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Lack of Association Between Temporal Lobe Epilepsy and a Novel Polymorphism in the Alpha 2 Subunit Gene (ATP1A2) of the Sodium Potassium Transporting ATPase

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Genetic linkage studies in rodents and humans have identified specific chromosomal regions harboring seizure susceptibility genes. We have identified a novel polymorphism in the human alpha 2 subunit gene (*ATP1A2*) of the sodium potassium transporting ATPase (NaK-pump), a candidate gene for human temporal lobe epilepsy (TLE) based on its chromosomal location and function in ion homeostasis. The polymorphism consists of a four base pair insertion 12 base pairs upstream of the start of exon 2. We performed an association study between this polymorphism and TLE. Our study did not find a significant difference in the frequency of this polymorphism between TLE patients and controls, indicating that this variation is not a major susceptibility factor. However, since the number of patients studied so far is small and the functional consequence of the polymorphism is unknown, the variation may yet be found to play a minor role in increased risk for seizure susceptibility. In contrast to the findings in TLE patients and controls, we did find a significant difference in the frequency of the variation between African Americans and persons of European descent. This finding demonstrates the potential effect of population stratification on studies of this type and supports the growing use of parental and familial samples for controls in association studies. Further study of this polymorphism is warranted as it may be involved in other disease processes for which there are known ethnic-specific susceptibilities. Am. J. Med. Genet. (Neuropsychiatr. Genet.) 96:79–83, 2000.

KEY WORDS: temporal lobe epilepsy; sodium potassium transporting ATPase; association study; polymorphism; genetics.

INTRODUCTION

Human diseases such as hypertension, Alzheimer's disease and epilepsy are multifactorial in nature [Risch and Merikangas, 1996]. Predisposing genetic factors and their interaction with environmental influences contribute to the onset and progression of these diseases in complex ways that vary among individuals [Risch and Merikangas, 1996]. Advances in molecular and statistical genetics have led to the development of means to examine the relationships between specific gene sequence variation and signs and symptoms of disease [Spielman and Ewens, 1996; Spielman et al., 1993]. One means of focusing the search for human disease susceptibility genes involves using information from genetic animal models of homologous human phenotypes. In our laboratories the study of genetic susceptibility to seizures in mice [Ferraro et al., 1997, Ferraro et al., 1999) has led to the identification of candidate genes for epilepsy. These data from murine quantitative trait loci mapping and congenic animal studies identify a region of distal murine chromosome 1 as harboring a gene or genes that contribute to both generalized and focal seizure susceptibility. These results are in agreement with recent human epidemiological evidence demonstrating the existence of com-

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mon predisposing factors between generalized and localized seizure phenotypes [Ottman et al., 1998]. This region of murine chromosome 1 is syntenic with a region of human chromosome 1 (1q21-23 and 1q41-44), and interestingly, humans with deletions in this region suffer from benign Rolandic epilepsy, a focal seizure disorder [Vaughn et al., 1996]. Therefore, we have begun to identify chromosome 1 candidate gene polymorphisms in humans with both focal and generalized seizure disorders. Although our patients with temporal lobe epilepsy exhibit distinctly different clinical phenotypes compared to those individuals with chromosome 1 deletions, these combined data from murine and human studies support the notion that a seizure susceptibility locus is present on mouse and human chromosome 1.

One prominent candidate gene that has emerged from the murine models in our lab is *ATP1A2*, an alpha subunit of the NaK-pump. The *ATP1A2* gene maps to the seizure susceptibility region on distal murine chromosome 1 and to the syntenic region of human chromosome 1 [Yang-Feng, et al., 1988]. In addition, *ATP1A2* is a viable candidate gene based on published murine and human epilepsy studies that document a key role for genes that code for proteins involved in ion homeostasis [reviewed in Ferraro and Buono, 1999].

The NaK-pump regulates cation transport across the plasma membrane and is essential for survival and function of neural, cardiac, renal, hematopoetic, and other cell types [Gloor 1997; Lingrel and Kuntzweiler, 1994; Stengelin and Hoffman, 1997]. The NaK-pump contains three subunits (alpha, beta, gamma) that associate in the plasma membrane and control the active transport of sodium and potassium ions coincident with ATP utilization. The alpha subunit contains the ATP hydrolysis domain as well as the cation binding and transport sites [Lingrel and Kuntzweiler, 1994; Wang et al., 1996]. The beta subunit is responsible for membrane anchoring, proper folding of the alpha subunit, and also affects cation binding and transport [Ueno et al., 1997]. The gamma subunit has a putative functional role of modulating the K+ activation of the NaK-pump [Beguin et al., 1997; Kim et al., 1997]. Three separate alpha subunits (possibly a testes-specific fourth) and three beta subunits have been identified; all are encoded by separate genes [Yang-Feng et al., 1988].

The potential association of a NaK pump gene subunit polymorphism with epilepsy is provocative given the fundamental role of the ATPase enzyme in regulating neuronal membrane potential and hence neuronal firing [Gloor, 1997; Lingrel and Kuntzweiler, 1994]. Since the NaK pump plays a critical role in ion homeostasis in most neural and epithelial cell types, it is reasonable to suggest that polymorphisms in genes encoding subunits of that protein may be associated with diseases such as epilepsy and hypertension. To further investigate this hypothesis we have begun to evaluate variations in the *ATP1A2* gene in individuals with temporal lobe epilepsy (TLE). During the course of identifying variations in this gene, a four base pair insertion was detected at an intron/exon boundary, 12 bp 5′ to the first coding nucleotide of exon 2. This intronic region, known as a poly-pyrimidine tract, consists of 10–12 consecutive pyrimidine (T or C) residues immediately preceding an exon and is critical for proper mRNA splicing. In the present study we report lack of association between the insertion polymorphism and TLE; however, we did find that the polymorphism is overrepresented in African Americans compared with persons of European descent.

MATERIALS AND METHODS

Blood samples (~ 50 mL) were taken from normal, healthy volunteers with no history of seizure, and DNA was extracted using Qiagen column purification following the manufacturer's protocols. Tissue samples were obtained from patients with TLE who underwent anterior temporal lobectomy. Inclusion criteria and surgical procedures for anterior temporal lobectomy have been described [Engle et al., 1997; Sperling et al., 1996]. Patients had medically refractory complex partial seizures and magnetic resonance imaging of the brain usually showed unilateral temporal lobe atrophy. Specimens were collected during anterior temporal lobectomy from the second and third temporal gyri and underlying hippocampus. Resection began 2–3 cm caudal to the temporal pole and approximately 2–3 cm of tissue was removed. The specimens were immediately frozen in liquid nitrogen in the operating room and stored at −70°C for subsequent DNA, RNA, and protein extraction. DNA was subsequently isolated from frozen tissue by standard methods of phenol/chloroform extraction and ethanol precipitation [Sambrook et al., 1989]. Additionally, some tissues were fixed for 1 hour in 4% para-formaldehyde in a 0.2 M Na cacodylate buffer (pH 7.2) on ice then stored at 4°C in buffered saline for subsequent embedding and sectioning for in situ protein and RNA localization.

Proper informed consent was obtained from all individuals in accordance with Institutional Review Board (IRB) procedures. During the course of taking family histories 18 of the 56 TLE patients (32%) reported a positive family history for epilepsy and identified nine first-degree relatives with seizure disorder.

Total genomic DNA was used as a template for the polymerase chain reaction (PCR), and primers were derived using the genomic sequence of the human *ATP1A2* gene contained in the Genbank database (accession #J05096). Primers flanking exon 2 were synthesized as follows: forward primer beginning at bp 6687, 5′ TGA CTC TCC GGC TCT CCC 3′; reverse primer beginning bp 6835, 5′ GCC CAG CAG TTC CCT CAC 3′. PCR was performed in a HybAid thermocycler (OMN-E) using 100–200 ng of template DNA in the presence of 100 ng of each primer, 2-mM dNTPs, 1.5 mM MgCl, and 1 unit of Taq polymerase in 50 μL total volume. The PCR program began with a 5-min incubation at 94°C for DNA denaturation followed by thermocycling for 35 cycles of 94°C, 55°C, and 72°C steps. Each step was held for 1 min. A final extension step was performed at 72°C for 7 min. PCR products were analyzed by nondenaturing polyacrylamide gel electrophoresis and visualized by ethidium bromide staining. Amplicons of the appropriate size (148 bp) were iso-

lated from the gel by diffusion and ethanol precipitation and ligated into the pGEMT vector (Promega) following manufacturers protocols. After ligation, recombinant clones were propagated by transformation of JM109 *E. coli* and minipreps were performed on bacterial cultures (Promega Wizzard) to isolate recombinant plasmid. Plasmids were sequenced by the University of Pennsylvania Genetics Core Sequencing Facility using ABI/Perkin Elmer automated fluorescent dideoxy-terminator cycle sequencing equipment. Plasmid sequencing was primed using the T7 and SP6 sites on the pGEMT vector and each clone was isolated at least two times, by two separate PCR reactions.

Once the four base insertion was identified and confirmed by sequencing, the screening procedure was modified so that PCR products were radiolabeled and analyzed by denaturing polyacrylamide sequencing gels (7 M urea, 8% acrylamide) and autoradiography. Under these conditions, four base differences in PCR products are readily detectable, providing a rapid method of screening individuals for the polymorphism (see Fig. 1). For this analysis, the forward primer was end labeled using gamma P^{32} ATP (3000Ci mM NEN/Dupont) and T4 polynucleotide kinase following manufacturers protocols (Promega End Labeling Kit). End-labeled primer was separated from unincorporated P^{32} by passage through a Sephadex G-25 5prime-3prime Inc. Boulder CO, USA spin column and used in the PCR mix described above except that the final reaction volume was 11 μL and 45 ng of template DNA was used. Sequencing gels were poured and run according to standard protocols. Gels were scored independently by two readers, and data were analyzed using chi-square distribution statistics.

RESULTS

The insertion polymorphism was first discovered in DNA isolated from an African American epilepsy patient who had undergone anterior temporal lobectomy. DNA extracted from biopsied tissue was amplified by PCR and the amplicon was subcloned and sequenced in both directions. Four additional bases (TTCC) were found inserted at base pair position 6704 of the human ATP1A2 sequence and we designated this polymorphism as ins-6704/TTCC. PCR reactions were then performed on DNA from this individual and analyzed again both by direct sequencing and by the radiolabeled, sequencing gel method as described above to confirm the presence of the insertion. The insertion was identified by the electrophoretic method in an additional group of individuals ($n = 6$) and was further analyzed by subcloning and sequencing exon 2 and the surrounding intronic regions. In each case the variation was confirmed as being ins-6704/TTCC.

Currently our data do not provide support for a significant difference in the occurrence of the insertion in TLE patients 15/56 (4 African Americans, 52 Caucasians) compared with controls matched for ethnic background 16/56 (four African Americans, 52 Caucasians) chi-square = 0.013, $p = 0.90$. However, as control individuals were genotyped for the insertion it became evident that the frequency of occurrence in African Americans (50/61, 82%) was significantly greater than that found in persons of European descent (23/63, 37%, chi-square = 24.6, $p < 1 \times 10^{-6}$). Distribution of the alleles in these populations was consistent with calculated Hardy-Weinberg equilibrium.

DISCUSSION

The documentation of genetic variation in human genes is of growing importance. Naturally occurring variations account for both genetic susceptibility to human diseases and individual differences in drug efficacy, tolerance, and side effects [Kleyn and Vessell, 1998]. Only two studies attempting to associate candidate gene polymorphisms with common forms of epilepsy have been published, and candidates chosen for study encoded receptor subunits for the major excitatory [glutamate, Sander et al., 1997] and major inhibitory [GABA, Guipponi et al., 1997] neurotransmitters in the CNS. Although polymorphisms in the kainate type glutamate receptor subunit gene were positively associated with juvenile absence epilepsy [Sander et al., 1997], a lack of association was reported between polymorphisms in the *GABAA5* and *GABAB3* genes and juvenile myoclonic epilepsy [Guipponi et al., 1997]. In our laboratories we have concentrated on identifying chromosomal regions linked to seizure susceptibility phenotypes in murine models that mimic both generalized and focal epilepsy. These data have provided an alternative method for generation of compelling candidate genes for analysis in human epilepsy patients; one

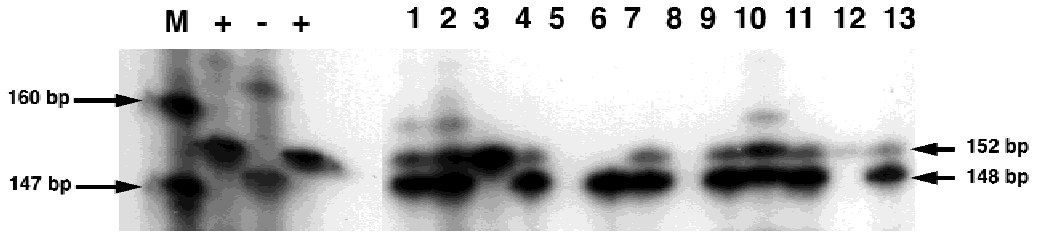

Fig. 1. Human genotypes for the *ATP1A2* ins-6704/TTCC analyzed by denaturing sequencing gel electrophoresis. Radiolabeled PCR products including exon 2 and ~30 bp of 5′ and 3′ intronic DNA were amplified yielding allele sizes of 148 and 152 bp (insertion). This scanned X-ray film shows the results of analyzing DNA from 13 African Americans with no history of seizure disorder **(lanes 1–13).** The three possible genotypes are shown: homozygous for the insertion (152 bp), **lanes 3** and **12;** homozygous for the common sequence 148 bp), lane 6; and heterozygous, **lanes 1, 2, 4, 7, 9–11,** and **13. Lane M** contains DNA molecular weight markers, radiolabeled *Msp*I-digested pBR322 fragments. Lanes marked **+** show the 152-bp allele amplified from a fragment with the insertion after it was subcloned into pGEMT; the lane marked **-** shows the 148-bp allele amplified from a recombinant plasmid clone containing the common sequence.

such region of interest is found on murine and human chromosome 1. We have begun collecting DNA from patients with either generalized or focal seizure disorders to identify polymorphisms in these chromosome 1 candidate genes. DNA is usually extracted from a blood sample; however, the resected brain material from the TLE patients not only provides us with genomic DNA, but, more important, the tissue is a source of RNA. This RNA is extremely valuable because the genomic structure is unavailable for many of the candidate genes we are examining. Thus, reverse transcription of the mRNA from human brain samples allows us to perform mutation scanning of candidate gene cDNAs.

Whereas the existence of an inherited component for generalized epilepsy is not in question, the evidence for a genetic component to focal epilepsy is less clear. Recent epidemiological evidence [Ottman et al., 1997] combined with our own observations of positive family history in our TLE patients supports the hypothesis that there are common genetic influences underlying susceptibility to both generalized and focal epilepsy. For example, in this study we found that nine first-degree relatives of the 56 TLE probands had a seizure disorder. If we assume that epilepsy occurs in 1–2% of the general population, and that each proband has on average five first-degree relatives, then we would have expected to see only 3–6 affected first-degree relatives.

We have identified a novel polymorphism in a candidate gene, *ATP1A2*, and have tested the association of that variation with TLE. At the present time, our data do not support an involvement of the ATP1A2 ins-6704/TTCC in temporal lobe epilepsy; however, the numbers of patients examined to date are relatively low. In the future, it will be important to confirm this preliminary conclusion with greater numbers of TLE patients as well as evaluate the potential role of this polymorphism in other types of epilepsy. Furthermore, the functional significance of the polymorphism remains to be determined. The insertion may affect proper mRNA splicing and hence expression of the *ATP1A2* gene. Experiments to test this hypothesis are in progress. Thus, it is possible that this variation may yet be found to subtly increase the risk for vulnerability to seizures.

In the course of studying the potential involvement of ATP1A2 polymorphisms in temporal lobe epilepsy, we discovered a striking disparity between the frequency of the ATP1A2 insert in persons of European descent and African Americans. The existence of ethnic differences in gene sequence variation carries several implications for human genetic studies of disease susceptibility. First and foremost it emphasizes the need for careful matching of experimental and control individuals with regard to ethnic background. This point cannot be overemphasized since mismatching could easily lead to false positive results in case-control population genetic studies. Additionally, the existence of ethnic differences in gene variation opens new avenues of inquiry regarding disease susceptibility genes since a number of medical conditions are found at much higher incidence in specific populations distinguished by genetic background [Lockette et al., 1995]. In light of present results, it could be hypothesized that the difference in frequency of the ATP1A2 ins-6704/TTCC between persons of European descent and African Americans is related to the well-documented increased incidence of hypertension in African Americans [Lockette et al., 1995]. The role of the NaK pump in renal ion homeostasis makes genes encoding subunits of the ATPase compelling candidates for genetic studies of blood pressure regulation. In fact, previous work in a genetic animal model of hypertension identified NaK pump genes as candidates underlying quantitative trait loci for control of blood pressure [Kobayashi et al., 1995; Samani et al., 1996]. Thus, evaluation of the ATP1A2 ins-6704/TTCC in African Americans with and without high blood pressure appears warranted and may provide insight into genetic control of cardiovascular function and mechanisms of hypertension.

In summary, we have identified a novel polymorphism and tested its association with TLE. Although this specific polymorphism does not appear to be associated with TLE phenotype, the gene remains a viable candidate, as neither gene regulation nor function was tested. We have identified several other polymorphisms in this gene and will expand our analysis to search for association of complex haplotypes with TLE and generalized epilepsies by using linkage disequilibrium between these novel markers in the *ATP1A2* gene.

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