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RESEARCH ARTICLE

Rare Missense Variants in *ATP1A2* in Families With Clustering of Common Forms of Migraine

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Migraine is a recurrent neurovascular disease. Its two most common forms—migraine without aura (MO) and migraine with aura (MA)—both show familial clustering and a complex pattern of inheritance. Familial hemiplegic migraine (FHM) is a rare monogenic subform caused by mutations in the calcium channel gene *CACNA1A* or the Na^+/K^+-ATPase gene *ATP1A2*. An involvement of FHM genes in the pathogenesis of common forms of migraine is not proven. We therefore systematically screened *ATP1A2* in families with several members affected by MA and/or MO. We identified two novel missense alterations [c.520G > A (p.E174 K) and c.1544G > A (p.C515Y)] in two out of 45 families, which were not found in 520 control chromosomes. Functional studies of these variants in *Xenopus* oocytes by two-electrode voltage clamp measurements and radiochemical determination of ATPase activity showed that C515Y leads to a complete loss of function comparable with the effect of FHM-mutations whereas for E174 K no functional alteration could be found in the in vitro assays. In conclusion we propose that rare variants in *ATP1A2* are involved in the susceptibility to common forms of migraine, because of 1) the absence of alterations in controls, 2) the particular pattern of segregation in both families, 3) the high conservation of mutated residues in Na^+/K^+-ATPases, 4) the functional effect of C515Y, and 5) the involvement of *ATP1A2* in a monogenic form of migraine. Hum Mutat 26(4), 315–321, 2005. **© 2005 Wiley-Liss, Inc.**

KEY WORDS: Na^+/K^+-ATPase; *ATP1A2*; migraine; complex diseases

INTRODUCTION

Migraine is a frequent disorder of the central nervous system, with a 1-year prevalence of about 12% [Lipton et al., 2001; Rasmussen and Olesen, 1992], and according to the Global Burden of Disease Study by the World Health Organization it is one of the leading causes of disability [Menken et al., 2000]. It is characterized by severe headache attacks and reversible autonomic and neurological symptoms [Goadsby et al., 2002; Palotie et al., 2002; Silberstein, 2004]. There are different forms of migraine, which are classified by the International Headache Society (IHS) criteria [Olesen, 1988; Olesen et al., 2004]. The two most common types are migraine without aura (MO, 70–80%) and migraine with aura (MA, 20–30%). Both forms have a strong genetic basis with complex inheritance [Estevez and Gardner, 2004; Palotie et al., 2002], but according to recent epidemiological data the genetic background of MA is stronger than that of MO [Russell and Olesen, 1995]. Recently, several loci for MA and MO have been identified by genome-wide linkage analyses in single extended pedigrees [Carlsson et al., 2002; Soragna et al., 2003] or a collection of multiplex families (MIM#s 157300, 607498, and 607501) [Bjornsson et al., 2003; Cader et al., 2003; Wessman et al., 2002]. Yet the genes involved in common forms of migraine are unknown. Candidate gene studies have resulted mainly in conflicting or unreplicated results [reviewed in Estevez and Gardner, 2004; Palotie et al., 2002]. Familial hemiplegic migraine (FHM; MIM#s 141500 and 602481) is a rare autosomal-dominant subform of migraine that is mainly characterized by additional episodes of reversible hemiplegia. Two causative FHM genes have been identified: 1) the α-1 subunit of the voltage-gated Ca^{2+}-channel CACNA1A (MIM# 601011) [Ophoff et al., 1996], and 2) the α-2 subunit of the Na^+/K^+-ATPase ATP1A2 (MIM# 182340) [De Fusco et al., 2003], (MIM# 182340). The role of

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CACNA1A in common forms of migraine has been studied primarily by linkage analyses, and the results have been inconclusive [reviewed in Estevez and Gardner, 2004; Palotie et al., 2002]. In a recent study [Jen et al., 2004], no mutations in *CACNA1A* or *ATP1A2* were found by DHPLC analyses in approximately 40 families with MA or MO, and no further studies regarding the role of *ATP1A2* in common forms of migraine have been performed to date.

MATERIALS AND METHODS

Patients

All of the participants in this study were diagnosed according to the revised criteria of the IHS [Olesen et al., 2004]. They provided written informed consent and the study was approved by the local ethics committees in Bonn and Kiel. All patients and family members were interviewed personally or by telephone by an experienced neurologist, and a detailed questionnaire was filled out for each of them. The questionnaire included a comprehensive assessment of 1) the type, frequency, location, and duration of aura symptoms; 2) the possible existence of motor symptoms, such as weakness or hemiplegia; 3) the properties, frequency, location, and duration of the headache; 4) the patient's medication history; 5) the existence of possible other diseases; and 6) the family's medical history. All families were enrolled by an index case with MA. All of the families had at least one further member affected by MA and/or MO, and in all but one family there were at least two members affected by MA. The average and maximum number of affected individuals in one family was 3.5 and 10, respectively. Blood samples were taken from all of the patients and participating family members nationwide, and genomic DNA was extracted by standard techniques.

Family A. The index patient (Fig. 1A; Patient IV-1) suffers from MA. The visual aura consists of unilateral flickering (seeing white spots) for about 10 min. Furthermore, she has unilateral side-changing tingling sensations (paresthesias) for 5 min on her arms (particularly fingers and hands), legs, and face (particularly in the perioral region). These symptoms precede every migraine headache. In addition, in 20% of the attacks she has speech difficulties (dysarthria) for 60 min. She has never experienced any kind of motor weakness or hemiplegia. The unilateral headache follows the aura after 20 min. Her migraine attacks (12 attacks per year) last up to 12 hours and are accompanied by nausea, vomiting, and photo- and phonophobia. Her monozygotic twin (Fig. 1A; Patient IV-2) has unilateral visual disturbances (seeing black and white spots and a scintillating scotoma) for 30 min that precede every migraine headache. Furthermore, she has unilateral paresthesias on her arms (side-changing) and face. In 10% of her

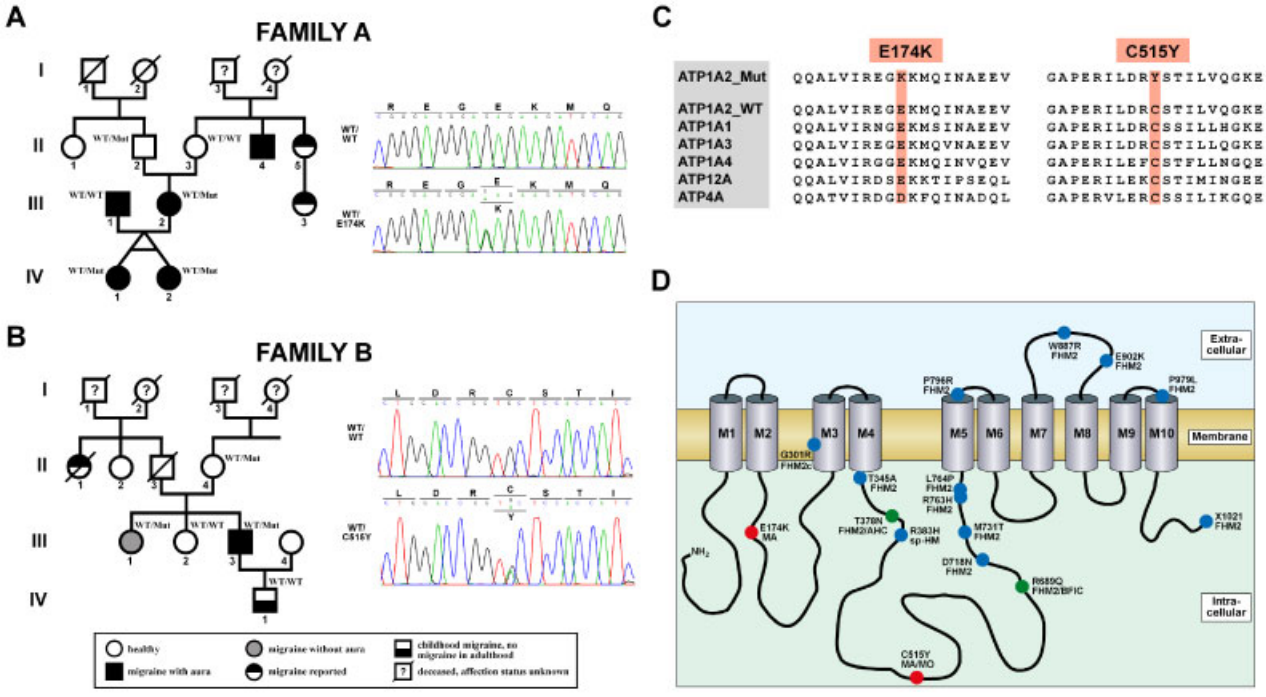


FIGURE 1. **Sequence alterations in *ATP1A2* in families with common forms of migraine. A and B: Pedigrees of Families A and B (left part) and direct sequencing results of mutation carriers (lower right) and controls (upper right). Open triangle in Family A denotes monozygotic twins, squares indicate males, circles denote females, and other pedigree symbols are explained in the box at the bottom. For all family members analyzed, the mutation status for E174 K (Family A) and C515Y (Family B) is given next to the symbol, WT/WT = wild-type on both alleles, WT/Mut = heterozygous mutation carriers. Above sequencing traces the amino acid translation is given in the one-letter code. C: Multiple sequence alignment of the four different human α Na^+/K^+-ATPase subunits (ATP1A1-ATP1A4) and the human nongastric (ATP12A) and gastric α H^+/K^+-ATPase (ATP4A), all belonging to the P_{2C}-ATPase family (see also Axelsen and Palmgren [1998] and the P-type ATPase database). Concerning residue 174 of ATP1A2 the negative charge is conserved, and concerning residue 515 the cysteine is perfectly conserved. The alignment was performed with the use of Clustal_X software with default parameters. D: Predicted transmembrane topology of ATP1A2 [Kaplan, 2002] and location of known mutations [Bassi et al., 2004; De Fusco et al., 2003; Jurkat-Rott et al., 2004; Kaunisto et al., 2004; Spadaro et al., 2004; Swoboda et al., 2004; Vanmolkot et al., 2003]. Novel alterations identified in common forms of migraine are depicted in red, and published FHM2 mutations are shown in blue. This includes a variant (R383 H) identified in a patient with sporadic hemiplegic migraine (sp-HM) [Jurkat-Rott et al., 2004] and a mutation (G301R) leading to FHM with cerebellar signs (FHM2c) [Spadaro et al., 2004]. A mutation (R689Q) leading to FHM and a form of epilepsy (benign familial infantile convulsions (BFIC), [Vanmolkot et al., 2003] and a mutation (T378N) leading to FHM and/or alternating hemiplegia of childhood (AHC) [Bassi et al., 2004; Swoboda et al., 2004] are given in green.**

attacks she has speech problems for 60 min. Like her sister she has never experienced any motor weakness or hemiplegia. After 30 min the unilateral side-changing pulsating headache begins in the frontal and temporal areas and is worsened by physical activity. The headache is accompanied by nausea, photo- and phonophobia, and sensitivity to strong odors. The migraine attacks (six attacks per year) usually last 12 hours. Her mother (Fig. 1A; Patient III-2) is also affected by MA. Her attacks (12 attacks per year) are characterized by distorted vision ("looking through a broken mirror") for 30 min and occasionally are accompanied by unilateral, side-changing numbness in the arms, legs, and face. Frequently a disturbance of language occurs with her migraine attacks. The pulsating, unilateral side-changing headache may last up to 12 hours, during which time she is sensitive to light, sound, and odors. The pain is worsened by physical activity. She has never had a motor weakness or hemiplegia. The maternal grandfather (Fig. 1A; Patient II-2) is healthy and never suffered from headaches or hemiplegia, and no cases of migraine are known in his family. The maternal grandmother (Fig. 1A; Patient II-3) has never reported having headaches; however, she is affected by late-onset dementia. In her family, a brother (Fig. 1A; Patient II-4) is affected by MA, and a sister (Fig. 1A; Patient II-5) and her daughter (Fig. 1A; Patient III-3) are reported to have migraine; however, neither of them wanted to participate in the study. The father of the index (Fig. 1A; Patient III-1) has a history of MA since age 18 characterized by an aura of "black and white spots" for 45 min. He has had no motor weakness or hemiplegia. The pain, which begins 30–60 min after the end of the aura, localizes mainly in the fronto-temporal area and is worsened by physical activity. He has six attacks a year, and a single attack lasts about 5 hours. No other cases of migraine in his family were reported.

Family B. The index (Fig. 1B; Patient III-3) has migraine with an aura including aphasia and acalculia, and sometimes has additional visual symptoms with flickering scotoma. He has never had any paresthesias or signs of motor weakness. The aura symptoms typically last for 60–120 min, occur about twice a year, and are accompanied by nausea. The headache is unilateral and pulsating, and lasts for up to 3 days. His son (Fig. 1B; Patient IV-1) suffered from childhood migraine without aura between the ages of 6 and 12 years, which stopped after he reached puberty. His headache was mainly bilateral and occipitally located. A sister of the index (Fig. 1B; Patient III-1) is affected by migraine without aura, which first manifested at the age of 20 years and occurs at least twice a month for up to 48 hours. Her holocephalic or unilateral headache is severe, pungent, and aggravated by physical activity, and is accompanied by nausea, vomiting, and phono- and photophobia. She has never experienced any signs of visual or sensory aura and has never had a motor weakness or hemiplegia. The mother (Fig. 1B; Patient II-4) is 89 years old and does not remember having any kind of chronic headache or typical migraine or aura symptoms. Moreover, she has had no motor weakness or hemiplegia in her life. Since she was adopted, no information about her family is available. The deceased father of the index (Fig. 1B; Patient II-3) did not have migraine. A deceased sister of the father (Fig. 1B; Patient II-1) was reported to suffer from heavy migraine. No member of either family was affected by hemiparesis or hemiplegia.

Mutation Screening

Amplification primers were chosen using the UCSC Human Genome Browser, and the primer sequences and PCR conditions are available from the authors. All exons were amplified and directly sequenced in all 45 index cases (36 females and nine males, average age = 43 years, average age of first migraine attack = 15 years, average frequency of migraine attacks = 30/year), and the sequences were compared with the *ATP1A2* GenBank entry NM_000702.2. The mutation numbering is based on this cDNA sequence, and position +1 corresponds to the A of the ATG translation initiation codon. A control sample consisting of 260 DNA samples from unrelated German individuals from the same ethnic background was tested for each of the identified missense variants by direct sequencing and/or allele specific digests.

Functional Analyses

Human Na^+/K^+-ATPase α2 and β1 subunits were subcloned into a modified pCDNA3.1 vector (Invitrogen), which contained the 5′ and 3′ untranslated regions of the *Xenopus* β-globin gene flanking the multiple cloning site (a kind gift of Dr. Renate Gauss, IonGate Biosciences GmbH, Frankfurt am Main, Germany). Site-directed mutagenesis was carried out by recombinant PCR techniques, and all PCR-derived fragments were verified by sequencing. To distinguish the activity of the heterologously expressed Na^+/K^+-ATPase from the endogenous oocyte Na^+/K^+-ATPase, the mutations Q116R and N127D were introduced into the extracellular loop between transmembrane segments M1 and M2 to obtain an ouabain-resistant protein with an IC_{50} in the mM range [Price and Lingrel, 1988]. For heterologous expression in the oocytes, each cell was injected with 15–25 ng α2 subunit and 2.0–2.5 ng of β1 subunit cRNAs. Stationary currents of the Na^+/K^+-ATPase were recorded by the two-electrode voltage clamp configuration using a Dagan CA-1B amplifier (Dagan Corp., Minneapolis, MN) and pClamp 8 software (Axon Inst., Union City, CA). Data analysis and presentation were carried out with Origin 5.0 (Microcal Software, Northampton, MA). Stationary Na^+/K^+-pump currents of the heterologously expressed α2 subunit were measured as the current difference assessed upon addition of 10 mM extracellular K^+ (solution exchange from Na-buffer [100 mM NaCl, 1 mM $CaCl_2$, 5 mM $BaCl_2$, 5 mM $NiCl_2$, 10 μM ouabain, 5 mM MOPS/TRIS, pH 7.4] to Na-K-buffer [90 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 5 mM $BaCl_2$, 5 mM $NiCl_2$, 10 μM ouabain, 5 mM MOPS/TRIS, pH 7.4]) at –40 mV holding potential. In addition to the analysis of the two newly identified mutations, we also included the mutant W887R (De Fusco et al., 2003) to compare a possible in vitro effect of FHM and MA/MO mutations.

For direct measurement of ATPase activity, total membranes of injected *Xenopus* oocytes were added to medium containing 50 mM TRIS-acetic acid (pH 7.0), 0.2 mM EDTA, 0.1 mM EGTA, 1 mM TRIS-N_3, 1.3 mM $MgCl_2$, 100 mM NaCl, 100 μM [γ^{32}P]ATP, with and without 10 mM KCl. After incubation at 37°C, the reaction was stopped by the addition of 500 μl 10% (w/v) charcoal in 6% (w/v) trichloroacetic acid. The mixture was centrifuged for 30 s (10,000 × *g*) and the supernatant containing the liberated inorganic phosphate ($^{32}P_i$) was analyzed by liquid scintillation analysis.

Isolation of Total Membranes and Western Blotting

For the isolation of total membranes, 10–20 oocytes were homogenized in 100–200 μl of buffer (250 mM sucrose, 2 mM EDTA, and 25 mM HEPES/Tris; pH 7.0) and centrifuged 3 min at 1000 × *g* and 4°C. Next, the membranes were isolated by centrifugation of the supernatant for 30 min at 16,000 × *g* and 4°C. The total membrane samples were solubilized in SDS-PAGE

sample buffer and separated on SDS gels containing 10% acrylamide, as described by Laemmli [1970], and blotted on immobilon polyvinylidenedifluoride membranes (Milipore Co., Bedford, MA). The α-subunits of Na^+/K^+-ATPase were detected with the polyclonal antibody C356-M09 [Koenderink et al., 2003].

RESULTS

Identification of Rare Missense Variants

We performed a systematic mutation screening of the 23 coding exons and adjacent splice sites of *ATP1A2* in index cases from 45 families with two or more members affected by MA and/or MO but with no motor symptoms or hemiplegia. In addition to some common intronic and silent exonic polymorphisms, we identified two different missense variants in two index cases (a summary of all of the variants is given in Table 1). In one of the patients (Patient IV-1; Family A) we found a unique G>A transition at nucleotide position 520 in exon 6 (c.520G>A). This alteration predicts an amino-acid exchange from the negatively charged glutamate to the positively charged lysine shortly after the second transmembrane domain (p.E174 K, Fig. 1A and D). The negative charge at this position is conserved in all P_{2C}-type ATPases (Fig. 1C) [Axelsen and Palmgren, 1998]. Except for the gastric H^+/K^+-ATPase ATP4A, which has an aspartate at this position, all P_{2C}-type ATPases have a glutamate at this position. This is also true for the known species orthologs from rat, mouse, chicken, and frog (data not shown). Since E174 K was not present in 520 control chromosomes of German origin, it is unlikely that it represents a rare polymorphism. The mutation is also present in the affected monozygotic twin (monozygosity proven by microsatellite genotyping; data not shown), the affected mother, and the unaffected maternal grandfather (Fig. 1A).

In another family (Family B), we identified a different missense alteration in the index who is affected by MA (Patient III-3; Fig. 1B). It is a G>A transition at nucleotide position 1544 in exon 12 (c.1544G>A) predicting an amino-acid exchange from cysteine to tyrosine (p.C515Y). The cysteine is perfectly conserved among all P_{2C}-type ATPases (including the known species orthologs) and is located in the intracellular loop between M4 and M5 (Fig. 1C and D). Again, the mutation was not found in 520 control chromosomes. It is also present in the sister of the index who is affected by MO, and in the healthy mother.

Functional Studies in *Xenopus* Oocytes

To investigate whether the observed sequence alterations affect the function of ATP1A2 in vitro, ouabain-resistant wild-type (WT) and mutant human Na^+/K^+-ATPase α2 subunits were expressed in *Xenopus* oocytes together with the human Na^+/K^+-ATPase β1 subunit. Figure 2A shows typical recordings of stationary currents of the WT Na^+/K^+-ATPase in response to a solution exchange from 0 to 10 mM K^+. The specificity is demonstrated by the inhibitory action of 10 mM ouabain, which reduces the stationary current of the ouabain-resistant WT enzyme to ~20% (Fig. 2A), which is in accordance with previous observations [Horisberger and Kharoubi-Hess, 2002]. In independent experiments, each of which used data from six different oocyte and three different cRNA preparations, we obtained the following mean currents: WT: 112 ± 8 nA (n = 64), E174K: 116 ± 10 nA (n = 58), C515Y: 5.2 ± 7.2 nA (n = 31), and W887R: 3.8 ± 3.5 nA (n = 25). The mean stationary currents for the WT, the two novel missense alterations, and the FHM2 mutation W887R are shown in Fig. 2B. Whereas E174 K is indistinguishable from WT (P = 0.7704, *t*-test for two independent samples), C515Y leads to a functional loss of ATP1A2 that is similar to FHM mutations (significance of C515Y as compared to WT was P = 0.0007, *t*-test for two independent samples).

We also performed a direct measurement of ATPase activity. In independent experiments, each of which used data from six different well-expressing oocyte preparations (as controlled by Western blotting; Fig. 2C bottom), E174 K again showed no significant loss of function (Fig. 2C). In contrast, and in accordance with the electrophysiological measurements, C515Y and W887R showed a complete loss of function as compared to WT activity (significance of WT vs. C515Y P = 0.0010), which was comparable to uninjected oocytes (Fig. 2C). In contrast to E174 K and W887R, Western blotting showed a lower expression of C515Y as compared to WT. In addition, we also tested the mutants in an ouabain survival assay, as described previously (Price

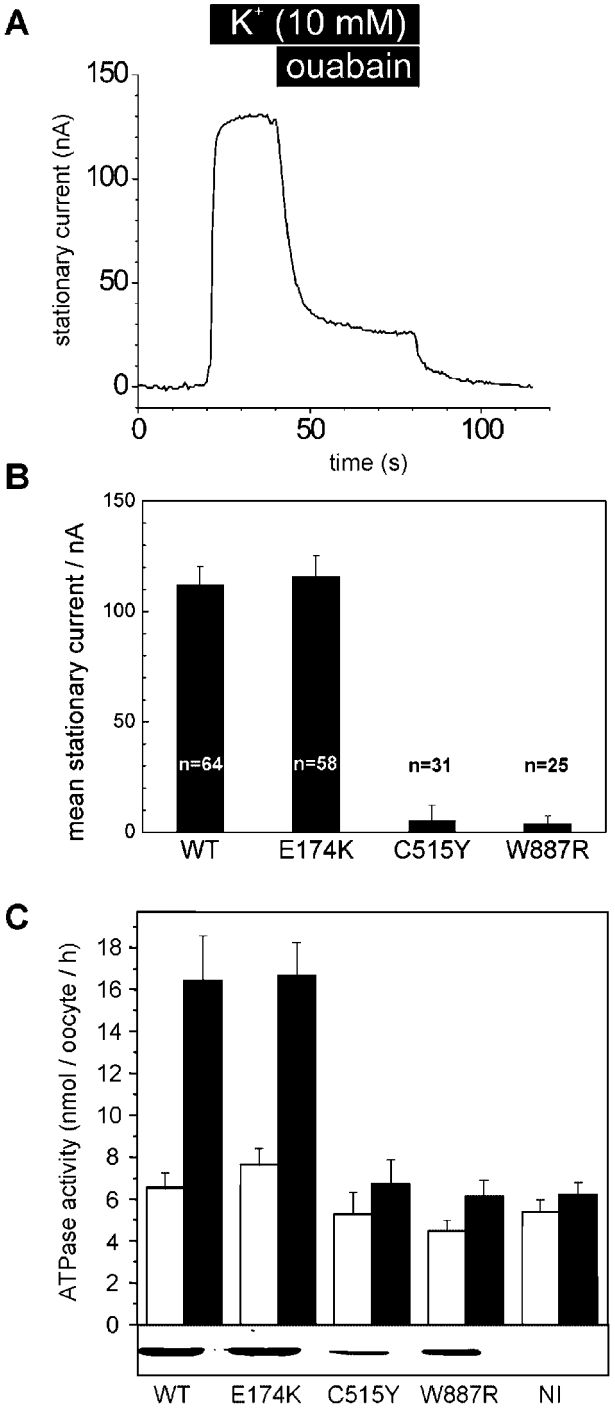
TABLE 1. **Sequence Variants Identified in *ATP1A2****

Nucleotide change	Localization	Protein consequence	Patient genotype frequency[a]
c.13–9_–8insTTCC	Intronic		33/11/1
c.177+95T>C	Intronic		34/10/1
c.495+81T>A	Intronic		23/17/5
c.495+96G>A	Intronic		28/15/2
c.520G>A	**Exonic**	**p.E174K**	**44/1/0**
c.749–27C>A	Intronic		37/7/1
c.1017+56A>G	Intronic		32/12/1
c.1119G>A	Exonic	Silent	28/15/2
c.1216+49_+50delG	Intronic		33/12/0
c.1461+91G>C	Intronic		42/3/0
c.1544G>A	**Exonic**	**p.C515Y**	**44/1/0**
c.1651–11C>G	Intronic		42/3/0
c.1704C>T	Exonic	Silent	43/2/0
c.2259C>T	Exonic	Silent	33/11/1
c.2841–20_–19insC	Intronic		32/11/1
c.2943–27G>C	Intronic		33/11/1
c.2943–47C>G	Intronic		33/11/1

*DNA numbering is based on GenBank cDNA sequence NM_000702.2; nucleotide +1 is the A of the translation initiation codon.
[a]Genotype frequencies of each variant were determined by sequencing of the same set of 45 index cases, the numbers given are: homozygotes for allele 1/heterozygotes/homozygotes for allele 2.
The two missense variants probably involved in the susceptibility to common forms of migraine and which are studied in more detail are given in bold.

and Lingrel, 1988). HeLa cells were transiently transfected with ouabain-resistant wild-type or mutant Na^+/K^+-ATPase α2 subunits. Two days after transfection, 1 μM ouabain was added to the media, and 5 days later the surviving cells were analyzed. The E174 K mutant gave a cell survival comparable to that of the wild-type enzyme, whereas the C515Y mutant gave no cell survival at all (three independent experiments, data not shown).

In summary, in three independent in vitro assays one of the newly identified variants (E174 K) showed no functional consequence, whereas the other one (C515Y) showed a loss of function comparable to that of an FHM mutation.



DISCUSSION

It has long been debated whether rare variants may be responsible for a person's susceptibility to common diseases. There appear to be good arguments for this hypothesis from both theoretical considerations [Pritchard, 2001] and very recent experimental observations [Cohen et al., 2004; Fearnhead et al., 2004], which may also strengthen our hypothesis about the role played by rare alleles of *ATP1A2* in common forms of migraine. Although the in vitro data could not prove the pathogenicity of E174 K, nor could we find a genotype–phenotype relationship for C515Y vs. typical FHM mutations in three functional assays, we still propose that rare variants in *ATP1A2* are involved in the susceptibility to common forms of migraine, for a number of different reasons:

1. Both alterations were absent in a large number of controls, which makes it more unlikely that they represent rare neutral polymorphisms.

2. The pattern of segregation in both families is compatible with a complex mode of inheritance, which is supposed to underlie the common forms of migraine. If an autosomal-dominant inheritance pattern (with incomplete penetrance) is assumed, in Family A the mutation would more likely be expected to be inherited by the unaffected maternal grandmother (Patient II-3; Fig. 1A), whose family includes several members with migraine. In contrast, E174 K was inherited from the healthy grandfather (Patient II-2), in whose family no cases of migraine are known. For this family, a possible explanation is that the mother of the index (Patient III-2) has MA because she inherited the E174 K mutation from her father and at least one unknown variant in another migraine susceptibility gene from her mother, in whose family a migraine susceptibility is evident. Neither this variant nor the E174 K alone seem to be sufficient to cause MA. The index and her sister (Patients IV-1 and IV-2) both developed MA (the concordance rate of MA in monozygotic twins is 50–60% [Palotie et al., 2002]) because they both got the E174 K mutation from her mother and at least one unknown variant from her father (Patient III-1), who is also affected by MA. In Family B, the mutation was likewise inherited from the asymptomatic mother (Patient II-4; Fig. 1B), whereas there is a history of migraine in the sibling (Patient II-1) of

FIGURE 2. **Stationary current and ATPase activity measurements of ATP1A2 and the mutants in *Xenopus* oocytes. A: Stationary currents recorded on oocytes expressing the ouabain-resistant human Na^+/K^+-ATPase α2 subunit (hNaKα$_2$-OuaR) and human Na^+/K^+-ATPase β1 (hNaKβ$_1$) subunit upon extracellular solution exchanges from Na-buffer (100 mM Na^+) to Na-K-buffer (90 mM Na^+/10 mM K^+) and Na-K-buffer plus 10 mM ouabain, as indicated by the perfusion scheme above the signal traces. The holding potential was −40 mV. B: Mean stationary current amplitudes (stimulated by extracellular addition of 10 mM K^+ at −40 mV) from oocytes expressing hNaKα$_2$-OuaR and the mutants hNaKα$_2$-OuaR(E174 K), hNaKα$_2$-OuaR(C515Y), and hNaKα$_2$-OuaR(W887R). Data for different mutations were obtained in independent sets of experiments from six different oocyte preparations (mean ± SE). C: K^+-stimulated ATPase activity from oocytes expressing hNaKα$_2$-OuaR and the mutants hNaKα$_2$-OuaR(E174 K), hNaKα$_2$-OuaR(C515Y), and hNaKα$_2$-OuaR(W887R). Data for different mutations were obtained in independent sets of experiments each from six different well-expressing oocyte preparations (mean ± SE), and data from experiments with no expression in one or more constructs were discarded. The expression level was controlled by Western blotting, as indicated by a representative Western blot shown below the bars. Open bars: ATPase activity without K^+-stimulation (i.e., background activity); black bars: ATPase activity in presence of K^+. NI = non-injected oocytes.**

the father who had no migraine. There is no information about the family of the mother, who grew up in an asylum, and we therefore cannot exclude the possibility that some of her family members were affected by migraine (perhaps even by FHM2, which is assumed to have a penetrance of about 90% [Jurkat-Rott et al., 2004]). The mother is 89 years old and has never experienced any form of migraine, and furthermore, C515Y is not fully penetrant. Therefore, the possibility of a non-Mendelian inheritance pattern in this family is reasonable. The identity of the suspected additional variants in other migraine susceptibility genes remains speculative at the moment. However, directly interacting protein subunits (such as the β-subunits and/or γ-subunits of the FXYD family) that are expressed in the central nervous system and modify the trafficking and/or biophysical properties of ATP1A2 should especially be considered. A number of other ion transporting proteins from different protein families are also crucially involved in the regulation of ion homeostasis in the brain, and therefore may also be good functional candidate genes.

3. The high conservation of the amino acid residues at which the alterations occurred, and the exchange of a negative for a positive charge in E174K argues for a possible impairment or disturbance of functional properties, even in the case of E174K, which may not have been apparent in the functional assays we applied. In this context it seems noteworthy that the mutation T345A, which has been found in a family with typical FHM [Kaunisto et al., 2004], did not show a complete loss of function like other FHM2 mutations, but rather showed only subtle kinetic alterations [Segall et al., 2004]. This means that even for highly penetrant FHM2 mutations there is no clear-cut genotype–phenotype correlation, and that susceptibility variants may have even more subtle functional effects that are not visible in commonly used in vitro assays. In this respect it may even be unrealistic to expect a strong functional alteration of a "pure" susceptibility variant. Such a variant alone is not sufficient to cause the phenotype but will just lead to the respective symptoms if it is present together with additional alterations in other susceptibility genes. Therefore, it may be that a functional effect will be observed only if these additional (currently unknown) variants are also included in the in vitro assay, whereas the single susceptibility variant is neither genetically nor functionally fully penetrant.

4. The observed functional effect of C515Y argues in favor of the notion that rare *ATP1A2* variants play a role in the pathogenesis of MA and MO, even if we cannot definitely exclude (due to the limited information about the maternal family) the possibility that this mutation may be an FHM mutation with complete penetrance.

5. In addition, the involvement of *ATP1A2* in a rather severe monogenic form of migraine (i.e., FHM) strongly reinforces our hypothesis about the involvement of this gene in milder and more common forms of migraine due to the extensive phenotypic overlap. Similar findings regarding the involvement of the same genes in severe monogenic and milder complex inherited forms of disease were recently reported for *ABCA1*, *APOA1*, and *LCAT* in disturbances of plasma levels of cholesterol [Cohen et al., 2004] and for *MSH2* in colorectal adenomas [Fearnhead et al., 2004], thereby establishing this concept of the rare variant-common disease hypothesis.

6. Finally, although the case has not yet been published in a peer-reviewed journal, it should be mentioned that in a large family with MO another *ATP1A2* variant was recently reported by Castro et al. in an abstract of the Annual Meeting of the American Society of Human Genetics 2004 (program no. 2089, www.ashg.org/genetics/ashg04s/index.shtml). This mutation is an arginine-to-histidine exchange at position 51 in the extreme N-terminus of the protein, which was also not found in a large number of controls. Notably, E174K is also exceptional in its extreme N-terminal localization (as compared to FHM2 mutations) and in being the only mutation located in the intracellular loop between M2 and M3 in the so-called actuator domain [Toyoshima et al., 2000]. The issue of whether a more N-terminal localization of alterations in *ATP1A2* is more likely to be involved in the susceptibility to common forms of migraine will have to be reevaluated after more mutations in *ATP1A2* are identified in future studies. In summary, we propose that rare *ATP1A2* alleles are a rare cause of familial clustering in common forms of migraine.

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