Three Candidate Genes and Angiotensin-Converting Enzyme Inhibitor–Related Cough
A Pharmacogenetic Analysis

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Abstract—Unexplained, persistent cough limits the use of angiotensin-converting enzyme (ACE) inhibitors in a significant number of patients. It has been speculated that occurrence of this adverse effect is genetically predetermined; in particular, variants of the genes encoding ACE, chymase, and B2-bradykinin receptor have been implicated. To investigate this question, we determined genotypes for common polymorphisms for these three genes in subjects with a history of ACE inhibitor–related cough. Specificity of the adverse effect was confirmed by a blinded, double-crossover design protocol in which subjects were rechallenged with either lisinopril or placebo. In 99 case subjects and 70 control subjects (who failed to develop cough on rechallenge with ACE inhibitor) thus selected, frequencies for the ACE D and I alleles were 0.56 and 0.44 (cases) and 0.56 and 0.44 (controls), respectively; frequencies for chymase A and B alleles (absence/presence of BstXI site) were 0.56 and 0.44 (cases) and 0.46 and 0.54 (controls), respectively; frequencies for B2-bradykinin receptor + and − alleles (presence/absence of a 21 to 29 nonanucleotide sequence) were 0.52 and 0.48 (cases) and 0.53 and 0.47 (controls), respectively. All observed genotype frequencies were in Hardy-Weinberg equilibrium. There was no evidence for association between genotype at either gene examined and cough (adjusted for gender and age). Our data indicate that common genetic variants of ACE, chymase, and B2-bradykinin receptor do not explain the occurrence of ACE inhibitor–related cough. (Hypertension. 1998;31:925-928.)

Key Words: angiotensin-converting enzyme inhibitors • cough • angiotensin-converting enzyme inhibitors • chymase • receptors, bradykinin • polymorphism • genetics

Angiotensin-converting enzyme inhibitors are widely used for the treatment of hypertension and are presently the unchallenged drugs of choice for the treatment of congestive heart failure.1 The major adverse effect encountered with ACEI treatment, and the most frequent reason for withdrawal of the drug, is a persistent, dry (nonproductive) cough.2,3 So far, no specific risk factors or patient characteristics that would predict the occurrence of this cough have been identified, and the underlying mechanism remains unclear; however, it has been speculated that the kinogen-kinin (bradykinin) system may be involved: inhibition of ACE may result in a local accumulation of bradykinin, which in turn may lead to the activation of proinflammatory peptides (eg, substance P, phospholipase C and/or A2, prostaglandins, neuropeptide Y) and to local release of histamine in the airways.3,4 Because cough is a class effect of ACEIs, and because its occurrence is not predicted by any external factors, it seem reasonable to suspect that a primary, genetically determined characteristic resulting in alteration of drug action or drug metabolism may be responsible.4

A prime candidate for a protein that might interact differentially with ACEIs on the basis of genetic variation is, of course, ACE itself. Thus, the I/D polymorphism of ACE has recently been proposed as possibly being involved in ACEI-induced cough,4,5 a speculation fueled by the observation that the incidence of cough among patients under ACEI treatment is roughly similar to the proportion of the population that is homozygous for the ACE insertional allele (about 15% to 20%). More recently, a possible link between ACEI treatment–associated adverse effects and chymase has also been suggested: as chymase represents an alternative pathway for the activation of angiotensin II, it is possible that ACE inhibition may lead to an increased biological significance of this enzyme. Release of chymase from mast cells results in a range of profound proinflammatory changes; in the dermis, this is associated with skin rashes, whereas in the lungs bronchial and pulmonary infiltration with inflammatory cells and altered regulation of vasoactive peptides7,8 are observed. A recent report describing an association between a polymorphism of the gene encoding MCC and atopic eczema in a Japanese population,7 along with the fact that skin rashes are another adverse effect of ACEIs, therefore raises the possibility that such molecular variants of this enzyme may also play a role in ACEI-related cough.

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Even more recently, a possible link between ACEI treatment–associated adverse effects and B2-bradykinin receptor has also been suggested: since B2-bradykinin receptor mediates most of the inflammatory actions of bradykinin and is widely present in most tissues, a genetic defect of B2BKR may lead to altered biological activities of the functional protein. A recent report implicating an association of an exon 1 polymorphism on B2BKR and bradykinin-induced angioedema in patients with C1-inhibitor deficiency, as well as with the fact that angioedema is yet another side effect of ACEIs, strongly raises the possible involvement of B2BKR genotype in the underlying ACEI-induced cough mechanism and the clinical manifestations associated with ACEI treatment.

It was therefore of interest to test whether genetic polymorphisms of these three enzymes may be associated with incidence of ACEI-related cough. DNA samples collected in the course of a very carefully designed and executed clinical study allowed us to pursue this goal.

**Methods**

**Subject Selection**

Our study was based on the population entered into a previously described international multicenter trial that was set up to examine the association of cough with ACEI and with AT1 receptor blocker treatment, respectively. In brief, individuals who fulfilled the inclusion criteria (history of ACEI-associated cough that resolved when not taking medication) underwent a double-blinded, placebo-controlled crossover challenge with the ACEI lisinopril at a daily dose of 20 mg, for up to 6 weeks. Ninety-nine case subjects who developed cough during lisinopril challenge were classified as control subjects, placebo, were thus identified. Seventy individuals who did not develop cough during lisinopril challenge were classified as control subjects.

**ACE D/I Genotype Determination**

The details of ACE D/I genotype determination have previously been described. In brief, the D and I alleles were identified on the basis of PCR amplification of the respective fragments from intron 16 of ACE and by subsequent electrophoretic size fractionation and ethidium bromide visualization. Because the D allele in heterozygotes is preferentially amplified, all DD genotype samples were subjected to a second independent PCR amplification with a primer pair that recognizes an insertion-specific sequence to ensure accurate genotyping.

**MCC BstXI Genotype Determination**

Genotyping for the BstXI polymorphism, a G3255A variant in a region of the 5'-flanking sequence, involved a PCR-RFLP technique. The assay was carried out in whole blood with the use of GeneReleaser (Bioventures) according to the manufacturer’s recommendations. Reagent concentrations in the 15-mL PCR reaction were 330 mmol/L each for sense 5'-TGC CCC ACA TCA TTC ATT C-3' and reverse 5'-TCC GGA GCT GGA GAA CTC TTG T-3' primers, 166 mmol/L deoxynucleotide triphosphates, 2.5 mmol/L MgCl2, and 0.15 U of Taq polymerase. Samples were amplified for 36 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 56°C for 45 seconds, and extension at 72°C for 1 minute, followed by a final extension step at 72°C of 5 minutes. The resulting 645-bp PCR fragment was incubated at 55°C for 7 hours with 3 U of the restriction endonuclease BstXI (New England Biolabs), according to the manufacturer’s specification, and restriction fragments were identified on 2% ethidium bromide–stained agarose gels. Genotypic polymorphisms of MCC were defined as AA (homozygous for absence of restriction site), BB (homozygous for presence of restriction site), or AB (heterozygous).

**B2BKR +/- Genotype Determination**

Genotyping for the presence/absence of a 9-bp sequence, a 21 to 29 nonanucleotide variant 5'-GGTGTGGAC-3' in a region of the 5'-untranslated sequence, involved a radioactive-labeling/PCR technique. The assay was carried out in whole blood with the use of GeneReleaser (Bioventures) according to the manufacturer’s recommendations. Reagent concentrations in the 15-mL PCR reaction were 330 mmol/L each for sense 5'-CTG TTC CCG CCG CCA CTC CA-3' and reverse 5'-CAG AGG TGA GGC GCC TGG AG-3' primers, 166 mmol/L deoxynucleotide triphosphates, 3.0 mmol/L MgCl2, and 0.15 U of Taq polymerase. The sense primer was labeled at the 5' end with [32P]-γ-ATP. Samples were amplified for 36 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 62°C for 45 seconds, and extension at 72°C for 1 minute, followed by a final extension step at 72°C of 5 minutes. The reaction products were resolved over denaturing sequencing gels containing 6% polyacrylamide, 8 mol/L urea, and 30% formamide and visualized by autoradiography. Genotypic polymorphisms of B2BKR were defined as ++ (homozygous for presence of nonanucleotide sequence), -- (homozygous for absence of nonanucleotide sequence), or ++ (heterozygous).

To confirm genotype assignment, the PCR-RFLP procedure was performed on all samples on two separate occasions. PCR results were scored blinded as to case-control status.

**Statistical Analysis**

Alleles and genotype frequencies among case and control subjects were counted and compared by x² test with Hardy-Weinberg predictions. In addition, multivariate regression analysis was carried out, adjusting for age and gender, to determine the odds ratio for each genotype to predict the occurrence of cough. Data for each polymorphism were analyzed under assumptions of an additive, dominant, and a recessive model, respectively, using a two-tailed value of P<.05 as the criterion for statistical significance.

**Results**

**Study Sample Characteristics**

There were 47 men and 52 women among the case subjects and 24 men and 46 women among the control subjects. Mean±SD ages of case and control subjects were not different (57.0±10.1 and 58.4±10.1 years, respectively; P=.37).

**ACE D/I Polymorphism**

Allele frequencies for D and I alleles were 0.56 and 0.44 in case subjects and 0.56 and 0.44 in control subjects, respectively. Genotype frequencies were 0.232 for II, 0.414 for ID, and 0.354 for DD in cases; 0.171 for II, 0.529 for ID, and 0.300 for DD in controls; and 0.226 for II, 0.445 for ID, and 0.329 for DD in both groups combined. Genotype frequencies were scored blinded as to case-control status.
did not deviate from the Hardy-Weinberg equilibrium in controls \((\chi^2_{df}=0.30, P=0.86)\), cases \((\chi^2_{df}=1.33, P=0.51)\), or the whole study group \((\chi^2_{df}=0.30, P=0.86)\). No overall difference in genotype distribution was seen among cases and controls \((\chi^2_{df}=2.25, P=0.32)\). Logistic regression analysis assuming additive \((DD\text{ versus } DI\text{ versus } II)\), dominant \((DD\text{ and } DI\text{ versus } II)\), or recessive \((DD\text{ versus } DI\text{ and } II)\) inheritance likewise failed to reveal any significant association between phenotype and genotype, with ORs (95% CIs) of 0.196 for \(AA\) versus \(AB\) and 0.196 for \(BB\) versus \(AB\), respectively. Genotype frequencies were 0.333 for \(AA\) in case subjects and 0.46 and 0.54 in control subjects, respectively. Again, genotype frequencies did not deviate from the Hardy-Weinberg equilibrium in controls \((\chi^2_{df}=0.98, P=0.30, 0.64\text{ to }1.50)\), 0.69 (0.32 to 1.52), and 1.28 (0.66 to 2.46), respectively.

\(\text{MCC BstXI Polymorphism}\)

Allele frequencies for \(A\) and \(B\) alleles were 0.56 and 0.44 in case subjects and 0.46 and 0.54 in control subjects, respectively. Genotype frequencies were 0.333 for \(AA\), 0.444 for \(AB\), and 0.222 for \(BB\), respectively, in case subjects; and 0.196 for \(AA\), 0.518 for \(AB\), and 0.286 for \(BB\), respectively, in control subjects. Again, genotype frequencies did not deviate from the Hardy-Weinberg equilibrium in controls \((\chi^2_{df}=0.061, P=0.97)\), cases \((\chi^2_{df}=0.55, P=0.76)\), or the whole study group \((\chi^2_{df}=0.21, P=0.90)\). Also, no overall difference in genotype distribution was seen among cases and controls \((\chi^2_{df}=3.71, P=0.16)\). Logistic regression analysis assuming additive \((AA\text{ versus } AB\text{ versus } BB)\), dominant \((AA\text{ and } AB\text{ versus } BB)\), or recessive \((AA\text{ versus } AB\text{ and } BB)\) inheritance likewise failed to reveal any significant association between phenotype and genotype, with ORs (95% CIs) of 1.47 (0.94 to 2.27), 1.41 (0.68 to 2.91), and 1.98 (0.96 to 4.10), respectively.

\(\text{B2BKR +/− Polymorphism}\)

Allele frequencies for \(+\) and \(−\) alleles were 0.52 and 0.48 in case subjects and 0.53 and 0.47 in control subjects, respectively. Genotype frequencies were 0.252 for \(++\), 0.525 for \(++\), and 0.223 for \(+-\) in cases; 0.257 for \(++\), 0.543 for \(++\), and 0.200 for \(+-\) in controls; and 0.254 for \(++\), 0.532 for \(++\), and 0.214 for \(+-\) in both groups combined. Genotype frequencies did not deviate from the Hardy-Weinberg equilibrium in controls \((\chi^2_{df}=0.26, P=0.88)\), cases \((\chi^2_{df}=0.19, P=0.91)\), or the whole study group \((\chi^2_{df}=0.43, P=0.81)\). No overall difference in genotype distribution was seen among cases and controls \((\chi^2_{df}=0.12, P=0.94)\). Logistic regression analysis assuming additive \((−−\text{ versus } +−\text{ versus } ++)\), dominant \((−−\text{ and } +−\text{ versus } ++)\), or recessive \((−−\text{ versus } +−\text{ and } ++)\) inheritance likewise failed to reveal any significant association between phenotype and genotype, with ORs (95% CIs) of 1.03 (0.65 to 1.62), 0.99 (0.49 to 2.02), and 1.09 (0.51 to 2.34), respectively.

\section*{Discussion}

The present study represents the first genetic investigation of a possible association between genetic variants of the \(ACE\), \(MCC\), and \(B2BKR\) genes and ACEI-related dry cough. While there is no previous evidence for a familial aggregation or transgenerational transmission of this phenotype (understandably so, because of how recently the phenomenon has been recognized), failure to find any association between its occurrence and hitherto observed clinical variables suggests genetically determined susceptibility as one possible explanation. Our data fail to provide any evidence for the existence of such an association with the genes/polymorphisms tested.

Among the strengths of our study is the very careful design which, based on positive response to rechallenge with drug and on negative response to placebo, removes as much as possible a major factor of uncertainty (namely, the specificity of the ACEI causation of cough) from the investigation and therefore adds power to the study. On the other hand, the stringent criteria required for inclusion into the study also resulted in a sample of only modest size. Given the number of subjects available, therefore, we reach 80% power in the additive models to rule out ORs of 1.87, 1.87, and 1.94 for \(ACE\), \(MCC\), and \(B2BKR\), respectively; for dominant or recessive models, power is less. Thus, we can be confident that by measure of the association of the polymorphisms tested, none of the three genes contributes materially to the cough phenotype in this sample, which was highly selected to allow recognition of true pathogenic factors over random noise. The observation that allele frequencies for \(ACE\) and \(B2BKR\) variants are in agreement with previously reported data in white subjects\(^{10,12}\) provides additional support for the validity of our study.

It is important to recognize that association studies like the present one only examine the possible association between phenotype and the actually tested polymorphism; such studies cannot exclude that examination of a different polymorphism (which would of course by definition have to be in linkage disequilibrium with the one tested) might obtain different results.

The negative results regarding the \(MCC\) and \(B2BKR\) polymorphisms signal—within the constraints of statistical power—that these genes (or at least the particular polymorphisms studied) do not play an important role in ACEI-related cough in our study population. However, because the original observation of an association between the \(MCC\) marker and atopic conditions was made in Japanese subjects, and the \(B2BKR\) variant and bradykinin-induced angioedema was made in patients with C1-inhibitor deficiency, it is possible that racial/ethnic differences as well as selection of disease phenotypes may also account for our data. In addition, of course, the hypothesis that there may be a common denominator for the susceptibility to atopic skin rashes, to C1-inhibitor deficiency–associated angioedema, and to ACEI-related cough is a speculative one.

Because expression of ACEI-related dry cough likely represents a multifactorial phenomenon that involves a complex interaction of multiple interrelated factors, the possibility of the involvement of other genes that might be responsible for the proinflammatory cascade of actions exists, and larger studies will be needed to further explore the possibility of genetic predisposition in ACEI-related dry cough.

In conclusion, we found no association of the three polymorphisms tested with ACEI-related dry cough in a highly selected group of subjects. However, the present study certainly presents one of the potential applications in defining pharmacogenetics in drug therapy and will attract more attention to better understanding of drug design, as well as development, in the near future.
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