Implication of an AGT Haplotype in a Multigene Association Study With Pregnancy Hypertension

Sébastien Lévesque, Jean-Marie Moutquin, Carmen Lindsay, Marie-Claude Roy, François Rousseau

Abstract—Several association studies of candidate genes for preeclampsia and essential hypertension have led to discordant results, partly because of small sample sizes. Using a large population-based sample of pregnant women, we conducted an association study of 10 polymorphisms in 9 genes and aimed (1) to validate 10 published associations with preeclampsia or essential hypertension, (2) to investigate candidate polymorphisms previously associated with preeclampsia for association with essential hypertension and similarly with polymorphisms previously associated with essential hypertension. From a prospective sample of 3391 nulliparous French Canadian pregnant women, we identified 180 cases of preeclampsia, 203 cases of essential hypertension that were matched with normotensive control subjects (n=310 and 357, respectively). Polymorphisms were genotyped by allele-specific PCR. Among our candidate polymorphisms, the Met allele of Thr174Met of AGT was associated with preeclampsia (P=0.0033). Haplotype analysis revealed that the A-Met-Thr (G1035A-Thr174Met-Met235Thr) haplotype was associated with a 2.1-fold increased risk of preeclampsia (95% CI, 1.4 to 3.4; P=0.0008). In conclusion, we observed a strong association between a specific AGT haplotype and preeclampsia in our population, without replicating previous published associations with either preeclampsia or essential hypertension. Our data support a role for AGT in genetic susceptibility to preeclampsia. (Hypertension. 2004;43:71-78.)

Key Words: genetics ■ polymorphism ■ haplotypes ■ hypertension, pregnancy ■ preeclampsia ■ population

Pregnancy hypertension, including preeclampsia, gestational hypertension, and preexisting chronic essential hypertension, is the most prevalent obstetrical complication and a leading cause of maternal and perinatal death.1 Preeclampsia is a proteinuric form of hypertension, affecting 5% to 6% of pregnant women.2 Its underlying cause is still elusive. However, trophoblast cells have been found to fail to invade uterine spiral arteries,3 leading to a decreased perfusion with consequent placental hypoxia.4 Further vascular endothelial dysfunction results in systemic hypertension and proteinuria.4 Oxidative stress has been suggested to link placental hypoxia to endothelial dysfunction.5 Whatever the exact mechanism leading from the failure of trophoblast invasion to manifestations of preeclampsia, maternal susceptibility for development of preeclampsia must be a determinant factor, since abnormal placentation is not specific to preeclampsia.6 Heritability has been estimated as 54% by a study in Swedish twin pairs.7 Linkage studies conducted in families have identified several potential susceptibility loci; however, few have been confirmed. At the present time, only three distinct susceptibility loci identified on chromosome 2p13 in Icelandic families and on chromosomes 2p25 and 9p13 in Finns showed an lod score >3 in a genome-wide scan.8,9 Another study showed suggestive linkage on chromosome 2p in families from Australia and New Zealand.10 Other chromosomal regions that have showed suggestive linkage include 4,11 2q23, 8 11q23–24, 10 7q36, 12 12q, 3p, 15q, 10q, and 22q.13 In parallel, association studies have suggested the involvement of candidate genes in susceptibility to preeclampsia, mostly related to oxidative stress, vasoactive substances, and coagulation disorders: lipoprotein lipase (LPL),14 5–10 methylenetetrahydrofolate reductase (MTHFR),15 glutathione-S-transferase pi (GSTP1),16 factor V (F5),15,17 apolipoprotein E (APOE),18 plasminogen activator inhibitor-1 (SERPIN1),19 microsomal epoxide hydrolase 1 (EPHX1),20 endothelial nitric oxide synthase (NOS3),21 angiotensinogen (AGT),22 and tumor necrosis factor-α (TNF).23

Essential hypertension is an elusive disease. Hypotheses including the renin-angiotensin-aldosterone system in relation with salt-induced hypertension,24 the alteration of nitric oxide metabolism,25 the impact of oxidative stress on endothelial dysfunction, and vascular smooth muscle cell hypertrophy26,27 have been investigated, but none of these could fully explain the pathophysiology of essential hypertension. The genetic susceptibility to essential hypertension has been extensively investigated in samples of individuals of older

Received July 8, 2003; first decision July 24, 2003; revision accepted October 22, 2003.
From Unité de Recherche en Génétique Humaine et Moléculaire and Centre for the Development, Evaluation and Rational Implementation of New Diagnostic Tools (CEDERINDT), Centre de Recherche de l’Hôpital Saint-François d’Assise du CHUQ, and Département de Biologie Médicale, Faculté de Médecine, Université Laval (S.L., C.L., M.-C.R., F.R.), Québec, Canada; and Département d’Obstétrique Gynécologie, Centre de Recherche Clinique du CHUS, Université de Sherbrooke (J.-M.M.), Sherbrooke, Canada.
Correspondence to Dr François Rousseau, 10 rue de l’Espinay, Centre de Recherche de l’hôpital St-François d’Assise, G1L 3L5, Québec, Canada.
E-mail Francois.Rousseau@crsfa.ulaval.ca
© 2003 American Heart Association, Inc.
Hypertension is available at http://www.hypertensionaha.org DOI: 10.1161/01.HYP.00000104525.76016.77
age (mostly men >40 years old). It is commonly accepted that heritability of blood pressure ranges from ~30% to 50%. Many genes have been associated with essential hypertension. Some of these include angiotensinogen (AGT), angiotensin-converting enzyme (ACE), angiotensin II type I receptor (AGTR1), transforming growth factor β-1 (TGFβ1), endothelial nitric oxide synthase (NOS3), and G-protein β3 subunit (GNB3). To our knowledge, no study has so far investigated the genetic susceptibility to essential hypertension in young pregnant women, also termed chronic essential hypertension. Although these two hypertensive disorders are treated as distinct entities, there is some evidence suggesting that genetic susceptibility of these two conditions might be related. Noteworthy, the gene AGT has been associated with both preeclampsia and essential hypertension. Moreover, it is known that women with blood pressure levels in the normal upper range are more at risk for development of preeclampsia and essential hypertension. Moreover, it is known that women with blood pressure levels in the normal upper range are more at risk for development of preeclampsia and essential hypertension.22,29

The diagnosis of preeclampsia or chronic essential hypertension was defined as 2 measurements, at least 4 hours apart, of a diastolic blood pressure ≥90 mm Hg (phase 5 Korotkoff sound) reported before pregnancy or within 20 weeks of gestational age and persisting 6 weeks after delivery without proteinuria. Blood pressure measurements were taken with a standard mercury sphygmomanometer, with the patient seated, by a nurse trained for blood pressure measurement with cuff size covering at least two thirds of the arm. Proteinuria was evaluated in a 24-hour urine collection or by using Albustix (>1+ on random urine analysis). Most chronic hypertensive pregnancies displayed mild to moderate hypertension without medication and without target organ complication. Multiple pregnancies, preexisting secondary hypertension, and preexisting hypertension with superimposed preeclampsia were excluded from the present study.

Cases diagnosed with preeclampsia and chronic essential hypertension were compared with matched normotensive nulliparous control subjects. Matching variables included maternal age ≤5 years, gestational age at delivery ≤21 weeks, body mass index (kg/m²), <20, 20 to 24, 25 to 27, and >27, and month of the year at delivery. Each case was matched with 2 control subjects when possible. Diagnosis was ascertained by one investigator. This project was approved by the institutional ethics committee, and participants provided written informed consent.

DNA Purification and Genotype Analysis

Genomic DNA was isolated from 250 μL of whole blood, with the use of a QIAamp 96 DNA blood kit (Qiagen), according to the manufacturer’s instructions. Purified DNA (concentration of ~25 ng/μL) was stored at −20°C. At the time of genotype analysis, a 50-μL aliquot was diluted (Tris 10 mmol/L and EDTA 0.05 mmol/L) in 96-well plates (Axygen), with a final concentration of 5 ng/μL, and kept at 4°C. DNA from cases and control subjects, either for preeclampsia or chronic essential hypertension, were represented in each DNA plate with respect to their relative proportions to minimize the effect of potential genotyping errors on the results.

All polymorphisms were analyzed by means of allele-specific PCR except for ACE insertion/deletion polymorphism, which was done by PCR. Conditions and primers for each polymorphism can be found in the expanded methods (please see Table I and text in an online supplement available at http://www.hypertensionaha.org). PCR products were visualized on a 1.5% agarose gel through the use of ethidium bromide staining. Alternatively, products were quantified by adding Sybr Green I (Molecular Probes) to each reaction tube, then fluorescence was detected with a Fluoroskan Ascent (MTX Laboratory Systems), as shown in the online supplement (Figure I in an online supplement available at http://www.hypertensionaha.org). Until now, with the use of this method, duplicate analysis of 5000 genotypes from 15 different SNP assays showed an average reproducibility of 99.7% and an average rate of data rejection <1%.

For all polymorphisms, genotypes were interpreted independently by two readers blinded to the clinical outcome of the samples. Concordance of results between readers ranged from 98.6% to...
Validation of Published Associations

Five selected published associations between polymorphisms and essential hypertension and preeclampsia were tested for replication in our sample of women with chronic essential hypertension (Table 2). Also, five published associations of polymorphisms with preeclampsia were tested for replication in our preeclamptic sample (Table 3). No published association with essential hypertension or preeclampsia was replicated with the use of a significance level of \( \alpha = 0.05 \). However, association with the Thr allele of the polymorphism Met235Thr of the \( AGT \) gene was significant in a subset of preeclampsia cases (ie, HELLP syndrome). An increased risk of 2.1-fold (95% CI, 1.2 to 3.9; \( P = 0.015 \)) was observed. Estimates of odds ratios that could be detected in our sample of preeclamptic women with a power of 0.8, for genotype and allele frequencies in control subjects ranging from 0.03 to 0.5, varied from 2.9 to 1.6 and 2.2 to 1.4, respectively. For genotype or allele frequencies of 0.5 up to 0.95, estimates of detectable odds ratios increased from 1.6 to 4.33 for genotypes and from 1.4 to 2.6 for alleles. Slightly smaller but similar odds ratio values were observed with our sample of women with chronic essential hypertension.
Association Study of Candidate Genes

Polymorphisms/genes associated with preeclampsia in published studies were treated as candidates for chronic essential hypertension. Using the same genotypes or alleles that showed significant associations with preeclampsia in published studies, we investigated potential association with chronic essential hypertension (Table 2). Similarly, polymorphisms and genes previously associated with essential hypertension were tested for association with preeclampsia (Table 3). Although not associated previously with PE or CHT, an APOB polymorphism (Thr2488Thr, C>T) was added to the list of candidate polymorphisms for both diseases. The rationale for testing this polymorphism for PE consists in its association with serum level of LDL and apolipoprotein B, and the link between preeclampsia and increased serum concentration of small dense LDL. For chronic essential hypertension, the rationale was based on the fact that an association of another polymorphism of APOB with blood pressure was reported previously. Using a significance level of \( \alpha = 0.05 \), no significant association was observed in the CHT group with genes APOB, F5, GSTP1, MTHFR, TNF, nor in the PE group with genes ACE, AGTR1, APOB, and TGFB1. However, the Met allele of Thr174Met of the AGT gene conferred a 2-fold increase in the risk of preeclampsia, and this has not been reported before, to our knowledge (Table 3). To refine the AGT allele associated with preeclampsia, analysis of AGT haplotypes was performed. Based on a recent study of AGT in whites and...
Japanese subjects, 44 3 polymorphisms were selected (G1035A-Thr174Met-Met235Thr) to characterize the at-risk haplotype. Four major haplotypes (G1035A-Thr174Met-Met235Thr) were observed in our French Canadian population. The AGT A-Met-Thr haplotype was observed in 13.6% among case chromosomes compared with 6.9% in control subjects (P = 0.0008). This haplotype was associated with a 2.1-increased (1.4 to 3.4) risk of preeclampsia.

Discussion
Our study attempted to take into account some issues that could lead to lack of replication between studies. First, a large sample ensured replication with a power >0.8 for all but 2 tested associations. Second, the phenotypes were determined by an experienced obstetrician using established criteria. This process has been used since 1987 in the perinatal research unit in the course of other cohort studies, and no significant discrepancy occurred when compared with clinical assessment by 2 clinicians. Fourth, matching of cases and control subjects for several known risk factors of preeclampsia and/or essential hypertension minimized the influence of confounding factors. Fifth, genetic admixture,
which could artifactually cause apparent disease associations, was minimized because the population studied in the present project was composed of 98% whites and 93% French Canadians, according to a recent census. Moreover, we tested PE and CHT cases and control subjects separately for possible admixture with 9 unlinked polymorphisms by the method of Pritchard. This analysis revealed that the probabilities of only one subpopulation for PE cases and control subjects and CHT cases and control subjects were of 0.99586, 0.99590, 0.97064, and 1.0000, respectively.

Prevalence of chronic hypertension in our sample was estimated to be 6%. Chronic hypertension has been estimated to complicate 1% to 5% of pregnancies. Based on data from the National Health and Nutrition Examination Survey (1988 to 1991), prevalence among women between 18 and 29 years of age was estimated to be 0.6% to 2.0%, and it rises to 4.6% to 22.3% for women 30 to 39 years of age. Approximately 77% of women in our sample were younger than 30 years old, and the prevalence in this group of age was 5%, compared with 9% for women older than 30 years of age. The higher prevalence of chronic hypertension in the present sample might be explained by the fact that high-risk pregnancies in the eastern part of the province of Quebec were referred to our tertiary perinatal center.

Genotypes of Ile105Val of GSTP1 in CHT control subjects were not in Hardy-Weinberg equilibrium, nor were the genotypes of preeclampsia cases for SNP Thr2488Thr of APOB. This might be caused by sample bias, genotyping errors (not likely, given that Hardy-Weinberg equilibrium was observed in other groups with the same genotyping assay used and that all cases and control subjects were genotyped together), or alternatively, by small variations of genotype-associated disease risks. Further increase in sample size may permit identification of the at-risk genotypes, and this finding remains to be confirmed in an independent sample.

Validation of Published Associations
In this sample of young women, none of the tested associations previously reported was replicated neither for chronic essential hypertension nor for preeclampsia, although study of a subset of cases (HELLP syndrome) led to observation of a significantly increased risk for the Thr allele of Met235Thr. However, only 25 cases of HELLP syndrome were available for analysis. Noteworthy, our analyses showed adequate statistical power, as we had at least 0.8 power to detect odds ratios of studies that initially reported these associations, except for MTHFR and F5, which were, respectively, 0.75 and 0.52. However, for MTHFR, our sample was larger than that of initial study by 2-fold, and it was of similar size compared with the sample of the initial positive study of F5. Previous involvement of some genes in genetic susceptibility to PE or CHT may be attributed to small sample size. Association with these polymorphisms may still be present in the French Canadian population, but with a smaller relative risk than reported by previous studies in other populations. In addition, one may expect differences in the extent of linkage disequilibrium blocks between populations, and this could lead to a lower probability to replicate associations. In other words, if the initial study genotyped a polymorphism that is in linkage disequilibrium with the DNA variation truly responsible for the genetic susceptibility, then a decrease of linkage disequilibrium between these two polymorphisms in another population would prevent detection of the association. However, since published associations were reported in whites, and since linkage disequilibrium does not seem to vary substantially among white populations, it seems unlikely to explain our findings. However, variations in environmental factors between populations, such as salt intake, could have an effect on the expression of the susceptibility conferred by a particular genotype.

For chronic essential hypertension, since the present sample consists exclusively of women as opposed to variable proportions of men and women of other studies, gender could be part of the explanation for the nonreplication of published results. It is well known that men are more prone than women to have hypertension, and this might be due either to environmental and/or genetic influences. Furthermore, a study of ACE has shown an association with hypertension in men but not in women. Another explanation may be that hypertension has been studied mostly in adults older than reproductive age, whereas this study focused on younger women. These two groups are different in terms of time of disease onset. Consequently, different genetic susceptibility factors may explain early and late onset hypertension. However, a study investigating the polymorphism Met235Thr of AGT reported an association with essential hypertension in early-onset cases defined as <50 years old.

For preeclampsia, phenotype heterogeneity of the disease may have lowered our power to replicate previous studies. Inclusion in some studies of recurrent preeclampsia, pregnancy-induced hypertension without proteinuria, preeclampsia superimposed over chronic hypertension of various proportions of HELLP syndrome cases, and severe preeclampsia might have contributed to discrepancies between association studies. These subphenotypes differ in the evolution of the disease, which may involve different susceptibility genes, at least partly.

Association Study of Candidate Genes
We report the association of the polymorphism Thr174Met of the AGT gene with preeclampsia. We identified the frequent at-risk haplotype A-Met-Thr in our French Canadian population, but the use of the haplotypes did not bring much more information than the polymorphism Thr174Met alone, since this one is exclusively present on the identified at-risk haplotype in the studied population. Even if we applied a Bonferroni correction for the number of candidate polymorphisms tested for PE (5 polymorphisms, P=0.01), the association remains significant. Previous studies that linked the AGT gene to increased risk of preeclampsia reported an increased frequency of the Thr allele of the SNP Met235Thr, which was not replicated in the present study. Moreover, we observed the Thr allele of Met235Thr on 2 other major haplotypes in addition to the at-risk haplotype A-Met-Thr. Thus, polymorphism Met235Thr does not appear to account for association of Thr174Met with preeclampsia, and Thr174Met appears to explain the association with AGT in our population. This could evoke the involvement of multiple AGT susceptibility
haplotypes to preeclampsia, which expression in a given population depends on interactions with other genes and environmental factors. However, a single variant might explain the association with different polymorphisms, since in other populations, different polymorphisms of AGT may show significance, depending on the population structure and history. In that view, study of haplotypes should prove useful for a more comprehensive analysis of association results. Until the functional variant in AGT gene responsible for genetic susceptibility to preeclampsia is proven, all of the seemingly conflicting studies with different AGT polymorphisms may in fact replicate each other. Thus, our finding might be better viewed as a further supporting evidence for involvement of AGT in the susceptibility to preeclampsia. Actually, there is no functional data supporting Thr174Met as the AGT polymorphism responsible for susceptibility to preeclampsia. Moreover, the effect of a change of a threonine for a methionine at codon 174 of the AGT gene still remains to be established. However, the C allele of polymorphism A–20C is associated with an increase in plasma angiotensinogen levels and was shown to be almost exclusively associated with the at-risk haplotype identified in this study (data not shown). Therefore, A–20C could be a good candidate for the functional variant responsible for the association with preeclampsia. Interestingly, there is evidence of a local renin-angiotensin system near the uterine spiral arteries, and that decidual cells, usually surrounding these arteries, express renin and angiotensinogen. It could be speculated that expression of higher concentrations of angiotensinogen in decidual cells of women with the A-Met-Thr haplotype would lead to more pronounced vasoconstriction of spiral arteries and consequently to a higher risk of placenta hypoxemia. However, this will require inactivation and trophoblasts of spiral arteries, which leaves the arteries responsible to angiotensin II. But, as it is known that not every woman with failure of trophoblastic invasion will have preeclampsia, the AGT haplotype might predispose women with this abnormality to more severe placental hypoxemia and thus higher risk of preeclampsia. Alternatively, the A-Met-Thr haplotype might be associated with abnormality of spiral arteries, as previously suggested for allele Thr235.

Perspectives

Our study was unable to reproduce, in our population, previous findings of 10 associations with preeclampsia or essential hypertension, although it possesses adequate statistical power. This should emphasize the need for replication studies of sufficient statistical power. However, we report an association between haplotype A-Met-Thr (G1035A-Thr174Met-Met235Thr) of the AGT gene and preeclampsia. Consistently with other studies associating AGT polymorphisms with preeclampsia, our results support a role for AGT in the genetic susceptibility to preeclampsia. Further identification of linkage disequilibrium blocks around these polymorphisms should be performed in an effort to delimit sequences and genes containing the functional variant responsible for increased risk of preeclampsia.

Acknowledgments

The present study was supported by a grant of the Hospital for Sick Children Foundation (XG99-052) of Toronto, from the Canadian Institutes for Health Research (G1–60757) and Valorisation Recherche Québec (CETDeQ). S. Lévesque holds a Jessie Boyd & Charles Seriver MDC/PhD Stuentship Award granted by the Canadian Gene Cure Foundation, the Canadian Genetic Diseases Network (Federal Networks of Centers of Excellence), and the Canadian Institutes of Health Research. M.C. Roy holds a studentship of the Natural Sciences and Engineering Research Council of Canada. Dr Rousseau is a senior Scientist of Fonds de Recherche en Santé du Québec. We are thankful to Patricia Rouillard and Hugues Pelletier for expert technical assistance.

References


Implication of an AGT Haplotype in a Multigene Association Study With Pregnancy Hypertension
Sébastien Lévesque, Jean-Marie Moutquin, Carmen Lindsay, Marie-Claude Roy and François Rousseau

Hypertension. 2004;43:71-78; originally published online November 24, 2003;
doi: 10.1161/01.HYP.0000104525.76016.77
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/43/1/71

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2004/01/04/43.1.71.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/