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Molecular Analysis of Aquaporin Genes 1 to 4 in Patients with Menière's Disease

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Key Words

Menière • Vertigo • Hereditary hearing loss • Inner ear • Aquaporin

Abstract

Background: Menière's Disease (MD) is an episodic cochleovestibular dysfunction of unknown etiology, still lacking a specific test and therapy. The proposed theories on the pathophysiology include genetic factors and factors relating to inner ear homeostasis. Various aquaporins (AQP), water channels, expressed in the inner ear and the vestibular organ, are involved in homeostasis. Mutations in *AQP* genes could result in disturbed inner ear homeostasis and endolymphatic hydrops, and therefore be involved in the pathogenesis of MD. Aim: To search for mutations in *AQP1* to 4 in patients suffering from MD. Methods: In patients with definite MD, DNA was extracted from whole blood. The coding sequences of *AQP1* to 4 were amplified by PCR reaction and sequenced. Results: One sequence alteration, homozygous c.105G->C (conservative change without alteration of amino acid) in *AQP3* was detected in 11 out of 34 patients but not in 100 control chromosomes. Conclusion: By itself the detected alteration is unlikely to play a role in the pathogenesis of MD. However, together with an additional modifying gene an effect can not be excluded. Additional regions (introns, splice-sites) and other genes involved in inner ear homeostasis need to be analyzed to identify a possible molecular alteration in MD.

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Introduction

Menière's disease (MD) is a cochleovestibular disorder characterized by the classic triad of episodic vertigo, tinnitus and fluctuating hearing loss. No specific diagnostic test exists. The etiology of MD remains unclear. The proposed theories on the pathophysiology of MD include, among others, genetic factors related to inner ear homeostasis [1-4]. The strong evidence of a genetic predisposition to

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MD mainly stems from family studies with a reported incidence of 7%. An autosomal dominant inheritance with reduced penetrance and anticipation was described [1, 4, 5]. A potential candidate gene, *COCH*, has been excluded by sequence analysis [6]. Other genes, such as aquaporins (*AQP*) and potassium channels, playing a role in the control of intralabyrinthine fluid dynamics, have been proposed in the etiology of MD based on association and animal study [7, 8].

AQP, water channels as their name suggests, are integral membrane proteins, which selectively transport water and also some small solutes, such as glycerol, along the osmotic gradient. AQP are ubiquitous in flora and fauna. In humans 11 isoforms (*AQP* 0-10) are described with different distribution and individual mode of action [9]. In the mammalian inner ear AQP 0-6, 7 and 9 can be detected in different structures of the cochlea, the vestibular organ and the endolymphatic sac [7, 10-15]. Among the *AQPs*, isoforms 1-4 are the best studied in the inner ear and have been proposed to be involved in the pathophysiology of MD. AQP1 has been detected in the intermediate cells of the stria vascularis in rats and in nonsensory epithelial cells in the spiral ligament in humans [16-18]. Likewise, the immunolocalization of AQP4 in humans closely resembles the one seen in animal models [7, 17, 19]. AQP2, along with AQP3 and 4, is coexpressed in the endolymphatic sac [10]. The important role of these three inner ear structures, stria vascularis, spiral ligament and endolymphatic sac, for fluid movement in the inner ear has been demonstrated in guinea pigs and *in vitro* studies [20, 21]. *AQP2* is of special interest with regard to MD. The application of both Lithium and OPC-31260, two vasopressin-antagonists, led to a down-regulation of *AQP2*-mRNA in the inner ear and reduced endolymphatic hydrops, the histopathologic hallmark of MD, in experimental studies [22, 23]. However, a mutation screen of *AQP2* in 12 individuals with MD did not show any mutations within the four coding exons and their intron-exon junctions [14].

An additional line of evidence for the important role of *AQPs* in the inner ear stems from hearing research. *AQP4* knockout and transgenic mice showed impaired hearing but normal neural conduction time. Therefore, cochlear dysfunction was suggested as the primary cause for hearing impairment in these mice [15, 19]. However, in other knockout mice lacking *AQP1,3* and *5* auditory brainstem response thresholds were not increased [15].

For these reasons, *AQPs* represent good candidate genes for an etiologic involvement in the pathophysiology of MD. Molecular changes in these genes might lead to a disturbed homeostasis and development of endolymphatic hydrops in MD. The aim of this study was to search for mutations in *AQP* isoforms *1-4* in patients with MD.

Materials and Methods

Patients

34 patients (18 males and 16 females) with "definite" MD were included in the study. Definite MD was defined according to the diagnostic scale set up by AAO/HNS. Other causes of cochleovestibular pathology or concomitant illnesses were excluded by a questionnaire. All participants signed an informed consent, according to guidelines approved by the Ethics Committee, University Hospital of Basel and Cantonal Hospital of Aarau, before entering the study.

Aquaporin mutation analysis

Genomic DNA was isolated from a 5mL EDTA blood sample using the Qiamp DNA Blood Maxi Kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. The following primers were used to amplify the coding exons and intron-exon junctions of *AQP1* to *4* (genbank accession no. NM_198098, NM_012909, NM_001079794, NM_001650):

AQP1-F(orward) (Exon 1) ; 5'GCT CTC AGA GGG AAT TGA GC3'
AQP1-R(everse) (Exon 1): 5'AAC CAG TGC CTT TCA GCA TC3'
AQP1-F (Exon 2): 5'TAC CCT CCT CAC CAG TCC TC3'
AQP1-R (Exon 2): 5'CTT CCC CCA CCT CCA TCT 3'
AQP1-F (Exon 3): 5'CCC TCC TCT CAC TCT CTC TTC A3'
AQP1-R (Exon 3): 5'GCT TAC CAT GGG ACA CCA AA3'
AQP1-F (Exon 4): 5'CCA GGT GGG AAA AAC CTG T3'
AQP1-R (Exon 4): 5'AGT GAC TTT GGC CAG CTT GT3'
AQP2-F (Exon 1); 5'CAT CCT GGC CCT GAG ACA 3'
AQP2-R (Exon 1): 5'ATC CCT CCC ATC TCA TCC TT3'
AQP2-F (Exon 2): 5'AGA GGA AAG TGG GCT CAG TG3'
AQP2-R (Exon 2): 5'CTC CAG GGA GGC TGG AAC 3'
AQP2-F (Exon 3): 5'TGT CCT CAC CTC CCT TCT CT3'
AQP2-R (Exon 3): 5'ATC CCA TGC TAT TCC AGC TC3'
AQP2-F (Exon 4): 5'GAT TAA TGT CGG GGA GGA GA3'
AQP2-R (Exon 4): 5'CAC GTC CAG GAA GCA GCT A3'
AQP3-F (Exon 1); 5'TCC TAT AAA GGG AGC CAC CA3'
AQP3-R (Exon 1): 5'GCA AGG GCT GGA GAG AGA AG'
AQP3-F (Exon 2/3): 5'CCT GCC TGT TCT TCT TCC TG3'
AQP3-R (Exon 2/3): 5'ACA GGG TGG GGA ATG CTT A3'
AQP3-F (Exon 4): 5'CTC TCC CCC GAC TCA CTG T3'

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Uni-/bilateral	Positive family hystory	Hearing Impairment			Functional Level	Duration of disease (mean, range)	Age of onset (mean, range)
		Type of Frequency	*Severity*	*Progression*			
29/5	4	all frequencies = 20 low =10 high= 4	mild=4, mild to moderate =5, moderate=12, moderate to severe=8 Severe =5	14	15 working, 3 partially disabled, (rest: data not available)	10 years (2-28)	45 years (24-65)

Table 1. Clinical features of patients with MD.

AQP3-R (Exon 4): 5'CCA TGA GCT ACC AAG GCA AC3'
AQP3-F (Exon 5/6): 5'TGC AGC TCA CCC TGT TCT C3'
AQP3-R (Exon 5/6): 5'GGA CAG TCA GTG GAT GCT CA3'
AQP4-F (Exon 1); 5'CAG GCA ATG AGA GCT GCA C3'
AQP4-R (Exon 1): 5'TGC CTA AGA AGG CAC AAA CA'
AQP4-F (Exon 2): 5'CCT GGG TGT AGT GGC TTC TG3'
AQP4-R (Exon 2): 5'TGC AAG AAG CTT GGA GTC CT3'
AQP4-F (Exon 3/4): 5'CTC TTG CTT CAA TTC TGA TGG A3'
AQP4-R (Exon 3/4): 5'CTGTAGGAAGATGGGAACTATCA3'
AQP4-F (Exon 5): 5'GC TGC TCA ATG GAA TAG CTT 3'
AQP4-R (Exon 5): 5'TGG AAG GAA ATC TGA GGA CAG 3'

PCR conditions were as follows: AQP 1 (Exon 1-4), AQP 2 (Exon 1-4): initial denaturation at 94 °C for 10 minutes; 40 cycles at 94 °C for 15 seconds, 58°C for 20 seconds and 72°C for 40 seconds; final elongation at 72°C for 4 minutes.

The annealing temperatures for AQP 3 (Exon 2-6) and AQP 4 (Exon 1-5) were 56°, for AQP 3 (Exon1) 57°C. The PCR was carried out on an Eppendorf Mastercycler Gradient (Vaudaux-Eppendorf, Basel, Switzerland).

For DNA sequencing, PCR products were purified using the QIAquick PCR Purification kit (Qiagen, Basel, Switzerland). The sequencing reaction was performed using the Big Dye Teminator Cycle Sequencing kit (Applied Biosystems, Rotkreuz, Switzerland), according to the manufacturer's guidelines. After purification using the DyeEx 2.0 Spin Kit (Qiagen, Basel, Switzerland) sequencing products were analyzed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Rotkreuz, Switzerland). Each fragment was sequenced twice following independent PCR amplification to assure accurate sequence results. The sequences were analyzed using the Sequencher 4.0 software (Gene Codes Corporation, Ann Arbor, MI).

Results

Patients

The mean age at onset of disease was 45 (range 24 to 65). The mean duration was 10 years (2-28).

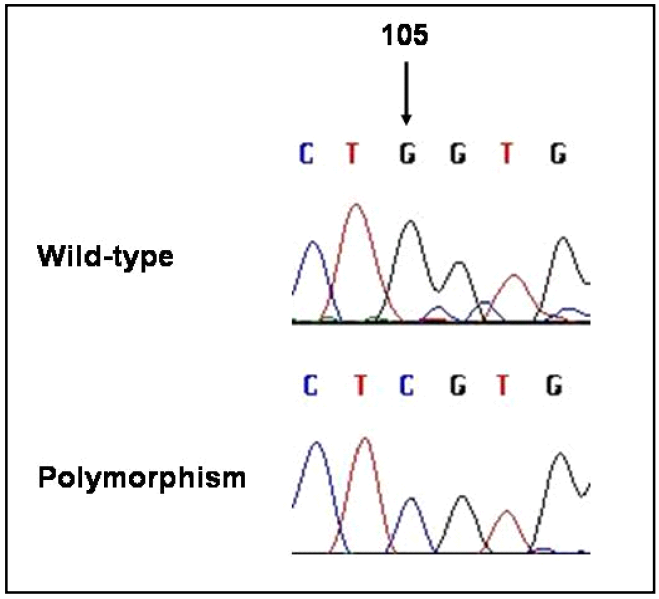

Fig. 1. Sequence analysis revealing the homozygous change c.105G->C (p.L35L) in exon 1 of *AQP3*.

The majority of patients were sporadic cases and suffered from unilateral disease. Family history was positive in 4 cases. The whole frequency range was affected in most patients with at least moderate severity. For the patients where data was available, working status was normal in about 80% (Table 1).

Mutation analysis

Mutation analysis of *AQP* subtypes *1- 4* revealed one sequence alteration in the coding sequences and intron-exon junctions of these genes after comparison with the published cDNA sequences. The homozygous change c.105G->C (p.L35L) in exon 1 of *AQP3* was found in 11 patients (Fig. 1). It was not detected

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in 100 control chromosomes and represents a novel variant. As it is not altering the amino acid, it was classified as a conservative change.

Discussion

Menière's disease is suggested to be of multifactorial origin, with one of the contributing factors being genetic [4, 7]. The distribution of AQPs in the cochlea and vestibular system, their known role as transporters of water and small solutes, the ability of vasopressin to induce endolymphatic hydrops are indicative of a possible role of *AQPs* in MD [7]. This study analyzed the coding sequences of the *AQP* isoforms *1-4* in patients with MD, the majority being sporadic cases. After sequencing analysis of the coding regions and exon–intron boundaries one single variation, c.105G->C, in *AQP 3* was found in 11 patients (32%). Although this percentage is intriguingly high, the amino-acid is not changed and therefore a mutational role must be considered with caution. However, it is possible that protein structure is slightly altered, that together with a modifying gene the function of *AQP3* is changed in a way, which leads to development of endolymphatic hydrops. The existence of a modifying gene would also explain the high clinical variability seen in patients with MD. Nevertheless, without functional analysis, which is difficult to perform in this context, this proposal remains a supposition. The selection of the patients was done with great care to achieve a homogenous sample size. However, it is possible that, in view of the lack of specific diagnostic tests, some patients might be wrongly classified as having MD. These patients will not necessarily exhibit genetic alterations. This might explain the number of patients in this study without the c.105G->C variation.

No other mutation was found in any of the patients. One explanation might be the fact that we cannot exclude either mutations in the *cis* transcription regulatory elements or promoter region, nor possible cryptic splice-site mutations. Splice-site mutations in the inner ear, for example, have been described for Pendrin, a deafness gene causing Pendred syndrome and enlarged vestibular aqueduct [24]. Also, in *AQP3* deficient humans, a homozygous transition in the 5'-donor splice site of intron 5 leading to the skipping of exon 5 has been found [25]. Other mutational mechanisms have been described for the *AQP* genes *1* and *2*. In *AQP1*, three individuals have been found with nonsense and missense mutations, encoding a nonfunctioning molecule but no obvious clinical defect [26]. Still, the total deficiency of AQP1 has been studied and subtle evidence for shortened red cell life span and reduced membrane surface area has been observed [27]. Additionally, *AQP1*-null individuals have limited capacity to concentrate urine [28]. Over 30 mutations in *AQP2* play a role in autosomal-recessive or autosomal-dominant nephrogenic diabetes insipidus (OMIM 222000 and 125800, respectively) [29]. Typically, missense mutations and small deletions have been found. In most cases, these AQP2 mutant proteins are misrouted and cannot be expressed at the luminal membrane [30]. However, Goji et al. reported non-functional channels not impaired in their routing to the plasma membrane [31]. In this study, sequencing analysis would have detected these types of mutations. In addition, homozygous deletions can be ruled out, as all exons of *AQP 1-4* could be amplified in all patients. A possible effect of the published mutations on hearing and balance cannot be evaluated, as neither hearing status nor vertigo have been described or measured in the patients with reported mutations in *AQP1-3*.

Quantitative differences in the expression of the different aquaporin subtypes as well as mutations in other - not yet analyzed - subtypes of *AQPs* might contribute to MD. The complex function of *AQPs* has been shown for the cochlea by Huang et al. [12]. The cellular localization and developmental expression were studied. The region-specific expression of AQPs have suggested compensatory and parallel pathways for water transport in the inner ear. The divergent expression might point to a concerted action of *AQPs* to maintain fluid regulation in the inner ear. The interaction and the high functional complexity of *AQPs* underlie the difficulty in studying a possible involvement of *AQPs* in human diseases. Further molecular-genetic research must be performed before a definitive statement concerning a possible etiologic role in MD can be made.

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Conclusions

The evidence from expression and functional studies points to a clear involvement of *AQP* genes in inner ear homeostasis. The detection of a conservative change in *AQP*3 in about a third of patients suffering from MD in this study and its absence in controls might be a first indication of a possible involvement of *APQs* in the development of MD. But the definite role of *AQPs* remains to be elucidated.

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