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ARTICLE

Two *de novo* mutations in the Na,K-ATPase gene *ATP1A2* associated with pure familial hemiplegic migraine

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Familial hemiplegic migraine (FHM) is a rare autosomal dominantly inherited subtype of migraine, in which hemiparesis occurs during the aura. The majority of the families carry mutations in the *CACNA1A* gene on chromosome 19p13 (FHM1). About 20% of FHM families is linked to chromosome 1q23 (FHM2), and has mutations in the *ATP1A2* gene, encoding the α2-subunit of the Na,K-ATPase. Mutation analysis in a Dutch and a Turkish family with pure FHM revealed two novel *de novo* missense mutations, R593W and V628M, respectively. Cellular survival assays support the hypothesis that both mutations are disease-causative. The identification of the first *de novo* mutations underscores beyond any doubt the involvement of the *ATP1A2* gene in FHM2.
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Introduction

Familial hemiplegic migraine (FHM) is a rare autosomal dominant subtype of migraine characterized by hemiplegia as part of the aura.[1,2] The majority of FHM families is linked to chromosome 19p13 (FHM1) and carry a mutation in the *CACNA1A* gene encoding the $Ca_v2.1$ subunit of neuronal voltage-gated P/Q-type calcium channels.[3,4] A smaller proportion of FHM families is linked to chromosome 1q23 (FHM2)[5–9] with mutations in the *ATP1A2* gene, encoding the Na,K-ATPase α2-subunit.[10–15] Recently, a third FHM gene, *SCN1A*, located on chromosome 2q24, encoding a neuronal voltage-gated sodium channel, was identified.[16] *ATP1A2* mutations have been associated with FHM, benign familial infantile convulsions (BFIC),[11] alternating hemiplegia of childhood (AHC)[17,18] and additional features like seizures, prolonged coma and cerebellar signs.[10–15]

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Functional studies of mutant Na,K-ATPases revealed a broad spectrum of abnormalities, including a complete loss of Na,K-pump function effect described for mutations T378N,[18] L764P and W887R.[10,19,20] Mutations R689Q and M731T resulted in a decreased catalytic turnover[21] whereas the T345A mutation showed a reduced affinity for potassium.[22]

Here we describe the first *de novo ATP1A2* mutations segregating with pure FHM. Moreover, in cellular survival assays where the endogenous Na,K-pump was blocked by ouabain, we could demonstrate that these mutations are disease-causative, since both mutant Na,K-ATPases are associated with reduced cellular survival.

Material and methods

Clinical data

Family 1 The proband of this Turkish family (III-6) (Figure 1) is a 47-year-old woman who has had, since the age of 13, attacks of headache and vomiting accompanied by homonymous hemianopsia and paresthesia of the ipsilateral upper and lower limbs, progressing to hemiplegia lasting up to 30 min. The attacks occurred twice a month but the last 8 years the frequency dropped to 4 attacks a year. Two of her children (IV-7 and IV-9) were also diagnosed with hemiplegic migraine. Her other two children (IV-6 and IV-8) were not diagnosed with migraine.

The elder sister (III-4) of the proband has hemiplegic migraine attacks, as well as all her five children. For the monozygotic twin brothers, a clinical description of attack characteristics is given in detail. Twin brother IV-1 (26 years of age) has 4–6 migraine with aura attacks per year. In addition, from age 18 until 20, he has had two migraine attacks with hemiplegia, but none since then. His twin brother (IV-2) has had 2–6 migraine attacks with hemiplegia every year from age 13 until 20, but none since then. Attacks in both brothers lasted 24–36 h and were associated with dysphasia, hemianopsia, motor weakness and ipsilateral sensory disturbances. The parents of the proband (II-1 and II-2), the grandparents (I-1 and I-2), the maternal aunt (II-4) and her offspring (III-8, IV-10, IV-11, IV-12), as well as her two brothers (III-1 and III-2) were not diagnosed with migraine. Of importance, all clinical diagnoses were made before genetic testing.

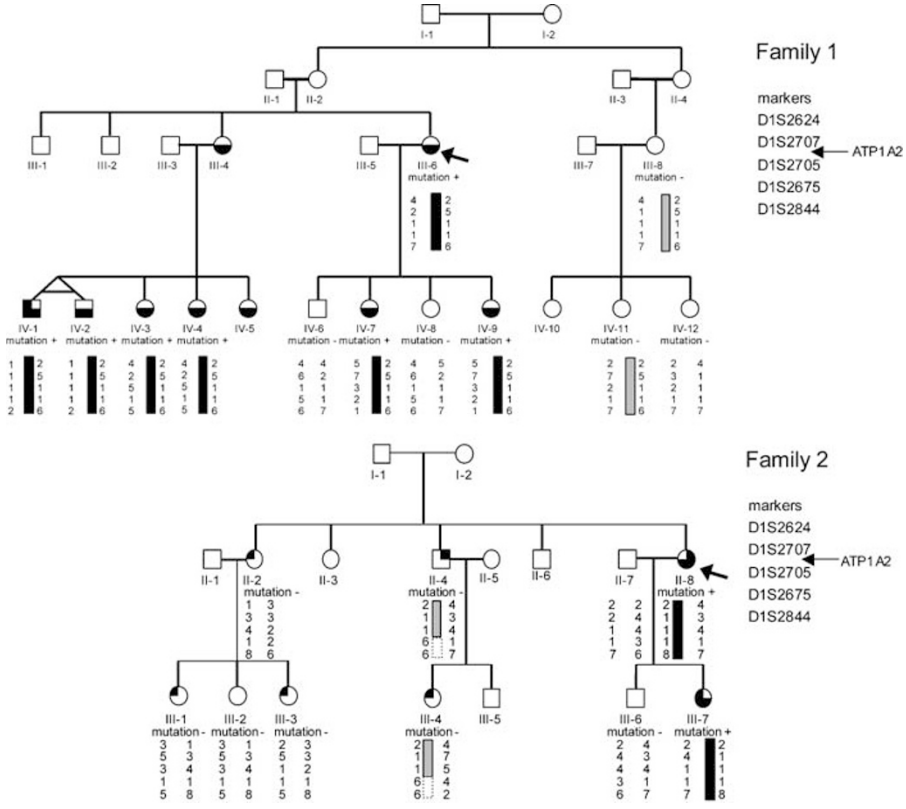

Figure 1 Pedigrees of *families 1* and *2*. The arrows indicate the probands. The following symbols are used to indicate the diagnosis: FHM: black lower half; MA: right upper square; MO: left upper square. Mutation + and Mutation− indicate carriers and noncarriers of the pathogenic mutations, respectively. Black bars show the haplotypes segregating with FHM; gray bars indicate the identical haplotype without the mutation; a dotted bar indicates a recombination. Genetic markers and the position of the *ATP1A2* gene are indicated on the right.

Family 2 The proband (II-8) of this Dutch family (Figure 1) is a 48-year-old woman who has monthly attacks of hemiplegic migraine from the age of 4. These attacks are accompanied by visual aura, with contralateral paresthesias around the mouth and paresthesias and motor weakness in one arm and leg for half an hour. She once had an atypical attack, for which she was admitted to a hospital. Prophylactic treatment using valproic acid was associated with an attack-free period of 2 years. Her daughter III-7 (23 years of age) has attacks of migraine without aura approximately once a week. In addition, during the last 6 years, she has had five attacks of hemiplegic migraine, starting with decreased hearing, followed by one-sided motor weakness and visual symptoms. She has had two episodes of unilateral motor weakness and temporary unconsciousness after a fall, not followed by headache. A sister of the proband (II-2) has migraine without aura, a brother (II-4) has migraine with visual aura, and three cousins have migraine without aura (III-1, III-3 and III-4). The parents of the proband (I-1 and I-2) never reported any headache attacks.

Genetic analysis

Blood samples were collected from 10 members of *family 1* and *family 2* each. Genomic DNA was isolated from peripheral blood using a standard salting out extraction method.[23] Microsatellite markers (D19S221, D19S1150, D19S226 and D1S2624, D1S2707, D1S2705, D1S2675, D1S2844) were selected to test the involvement of the FHM1 and FHM2 loci, respectively. Oligonucleotide primer sequences were obtained from the Human Genome Database (GDB) (http://www.gdb.org/). After amplification, PCR products were detected on automated sequencer (ABI 3700 DNA sequencer, Applied Biosystems, Foster City, CA, USA). All genotypes were analyzed and independently scored by KRJV and SKhK using Genescan and Genotyper 2.1 software (Applied Biosystems, Foster City, CA, USA). Haplotypes were constructed by inspection of segregation and assuming a minimal number of recombinations.

Proof of monozygosity of the twin brothers in *family 1* was established by monozygotic-probability calculation (MZ Probability > 99.99%) using 17 autosomal markers (results not shown).[24]

Mutation analysis of the *ATP1A2* gene was performed by direct sequencing of all exons and flanking intronic regions using genomic DNA of probands of both families as described in Vanmolkot *et al*.[11] Genomic DNA of 100 population-matched subjects was used as a control group to test each mutation. For detection of the V628M mutation (G > A, nt position 1987; Ac no. NM_000702) amplification refractory mutation system PCR (ARMS-PCR) was performed.[25] In brief, the wild-type allele was detected using forward primer 'Universal' (5′-GGGCTGAGGAAC CAGTCACAA-3′) and reverse primer 'G' (5′-AGGCCATTG CCAAAGGCG-3′), whereas the mutant allele was detected with reverse primer 'A' that differed at the ultimate 3′-base position (5′-AGGCCATTGCCAAAGGCA-3′). Both PCRs gave a product of 172 bps. The GG (wild type) genotype showed a PCR product only in case reverse primer 'G' was used; the AA (mutant) genotype gave only a PCR product with reverse primer 'A'. In case of a heterozygote (eg GA genotype') PCRs with either reverse primer gave a product. As an internal control for PCR, in every reaction, forward primer 'Control forward' (5′-TGTCATCTTGGATGGCA CTG-3′) and reverse primer 'Control reverse' (5′-TGCGTT GATCTGCATCTTCT-3′) were included resulting in a 500-bp PCR product.

For detection of the R593W mutation (C > T, nt position 1881, Ac no. NM_000702), exon 13 was amplified by PCR using primers 'exon13F' (5′-GGGATTCCCAAGCCTCTG-3′) and 'exon13R' (5′-TCTCTGAGTCAGTGGGAAGGA-3′), resulting in a 398-bp product. Subsequently, PCR products were digested with restriction enzyme *Sma*I using standard protocols, and electrophoresed on a 3% agarose gel. The R593W mutation causes a loss of a *Sma*I site, which results in an uncut band of 398 bps for the mutant allele, besides the wild-type bands of 172 and 226 bps.

Functional analysis

cDNA constructs Human Na,K-ATPase α2-subunit cDNA was subcloned into a modified pCDNA3.1 vector (originally from Invitrogen, Carlsbad, CA, USA), which additionally contained the 5′- and 3′-untranslated regions of the *Xenopus* β-globin gene flanking the multiple cloning site.[19] To distinguish endogenous Na,K-ATPase activity from that of transfected Na,K-ATPase, mutations Q116R and N127D were introduced in the original α2-subunit cDNA to express an ouabain-resistant isoform (α2-WT).[26] Next, mutations R953W and V628M were introduced into the ouabain-resistant wild-type α2-subunit construct by site-directed mutagenesis (Quikchange, Stratagene, La Jolla, CA, USA) to obtain mutants α2-R593W and α2-V628M, respectively. All constructs were sequence-verified.

Transfection and ouabain treatment HeLa cells (5×10^5) were transfected with 1.6 μg plasmid DNA of either α2-WT, α2-R593W or α2-V628M, using Lipofectamine 2000 Transfection Reagent in Opti-Mem medium (Invitrogen, Carlsbad, CA, USA) and cultured in DMEM-containing Glutamax and 10% FCS (Invitrogen, Carlsbad, CA, USA). At 2 days after transfection, one-third of the cells (1.7×10^5) were seeded on 10 cm Petridishes and subsequently 1 μM ouabain was added to the culture medium. After 5 days of ouabain challenge, colonies were stained with 1% methylene blue in 70% methanol, scanned and analyzed with Image Pro Plus (MediaCybernetics, Silverspring, MD, USA). Each transfection was performed nine times.

Electrophoresis and Western blot analysis At 2 days after transfection, two-third of the cells (3.3×10^5) were harvested for Western blot analysis.[19] In brief, proteins were resuspended in a mix of protease-inhibitor (Complete Mini, Roche, Basel, CH, USA) and DNaseI. Subsequently, Laemmli-loading buffer and 0.1 M DTT were added and the samples were heated for 10 min at 65°C. Next, equal amounts of protein as measured by Bio-Rad protein assay (Bio-Rad Laboratories, Munchen, Germany) were separated on 7.5% SDS-polyacrylamide gels for 40 min at 200 V. Proteins were electroblotted onto nitrocellulose membranes (Hybond, Amersham, Buckinghamshire, UK) and incubated overnight with the α2-subunit-specific polyclonal antibody HERED.[27] The primary antibody was detected with goat-anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma, St Louis, MO, USA). Protein bands were visualized with Super Signal Substrate (Pierce Biotechnology, Rockford, IL, USA).

Results

Our two pure FHM families were compatible with involvement of the 1q23 FHM2 locus, because single haplotypes consistently co-segregated in all tested patients with FHM (Figure 1). No indications for involvement of the 19p13 FHM1 locus were found either by haplotype analysis (in case of *family 1*) or sequencing of the *CACNA1A* gene (in case of *family 2*). However, sequence analysis of the *ATP1A2* gene identified two missense mutations, V628M and R593W, in *families 1* and *2*, respectively. The V628M mutation in exon 14 (nt 1987 G>A) causes a Valine to Methionine substitution, whereas the R593W mutation in exon 13 (nt 1881, C>T) causes an Arginine to Tryptophan substitution (Figure 2). Both mutations were not observed in a panel of 100 population-matched control individuals. Taxonomy analysis indicates a strong conservation of amino acids Arg^{593} and Val^{628} among several alpha subunits of the P_2-type ATPase subfamily (Figure 3). Haplotype analysis showed that both mutations occurred *de novo*. Haplotypes identical by descent, but without the mutations, were not associated with FHM (Figure 1). Interestingly, in *family 1*, we identified monozygotic twin brothers that carry the V628M mutation and have similar hemiplegic migraine attack characteristics and age of onset, although the attack frequency seems to vary between them.

To evaluate the functional consequences of both mutations, survival assays were performed. In these assays, the endogenous Na,K-ATPase activity was completely inhibited by ouabain challenge in HeLa cells. In such an assay, we assessed whether transfected Na,K-ATPase is able to compensate for this loss. For transfections we used either

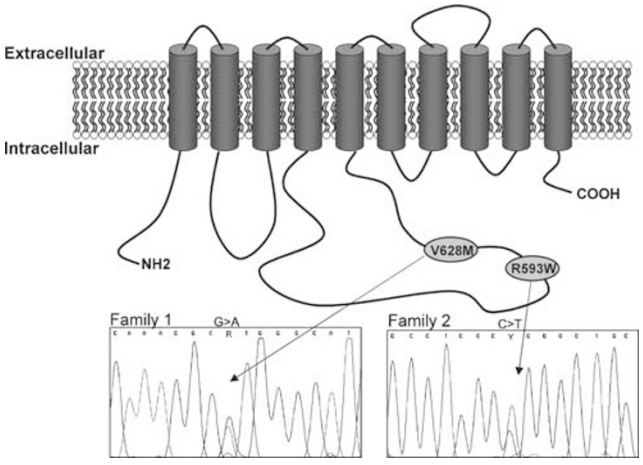

Figure 2 Transmembrane topology model of the Na,K-ATPase α2-subunit and location of novel mutations. The location of the amino-acid substitutions V628M and R593W are shown. Arrows point to electropherograms with the respective heterozygous mutations.

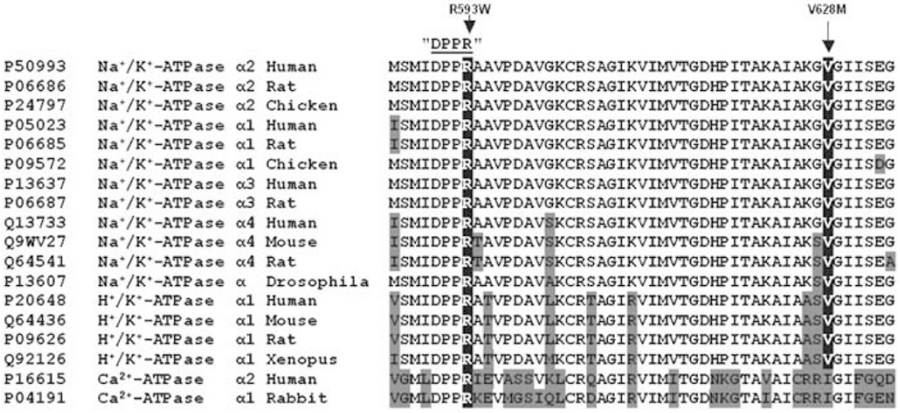

Figure 3 Amino-acid sequence alignments. Conservation of the Arg^{593} and Val^{628} residues (in black) in several subunits of various Na,K- ATPase, H,K-ATPase and SERCA ATPases. The 'DPPR' motif is indicated. Accession numbers are indicated on the left. Variations from the human *ATP1A2* sequence are given in gray.

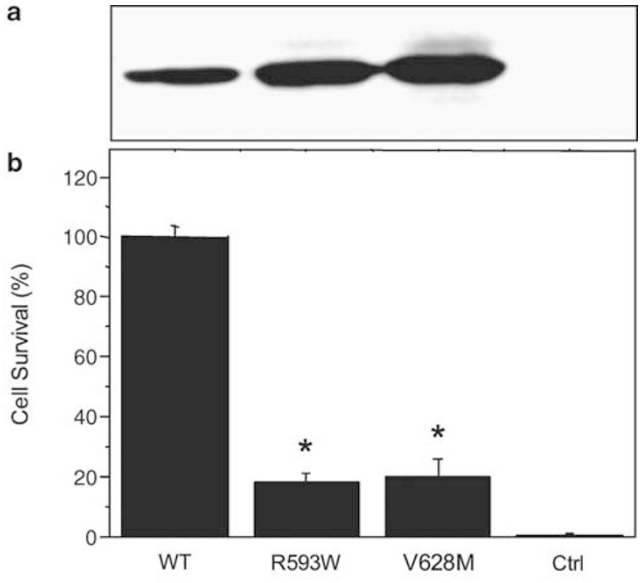

Figure 4 Ouabain survival assay. (**a**) Western blot analysis of transfected HeLa cells. Detection was carried out using a polyclonal antibody specifically directed against the Na,K-ATPase α2-subunit (anti-HERED). (**b**) Ouabain sensitivity of cells transfected with either wild type or mutant *ATP1A2* cDNA constructs. Graphic representation of cell survival after 5 days of ouabain treatment ($n=9$). Lane 1: WT: α2-WT construct; lane 2: α2-R593W; lane 3: α2-V628M; lane 4: Control: mock-transfected cells.

wild-type or mutant (α2-R593W or α2-V628M) Na,K-ATPase α2-subunits that were made insensitive to ouabain by mutagenesis.[26] Compromised rescue will lead to cell death, revealing clear functionality of the mutation. Importantly, Western blot analysis showed that our constructs were expressed at comparable, sufficient, levels (Figure 4a). In the survival assay (Figure 4b), cells expressing the wild-type construct were able to survive ouabain treatment. Cells expressing mutant constructs α2-R593W or α2-V628M showed a significantly reduced rate of survival, clearly indicating that both mutants are unable to compensate sufficiently for loss of endogenous Na,K-ATPase activity.

Discussion

We have identified two novel missense mutations, R593W and V628M, in the FHM2 *ATP1A2* gene in two families with pure FHM. Both mutations co-segregate consistently with the FHM phenotype. Haplotype analysis of each family revealed that both mutations had occurred *de novo*, as identical haplotypes without the mutation were present in several unaffected family members. *Family 1* is the first Turkish family reported with FHM. These are the first *de novo* mutations reported for *ATP1A2*, underscoring that mutations in the *ATP1A2* gene are indeed causing FHM.

Transfection studies with the *ATP1A2* cDNA constructs showed sufficient residual function of both mutants R593W and V628M to allow cell growth, although at a significantly reduced rate compared to the ouabain-resistant wild-type isoform. In this respect, they differ from the previously published T378N, L764P, W887R mutations that all produce pumps completely incapable of a functional rescue.[10,18–20] On the other hand, FHM2 mutants R689Q and M731T located in the same cytoplasmatic loop as R593W and V628M also allow partial or complete cell growth in survival assays, but show decreased catalytic turnover in additional functional studies.[21]

Both mutated amino acids are well conserved within the P_2-type subfamily. The Na,K-ATPase α2-subunit has a structure similar to Ca^{2+}-ATPases, which comprises 10 transmembrane helices and three cytoplasmic domains: the A-domain (actuator), the P-domain, which contains the residue of phosphorylation, and the N-domain that binds ATP.[28] The Na,K-ATPase Val^{628} is the equivalent amino acid of Ca^{2+}-ATPase Ile^{639} that resides in the P-domain. Ile^{639} is located at the border of one of the short conserved helices that together with the seven-stranded parallel β-sheet form a typical Rossman fold.[28] This residue is rather well conserved in all P-type ATPases.[29] We showed that the substitution of Valine with a Methionine decreases the cell survival. The Na,K-ATPase Arg^{593} that is equivalent to Ca^{2+}-ATPase Arg^{604} is located at the border of the P-domain, directly after the two prolines that form the hinge between the P- and N-domains. Large domain motions require the presence of a flexible hinge region, with an invariant DPPR motif. It has been reported that the hydrogen-bond network involving the conserved Gly^{354}, Arg^{604}, and Asp^{737} seems to link the movement of this hinge region to that of M5, thereby effectively transmitting the phosphorylation signal to the Ca^{2+} binding sites.[28] Abnormal Na,K-ATPase functioning observed with our R593W mutant is in line with abnormal functioning in Ca^{2+}-ATPase mutant R604M that revealed a 35% reduced Ca^{2+} transport.[30]

Until now, 12 variations in the intracellular domain of the Na,K-ATPase α2-subunit have been reported in FHM patients.[10–15] ATP1A2 mutations have been shown to result in a broad spectrum of functional abnormalities. Two mutations associated with pure FHM (eg L764P and W887R), and mutation T378N that causes AHC are incapable of a functional rescue in survival assays, implicating complete loss-of-function.[10,18–20] The T345A mutation does not lead to reduced rescue in survival assays, but additional experiments showed a functionally altered pump with reduced affinity for K^+.[22] Mutants R689Q and M731T, also allow partial or complete cell growth in survival assays, but show decreased catalytic turnover.[21] Therefore, the partial survival of our novel mutations (R593W and V628M) supports the hypothesis that they are disease-causative.

Both the R593W and the V628M mutation occurred *de novo*, underscoring that mutations in the *ATP1A2* gene are indeed causing FHM in these families. In addition, the

occurrence of *de novo* mutations indicates that such mutations may also be found in sporadic hemiplegic migraine (SHM), that is, in hemiplegic migraine patients with no other family members suffering from this disease. The genetic etiology of SHM is not well established and only a few causative mutations in FHM1 and FHM2 genes have been identified in SHM patients.[14,31] Therefore, our findings suggest that the *ATP1A2* gene is a good candidate for genetic testing in SHM cases.

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