may modify an individual's risk for developing essential hypertension. The underlying DNA alteration(s) and the ensuing changes in the angiotensin signaling pathways remain to be explored.

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