Linkage Analysis of Candidate Genes and Gene-Gene Interactions in Chinese Hypertensive Sib Pairs

Tianhua Niu, Xiping Xu, Heather J. Cordell, John Rogus, Yusheng Zhou, Zhian Fang, Klaus Lindpaintner

Abstract—Previous studies of hypertension in humans and experimental animal models have identified a number of candidate genes that have since been implicated as possibly contributing to essential hypertension. Among them are the genes encoding angiotensinogen, renin, the β- and γ-subunits of the epithelial sodium channel (β/γ-ENaC), α-adducin, and kallikrein (KLK). To examine the role of possible contribution of these genes in ethnic Chinese, as well as the epistatic interaction among them, we studied a large cohort of hypertensive sib pairs from China. DNA samples from 310 concordant affected sibling pairs with hypertension were tested for linkage with the use of excess allele–sharing algorithms based on genotyping with highly informative GT-repeat microsatellite markers localized in the immediate vicinity of the genes encoding angiotensinogen, renin, β- and γ-ENaC, α-adducin, and KLK. Affected sib pair analysis conducted according to 3 different methods (Statistical Analysis for Genetic Epidemiology [S.A.G.E.]/SIBPAL, MAPMAKER/SIBS, and affected pedigree member [APM] methods) revealed no evidence for linkage of any of these genes to primary hypertension in the population studied. Moreover, 2-locus sib pair linkage analyses to test for gene-gene interactions among each possible pair of candidate genes failed to yield any statistically significant results. Our findings provide no support for a significant contribution of the angiotensinogen, renin, β/γ-ENaC, α-adducin, or KLK genes, alone or in concert, to the pathogenesis of essential hypertension among Chinese. Our results emphasize the possible role of ethnic differences for complex disease genetics, as well as the need for large, well-characterized investigations. (Hypertension. 1999;33:1332-1337.)

Key Words: renin ■ sodium channels ■ adducin ■ kallikrein ■ hypertension, essential ■ genetics ■ Chinese

Molecular variations in genes encoding blood pressure–relevant proteins, in concert with specific gene-environment effects and gene-gene interactions, are assumed to play a pivotal role in the pathogenesis of hypertension, given the well-established familial nature of this disorder. A number of candidate genes—derived from experimentation with experimental animal strains, from studies in rare, familial forms of hypertension, and from linkage and association studies in more heterogeneous cohorts—have recently received considerable attention. The majority of studies addressing the role of these genes in human essential hypertension have been of limited size, have primarily targeted whites, and have not considered gene-gene interactions. Given current knowledge of differential allele frequencies of many single nucleotide polymorphisms among different ethnicities, the importance of validating the data in these groups is obvious. We recently reported that among Chinese hypertensives, the angiotensinogen gene (AGT), the most thoroughly studied of hypertension candidate genes, was found not to contribute to blood pressure. In the present set of investigations we tested for possible gene-gene interactions between AGT and a number of other candidate genes that may shed light on this finding, and we report the results of linkage analyses for these additional genes and for all pairwise interactions among them.

Renin, the rate-limiting enzyme involved in the generation of the potent vasoactive hormone angiotensin II and therefore an attractive candidate for hypertension, has recently been reported to be associated with differential blood pressure in subjects of Afro-Caribbean heritage on the basis of a restriction fragment length polymorphism (RFLP) analysis. A study conducted among Taiwanese, enrolling 86 hypertensive and 107 normotensive subjects, demonstrated a significant association of the renin HindIII polymorphism with essential hypertension. In contrast, several previous case-control studies and linkage studies failed to support an etiologic role for the renin gene in human hypertension.

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Liddle’s syndrome (pseudohypoaldosteronism) is a hereditary form of hypertension with autosomal dominant inheritance characterized by severe hypertension, hypokalemia, and suppressed secretion of aldosterone; it has recently been shown to be caused by mutations in the genes encoding the β- and γ-subunits of the epithelial sodium channel (βγ-ENaC) that result in excessive reabsorption of sodium in the distal nephron.10,11 Although the mutations causing the severe phenotype of Liddle’s syndrome are very rare, it has been suggested that less dramatic genetic variants of these genes may be more frequently represented as one of the contributing genes in polygenic forms of hypertension.12 Indeed, Baker and colleagues13 recently demonstrated a significant association of the T594M mutation of β-ENaC with hypertension in blacks. However, in a Japanese cohort of both hypertensive and normotensive subjects, no mutations of the carboxyl-terminal portion of β-ENaC were detected.14

Adducin, a heterodimer composed of an α- and a β-subunit, is a cytoskeletal protein probably involved in cellular signal transduction.15 Point mutations of the rat homologues of the 2 genes have been linked to hypertension in the Milan rat strain.16 Genetic variants of α-adducin have been postulated to affect kidney function by modulating the overall capacity of tubular epithelial cells to transport ions.17 A number of previous studies showed that the Gly460Trp polymorphism of the α-adducin gene is significantly associated with hypertension,18–20 especially in relation to salt sensitivity. However, no such association was found in 2 Japanese populations.21,22

The kallikrein gene family encodes proteins with limited substrate specificity and high homology in their amino acid sequences. Two of these proteins may contribute to the regulation of blood pressure: tissue kallikrein, which specifically cleaves kininogens to generate kinins, and tonin, which cleaves angiotensin I and angiotensinogen to generate angiotensin II.23,24 A RFLP within the kallikrein gene (KLK) had previously been shown to cosegregate with increased blood pressure in recombinant inbred strains derived from the spontaneously hypertensive rat and the normotensive Brown Norway rat,25 and additional evidence points to a role of renal kallikrein in blood pressure regulation in animal models as well.26 Thus far, a limited number of small association analyses have failed to support a significant association of KLK polymorphisms with essential hypertension.27,28

None of these candidate genes has been studied in ethnic Chinese, and for none have initial results been replicated in large, well-characterized, family-based samples that avoid the possible selection bias inherent in case-control studies. Moreover, gene-gene interactions, although frequently postulated as being potentially of major impact, have only rarely, and never systematically, been taken into consideration. Using a cohort of 310 concordant affected sib pairs provided us with the opportunity to perform linkage analyses to address these issues.

Methods

A detailed description of the study sample collection has been published previously.1 In brief, 310 hypertensive sib pairs (223 sib pairs, 21 sets of 3, and 4 sets of 4 affected siblings) were selected from the Zongyang and Huaining counties of Anhui province on the basis of data collected in a community survey of 20,216 individuals from the same area.29 Individuals aged >15 years without evidence of secondary forms of hypertension such as diabetes, hyperthyroidism, or glomerular nephritis were selected if their systolic blood pressure was ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg regardless of treatment status. Five hundred fifteen (98%) of the 525 selected subjects had a previous history of physician-diagnosed hypertension according to medical records, including information about age of onset. In addition, 15 individuals who had a history of hypertension and who were on antihypertensive therapy but who had blood pressure <140 mm Hg systolic and 90 mm Hg diastolic were enrolled. The study was approved by the Institutional Review Committee of the Anhui Medical University, and all study subjects gave informed consent. All the procedures followed were in accordance with institutional guidelines. A questionnaire was administered, and a screening examination was performed with the use of standard protocols, as described previously.29

Blood Pressure Measurement

Triplicate blood pressure measurements were made by trained nurses using a mercury-gravity manometer with appropriately sized cuffs, as described previously.1

Phlebotomy

Forcemarm venous blood samples were then collected from each subject by venipuncture, and plasma was subsequently removed from the cell pellet by pipetting.3 All samples were frozen at −85°C.

DNA Extraction

High-molecular-weight DNA (200 to 400 kb) was extracted with the use of the QIAamp Blood Kit (QIAamp Inc), as described previously.1

Microsatellite Polymorphisms

Genotyping for the GT-repeat microsatellite markers D1S249 (remin), D4S43 and D4S126 (α-adducin), D16S403 and D16S420 (β- and γ-ENaC), and D19S246 (kallikrein) was performed as previously described.30–32 For each marker, 1 primer was labeled at the 5’ end with [32P]γ-ATP as previously described,30–32 and polymerase chain reaction was performed at annealing temperatures of 64°C (D1S249), 58°C (D4S43), 68°C (D4S126), 63°C (D16S403), 55°C (D16S420), and 57°C (D19S246) for 39 cycles. Reaction products were resolved over denaturing sequencing gels containing 6% polyacrylamide, 8 mol/L urea, and 30% formamide and visualized by autoradiography. Three control samples with known genotype were run on each gel, interspersed with unknown samples, to account for gel-to-gel variations. Scoring was performed by 2 independent observers, as described previously.33

Statistical Analysis

A multianalytical approach encompassing several different methods was used for the affected sib pair analysis.

S.A.G.E./SIBPAL Method

We used the Statistical Analysis for Genetic Epidemiology (S.A.G.E.)/SIBPAL program34 to perform a qualitative-trait linkage analysis of D1S249, D4S43, D4S126, D16S403, D16S420, and D19S246. Given the sibships we have and appropriate allele frequencies for the markers used, this nonparametric method estimates the proportion (π) of alleles identical by descent (IBD) that the hypertensive sib pair shares at that locus. Because we only have 4 sets of 4 affected siblings out of 248 sibships (1.6%), weighting by family size was considered unnecessary.

MAPMAKER/SIBS Method

A multipoint linkage analysis was implemented with the use of MAPMAKER/SIBS.35
Hypertension Candidate Genes in Chinese

Table 1: Results of Qualitative-Trait S.A.G.E./SIBPAL Analyses for REN, β/γ-ENaC, α-Adducin, and KLK

<table>
<thead>
<tr>
<th>Gene</th>
<th>Marker</th>
<th>Sib Pairs</th>
<th>Allele Sharing (Expected)</th>
<th>Allele Sharing (Observed)</th>
<th>SE</th>
<th>P</th>
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</thead>
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<tr>
<td>REN</td>
<td>D15S249</td>
<td>298</td>
<td>0.50</td>
<td>0.46</td>
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<td>NS</td>
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<td>β/γ-ENaC</td>
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<td>0.50</td>
<td>0.46</td>
<td>0.02</td>
<td>NS</td>
</tr>
<tr>
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<tr>
<td>KLK</td>
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<td>0.50</td>
<td>0.46</td>
<td>0.02</td>
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</tr>
</tbody>
</table>

Data calculated without weighting.

Affected Pedigree Member Method

The affected pedigree member (APM) method is a model-free approach that measures whether affected members of a pedigree have an excess of allele sharing of the genetic markers tested. They consisted of 335 males (64%) and 190 females, and

TWOLOC Method

The TWOLOC method is a multilocus linkage test based on maximum likelihood that accounts for the interdependency of 2 putative susceptibility genes and evaluates the support for an interaction between them. We used a modified version of TWOLOC that maximized the likelihood using the FORTRAN subroutine MAXFUN of S.A.G.E. Multilocus 2–1 IBD sharing probabilities for linked and unlinked markers were calculated with the use of the VITESSE and MAPMAKER/SIBS programs, respectively.

Power Simulation Method

Power simulations were performed with the linkage strategies described previously. For the D15S249 (polymorphism information content [PIC]=0.71, recombination fraction θ=0.015), the D16S403 (PIC=0.65, θ=0.001), the D4S43 (PIC=0.67, θ=0.013), and the D19S246 (PIC=0.73, θ=0.025), a 4-allele model with equal allele frequencies (PIC=0.70), is used; for the D16S420 (PIC=0.75, θ=0.000) and the D4S126 (PIC=0.79, θ=0.007), a 5-allele model with equal allele frequencies (PIC=0.77) is used. The hypothesis being tested in our study is whether the genes coding for renin, β- and γ-ENaC, α-adducin, or kallikrein individually play a major role in the causation of essential hypertension. Hence, a single-locus model was used for each of the tested candidate genes, given the gene-specific relative risk ratio, λ. Power simulations were performed for the total number of 310 affected sib pairs assuming identity-by-state (scheme 1: information on pairs only).

Results

The 525 study subjects, representing 248 sibships and 310 sib pairs, were exactly those subjects we previously investigated. They consisted of 335 males (64%) and 190 females (36%), and their clinical characteristics are shown elsewhere. Except for smoking, clinical parameters show very small variances, supporting the notion that the study population is relatively homogeneous. For all 6 markers studied, the distributions of genotype frequencies, determined from 1 randomly chosen individual from each sibship, were in agreement with Hardy-Weinberg equilibrium predictions.

Table 1 shows the results of linkage analyses using the S.A.G.E./SIBPAL program packages. No evidence for linkage was found for any of the markers studied. Likewise, APM analyses (shown in Table 2), using the recommended intermediate weighting function f_\text{B}(p), showed no evidence of significant linkage for any of the markers tested. Single-locus linkage analyses using MAPMAKER/SIBS yielded z_\text{2}:z_\text{1}:z_0 ratios not different from 0.25:0.50:0.25 for all markers tested.

The maximum lod score results of a 2-locus general model that uses linkage algorithms to test for the presence of epistatic interactions among AGT and the candidate genes studied, as well as for each possible pairwise combination of these loci, are presented in Table 3. No increased allele sharing was observed among affected sib pairs for any 2 loci, and there was no excess of sharing 2 alleles IBD simultaneously at both loci or either locus tested in each pairwise constellation.

Table 4 shows the power as a function of gene-specific λ, according to the PIC value of each of the 6 microsatellite markers tested (D15S249, D4S43, D4S126, D16S403, D16S420, and D19S246), on the basis of single-locus simulations assuming identity-by-state for all the 310 affected sib pairs.

Discussion

The primary goal of this family-based study was to test the possible epistatic modification of the previously reported association of AGT on hypertension by a series of additional candidate genes. In addition, we examined the contribution of these genes, which encode renin, β-γ-ENaC, α-adducin, and kallikrein, to essential hypertension in ethnic Chinese. To maximize the statistical power of our investigation, we used a combination of approaches discussed below. We found no support for a role of any of the genes, alone or in interaction with one another, in the sample population studied.

The contribution of a given disease-contributing gene variant in common complex disorders will vary among individuals with diverse environmental exposures or at various developmental stages. Only if select, genetically relatively homogeneous, large samples are studied, in whom extensive documentation on confounding factors exists (and thus the possibility to adjust for them), will there be sufficient statistical power to successfully conduct and interpret such studies. The sample size of our study (310 sib pairs) is, compared with other similar studies, quite substantial. Moreover, our study included a large majority of subjects whose blood pressure was in the extreme upper tail of the distribution of the source population. Only 15 individuals who had normotensive blood pressures while being treated with anti-
hypertensive drugs, and who had extensive and unambiguous documentation of pretreatment hypertensive values, were included to avoid the loss of 19 sib pairs. These 15 patients constitute 3% of the total sample and were also included in the previously published report on AGT.1 Because of limited means of transportation, the Anqing population has a quite stable base over thousands of years. Therefore, individuals of this study are relatively homogeneous with regard to ethnicity, socioeconomic status, and environmental, occupational, and dietary exposures. These particular characteristics favor the presence of relatively few genes/gene variants contributing to the disease and thus increase the power of the linkage analysis. In addition, our phenotype data—a common source of noise—are comparably robust, because all blood pressure data were verified on entry in the study. To quantify subjects’ deviation from the mean of the blood pressure distribution and thereby to confirm the relative severity of their hypertension, we calculated residualized values, adjusting for age, body mass index, gender, height, weight, cigarette smoking, and other environmental covariates, and were able to show indeed that the majority of our subjects fell into the distant upper tail of the overall distribution (data not shown; see Reference 1 for detail). For 2 of the candidate genes, β/γ-ENaC and α-adducin, we extended and verified the analysis by inclusion of an additional, highly polymorphic marker localized in close proximity to each other.

Complex traits such as essential hypertension are usually not controlled by only a single disease locus but often involve multiple genetic and/or environmental factors that are assumed, at least to some extent, to be subject to specific gene-environment and gene-gene interactions. Only a small number of genetic studies of complex disorders have to date considered 2 marker loci simultaneously to detect linkage, undoubtedly because of, at least in part, the complexity and

<table>
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<tr>
<th>Gene</th>
<th>Marker</th>
<th>Alleles</th>
<th>PIC</th>
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Values in the diagonal are single-locus maximum lod score statistics. Values in the other cells are 2-locus maximum lod score statistics assuming arbitrary epistatic components (ie, a general model) for the loci in the relevant row and column. Because AGT-GT, M235T, and T174M are tightly linked to the AGT gene, the 2-locus model was not applied with regard to interactions among these markers.
challenges of such analyses.\textsuperscript{43,44} With regard to hypertension, West and colleagues\textsuperscript{45} examined RFLPs of 5 genes (renin gene, haptoglobin gene, neuropeptide Y gene, and cardiac myosin $\beta$ heavy chain gene). There was no significant association between alleles at any of these loci and the presence of hypertension. Huggard and coworkers\textsuperscript{46} also investigated multiple candidate genes ($AGT$, insulin-like growth factor receptor gene, insulin receptor gene, angiotensin II receptor gene, elastin gene, and $KLK$), and no significant association of any of the markers used was found with hypertension status. Except for 1 limited attempt,\textsuperscript{44} none of these studies assessed the joint effects of 2 (or >2) candidate loci simultaneously.

Classic genetic statistical analysis typically investigates only a single marker locus, accounting for the effects of additional loci through assumptions of reduced penetrance or phenocopy. Recently, a number of new methods have been proposed to investigate the joint action of 2 genes in a disease by using information on 2 genetic markers simultaneously.\textsuperscript{37,47–49} The parametric, 2-locus lod score method of Schork et al\textsuperscript{48} requires the specification of the mode of inheritance and involves a large burden of computation. Affected sib pair tests are computationally simple and do not require an explicit specification of the disease model. In the past, however, use of these tests has been restricted to data with a single marker locus. Cordell and colleagues\textsuperscript{37} extended the maximum lod score method of Risch,\textsuperscript{42} which allows the simultaneous detection and modeling of 2 disease loci unlinked to each other but potentially interacting in their linkage to affection status. Using simultaneous information on 2 markers can lead to increased power for detection of an effect over using a single-locus method if the effect of the 2 genes is not multiplicative.\textsuperscript{43,49} We have performed the sib pair linkage analyses by modeling 2 candidate loci at the same time for all marker pairs, and no significant findings were obtained. In addition, single-point approaches (APM, S.A.G.E./SIBPAL, MAPMAKER/SIBS) were also performed in our affected sib pair linkage analyses. The results of our analyses remain consistent whether or not weighting for rare alleles is used; this raises our level of confidence in our data.

As documented previously,\textsuperscript{1} we were in the unique position, in the Anhui population, to have access to a contemporaneously established, large, cross-sectional database collected in 20,216 subjects from the same province 1 year earlier.\textsuperscript{29} This allowed us to validate our selection of probands and to determine a population-specific index of heritability of hypertension for direct assessment of the power of our study. Thus, using the 90th percentile of the distribution of residuals (adjusted for blood pressure–relevant covariates such as age, gender, height, socioeconomic status, exercise, and smoking) as cutoff for defining hypertension, we found the sibling recurrent risk ratio, $\lambda_s$, to be 2.4.\textsuperscript{1} Because our study yields negative results, it raises the concern of statistical power. The most critical factor in determining power is the PIC.\textsuperscript{42} Four equally frequent alleles yield a PIC of 0.70, and 5 equally frequent alleles correspond to a PIC of 0.77, both reasonable for the markers we used. For the total sample, the estimated power was $>85\%$ with respect to any given marker tested (Table 4). However, because the gene-specific sibling recurrent risk $\lambda_s$ for each of the hypertension-causing genes is less than the aggregate recurrent risk $\lambda_{s,\text{total}}$ (it is assumed that given N genes contributing to essential hypertension, $\lambda_{s,\text{total}}=\lambda_{s,1}+\lambda_{s,2}+\ldots+\lambda_{s,N}$ under the additive model), the actual power of our sample may be less than the power derived from our simulation algorithm for any given gene of interest, because we have to assume a polygenic etiology.

In summary, we found no interaction between the $AGT$ locus and the genes encoding renin, $\beta$-$\gamma$-ENaC, $\alpha$-adducin, and $KLK$ loci with respect to essential hypertension in Chinese, and neither was any of these candidate genes linked to the disease. Our results demonstrate that if genetic variants of these genes indeed contribute to essential hypertension, then their role may depend importantly on ethnic background. Thus, our findings may indicate important etiologic diversity in the genetic spectrum of primary hypertension.

**Acknowledgments**

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**References**

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<table>
<thead>
<tr>
<th>Assumed $\lambda_s$</th>
<th>D1S249</th>
<th>D16S420</th>
<th>D16S403</th>
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See Methods for details.
Linkage Analysis of Candidate Genes and Gene-Gene Interactions in Chinese Hypertensive Sib Pairs
Tianhua Niu, Xiping Xu, Heather J. Cordell, John Rogus, Yusheng Zhou, Zhian Fang and Klaus Lindpaintner

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