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First case of compound heterozygosity in Na,K-ATPase gene *ATP1A2* in familial hemiplegic migraine

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Familial hemiplegic migraine (FHM) is a rare autosomal-dominant subtype of migraine with aura, associated with hemiparesis during the aura. Here we describe a unique FHM family in which two novel allelic missense mutations in the Na,K-ATPase gene *ATP1A2* segregate in the proband with hemiplegic migraine. Both mutations show reduced penetrance in family members of the proband. Cellular survival assays revealed Na,K-ATPase dysfunction for both *ATP1A2* mutants, indicating that both mutations are disease causative. This is the first case of compound heterozygosity for any of the known FHM genes. *European Journal of Human Genetics* (2007) 15, 884–888; doi:10.1038/sj.ejhg.5201841; published online 2 May 2007

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Introduction

Familial hemiplegic migraine (FHM), a rare and severe form of migraine with aura, has an autosomal-dominant inheritance pattern and is characterized by transient motor weakness in addition to a wide range of other neurological aura symptoms.¹ Mutations for FHM have been identified in three genes; the *CACNA1A* calcium channel FHM1 gene, the *ATP1A2* Na,K-ATPase FHM2 gene and the *SCN1A* sodium channel FHM3 gene.^{2–5} Here, we report compound heterozygosity for two novel allelic missense *ATP1A2* mutations in the proband of a FHM family. Functional

consequences were shown for both mutations in cellular survival assays. This is the first case of compound heterozygosity for any of the FHM genes.

Patients and methods

Subjects

This family is of British Caucasian origin. The proband and available family members were diagnosed according to the International Classification of Headache disorders, second edition.¹ All family members were seen by a neurologist, who interviewed them directly and where relevant, examined the patients.

The proband (III-2, Figure 1) (aged 29 years) has, since the age of 8 years, typical hemiplegic migraine with an attack every 4–6 weeks that can last between 2 h and 9 days with an average duration of 2 days. Attacks are sometimes triggered by mild head injury. A typical attack starts with a visual aura of zigzag lines, which on occasion can progress to complete

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Figure 1 (a) Pedigree of the family. Symbols represent the following; lower black half: hemiplegic migraine; lower right quadrant: aura without headache; right upper quadrant: migraine with aura; left upper quadrant: migraine without aura; square: male; circle: female. The arrow indicates the proband. I286T and T415M indicate the presence of the *ATP1A2* allele harbouring the respective mutation. (b) Schematic representation of the *ATP1A2* protein. The location of mutations I286T and T415M is shown. Boxes with electropherograms show the respective heterozygous mutations.

hemianopia. Subsequently, she develops dysphasia and within 10 min complete right- or left-sided hemiplegia, followed by severe unilateral throbbing headache, associated with vomiting and photophobia. The nature of the headache can vary from a sharp, stabbing pain to an explosive thunderclap headache. Severe attacks are accompanied by disorientation, drowsiness and confusion. At the age of 20 (at 12 weeks pregnancy), she had a particularly severe attack associated with drowsiness, dysphasia and right hemiparesis lasting 9 days. During this attack, at the peak of her symptoms, MRI brain imaging including diffusion-weighted imaging showed no abnormalities, but perfusion imaging

showed hyperaemia of the left hemisphere (data not shown). EEG showed high voltage slow activity over the left hemisphere (data not shown).

The maternal aunt (II-1) of the proband also suffers from hemiplegic migraine. Her attacks started at the age of 13 and are characterized by mild weakness in hand and arm with an average duration of 30 min to 5 h. The headache is severe, unilateral, throbbing and aggravates by physical activity. The attack frequency ranges from once a week to twice a year. Her attacks are not provoked by head trauma. The proband's maternal grandfather (I-1) heteroanamnestically had severe migraine with possible hemiplegic

attacks and her maternal grandmother (I-2) suffered from migraine without aura. Other family members suffer from migraine with visual and sensory aura (IV-2, aged 8 years), a few isolated visual auras without headache (II-3), or do not suffer from migraine until now (IV-1, aged 6 years and II-2).

Mutation detection

Direct sequencing was performed for all 23 exons of the *ATP1A2* gene in the proband. For detection of the first heterozygous substitution (c.961T>C; I286T, Ac no. NM_000702), exon 8 was amplified by PCR using specific primers. Subsequently, PCR products were digested with restriction enzyme *Alw261* using standard protocols and electrophoresed on a 3% agarose gel. Detection of the second heterozygous substitution (c.1348C>T; T415M, Ac nr NM_000702) was performed by amplification of exon 10, and subsequently, digestion of PCR products with restriction enzyme *TaiI*. Family members and 180 control individuals were tested for the presence of both mutations.

Functional analysis

Human Na,K-ATPase $\alpha 2$ subunit cDNA was subcloned into a modified pCDNA3.1 vector.⁶ To distinguish endogenous Na,K-ATPase activity from that of transfected Na,K-ATPase, a cDNA encoding ouabain-resistant wild-type ($\alpha 2$ -WT)⁷ was used to introduce the I286T and T415M mutations with site-directed mutagenesis (Quikchange, Stratagene, La Jolla, CA, USA). For the assay, HeLa cells (5×10^5) were transfected with 1.6 μ g plasmid DNA of either $\alpha 2$ -WT, $\alpha 2$ -I286T or $\alpha 2$ -T415M, using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA). Two days after transfection, two-third of the cells were harvested for immunoblotting and $\alpha 2$ subunit was detected using specific polyclonal antibody HERED.⁸ The remaining one-third of the cells were seeded on 10 cm Petri dishes, and subsequently, 1 μ M 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 (Media Cybernetics, Silverspring, MD, USA). Each transfection was performed 15 times and the average values were calculated for each construct, respectively. The average number of colonies obtained with the WT construct was used as a reference (100% cell survival). Next, relative percentages of cell survival were calculated for both mutant constructs.

Results

In the proband with hemiplegic migraine, two heterozygous sequence variants I286T (exon 8; c.961T>C) and T415M (exon 10; c.1348C>T) were identified in the *ATP1A2* gene (Figure 1). The sequence variants are located on different alleles because I286T was inherited from the

mother (II-3) and T415M from the father (II-2). Both variants were absent in 180 Dutch control individuals. Sequence alignments indicate a strong evolutionary conservation of both amino acids Ile²⁸⁶ and Thr⁴¹⁵ among several α subunits of the P-type ATPase family (Figure 3).

Functional consequences of both variations were investigated in cellular survival assays in HeLa cells. In these assays, the endogenous Na,K-ATPase activity is completely inhibited by ouabain challenge. Transfected WT Na,K-ATPase $\alpha 2$ cDNA, that was made insensitive to ouabain by mutagenesis, is able to rescue cell survival (Figure 2b). We assessed whether mutant ouabain-insensitive Na,K-ATPase $\alpha 2$ cDNA ($\alpha 2$ -I286T or $\alpha 2$ -T415M) can do the same. Compromised rescue will lead to cell death, thus demonstrating that the mutations are functional-defective and can be considered pathogenic. Mutant T415M did not show cell survival at all. Cells expressing mutant I286T resulted in significantly reduced survival (Figure 2b). Western blot analysis showed that the constructs were expressed at levels comparable to the WT, indicating that

Figure 2 Ouabain survival assay. (a) Western blot analysis of transfected HeLa cells. Detection was performed using a polyclonal anti-Na,K-ATPase $\alpha 2$ subunit antibody. (b) Ouabain sensitivity of cells transfected with either wild-type (WT) or mutant *ATP1A2* cDNA constructs. Graphic representation of cell survival after 5 days of ouabain treatment ($n = 15$). Survival obtained with the WT construct was set to 100% and compared to the relative survival for both mutants constructs. Lane 1: WT: Na,K-ATPase $\alpha 2$ -WT construct; Lane 2: I286T: Na,K-ATPase $\alpha 2$ -I286T construct; Lane 3: T415M: Na,K-ATPase $\alpha 2$ -T415M construct; Lane 4: Control: mock-transfected. Error bars indicate SEM.

		I286T	T415M
ATP1A2	Human	EVGRTPIAEIEHFIQLITGV	SGATFDKRSPTWTALSRIAGL
ATP1A2	Rat	EVGQTPIAMEIEHFIQLITGV	SGATFDKRSPTWTALSRIAGL
ATP1A2	Chicken	EVGRTPIAEIEHFIRLITGV	SGATFDKRSPTWAALSRIAGL
ATP1A1	Human	EGGQTPIAAEIEHFIHIITGV	SGVSFDKTSATWLALSRIAGL
ATP1A1	Rat	EGGQTPIAAEIEHFIHLITGV	SGVSFDKTSATWFALSRIAGL
ATP1A1	Chicken	EGGKTPIAMEIEHFIHLITGV	SGASFDKSSATWLALSRIAGL
ATP1A3	Human	EVGKTPIAIEIEHFIQLITGV	SGTSFDKSSHTWVALSHIAGL
ATP1A3	Rat	EVGKTPIAIEIEHFIQLITGV	SGTSFDKSSHTWVALSHIAGL
ATP1A4	Human	AVGQTPIAAEIEHFIHLITVV	TGKTFTKSSDIWFMLARIAGL
ATP1A4	Rat	TMGKTPIATEIEHFIHIITAV	TGKTFFPKSSDIWFYLARIAGL
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Figure 3 Evolutionary conservation of the mutated amino acids Ile²⁸⁶ and Thr⁴¹⁵. Alignment of amino-acid sequence from several vertebrate Na,K-ATPase α subunits, showing evolutionary conservation of the amino acids Ile²⁸⁶ and Thr⁴¹⁵ (in boxes). Dashed lines indicate fully conserved amino acids. Protein sequences were obtained from Genbank. Human: P50993 (ATP1A2), P05023 (ATP1A1), P13637 (ATP1A3), Q13733 (ATP1A4); rat: P06686 (ATP1A2), P06685 (ATP1A1), P06687 (ATP1A3), Q64541 (ATP1A4); chicken: P24797 (ATP1A2), P09572 (ATP1A1).

compromised rescue was not due to a reduction in protein expression level (Figure 2a). These results clearly indicate that I286T and T415M mutant Na,K-ATPase pumps are unable to (fully) compensate for the loss of endogenous Na,K-ATPase activity, thereby indicating that both variations are disease causative.

Discussion

Here we describe a case of two allelic, novel, *ATP1A2* missense mutations in a patient with hemiplegic migraine. In fact, this is the first report of a hemiplegic migraine patient with compound heterozygosity, that is the presence of two different mutant alleles at a hemiplegic migraine gene locus. Several lines of evidence suggest that these two mutations are disease causative. First, both DNA variations were not present in 180 control individuals. Second, both affect amino acids that are highly conserved among α subunits of Na,K-ATPases from different species. Third, cell survival assays revealed that both mutations result in dysfunctional sodium–potassium pumps. The I286T mutation is located in the intracellular loop between M2 and M3 and showed a partial rescue of cell survival. Mutation T415M, resulting in complete *loss-of-function* on cell survival, is located in the large intracellular loop between M4 and M5, in which the majority of FHM2 mutations are found. This loop is critical for the function of the Na,K-ATPase pump since it harbours the phosphorylation and ATP-binding domains and undergoes major conformational changes during the enzymatic cycle.⁹

Interestingly, both mutations show reduced penetrance in family members of the proband. The proband's father (II-2), who has the T415M mutation (associated with complete loss of cellular rescue), only had non-migrainous headaches. His grandson (IV-2) who also carries the T415M mutation suffers from migraine with aura. However, he is still young (8 years old) and can still develop hemiplegic migraine. Although aunt II-1 who carries the I286T mutation (associated with partial cellular rescue) was

diagnosed with hemiplegic migraine and migraine with aura, the proband's mother (II-3) also carrying the I286T mutation only had a few isolated visual auras without headache. Clinical variation and reduced penetrance in FHM2 families has been reported before.^{10,11} In this respect, it is interesting to note that Todt *et al*¹² identified two *ATP1A2* variations that are possibly involved in the susceptibility to common forms of migraine.

Importantly, the patient described here shows that compound heterozygosity for *ATP1A2* is compatible with life. Apparently, *ATP1A2* mutations of various severities can cause FHM, but probably other mechanisms are still able to compensate partially for the loss of functional sodium–potassium pumps. In knock-out *Atp1a2* mice, however, homozygosity of two totally non-functional alleles is incompatible with life as pups die immediately after birth because they are unable to start breathing due to altered neuronal activity in respiratory neurons.¹³

The presence of two *ATP1A2* mutations in the proband causes a more severe phenotype compared to the milder FHM phenotype of her aunt, who carries only the I286T mutation. Hemiplegic migraine in the proband occurs at a lower age of onset, with a higher frequency and a longer duration of attacks that are always associated with hemiplegia. Although most likely due to the presence of two dysfunctional *ATP1A2* alleles, (part of) the difference in severity between these two individuals may reflect clinical variability that is not uncommon in FHM.¹⁴ Similarly, other genetic modifiers and environmental factors play a role in the expression of disease.

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