

## Analysis of Sequence Variations in the *ABCC6* Gene among Patients with Abdominal Aortic Aneurysm and Pseudoxanthoma Elasticum

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### Key Words

*ABCC6* · Abdominal aortic aneurysm · Gene polymorphism · MRP6 · Mutational analysis · Pseudoxanthoma elasticum

### Abstract

Abdominal aortic aneurysm (AAA) is characterized by dilatation of arterial walls, which is accompanied by degradation of elastin and collagen molecules. Biochemical and environmental factors are known to be relevant for AAA development, and familial predisposition is well recognized. A connective tissue disorder that is also associated with fragmentation of elastic fibers is Pseudoxanthoma elasticum (PXE). PXE is caused by mutations in the *ABCC6* gene and mainly affects dermal, ocular and all vascular tissues. To investigate whether variations in *ABCC6* are found in AAA patients and to determine mutations in PXE patients, we analyzed seven selected *ABCC6* exons of 133 AAA and 54 PXE patients subjected to mutational analysis. In our cohort of AAA patients, we found five *ABCC6* alterations, which result in missense or silent amino acid variants. The allelic frequencies of

these sequence variations were not significantly different between AAA patients and healthy controls. Therefore, we suggest that alterations in *ABCC6* are not a genetic risk factor for AAA. Mutational screening of the PXE patients revealed 19 different *ABCC6* variations, including two novel PXE-causing mutations. These results expand the *ABCC6* mutation database in PXE.

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### Introduction

Abdominal aortic aneurysms (AAAs) are localized dilations of the artery that are 1.5 times the diameter of the normal segment [1]. Rupture of an AAA is a significant cause of mortality and morbidity, and 1–6% of the population in the industrialized countries harbor aneurysms [2]. The survival rate after a ruptured aneurysm is low, and, therefore, the early diagnosis of AAAs and the prediction of risk factors for developing an aneurysm are important. AAAs are caused by an interplay of environmental, biochemical and genetic factors, each of which may modify the expression of the other [3, 4]. Smoking,

male gender and increasing age are the most powerful predictors of AAAs, and hypertension as well as atherosclerosis have been viewed as key factors in their development [5, 6]. In addition, familial aggregation of AAAs suggests that there are candidate genes that contribute to the pathogenesis of AAAs [1, 7]. The development of aneurysms is associated with a thinning of the vessel wall, which is accompanied by a remodeling of the connective tissue of the aortic wall, including the fragmentation of elastic and collagenous fibers [2, 8, 9].

A highly similar degradation of extracellular matrix components can be observed in skin biopsies from patients with Pseudoxanthoma elasticum (PXE, OMIM 177850 and 264800), an autosomally inherited connective tissue disorder characterized by accumulation of fragmented and calcified elastic fibers in dermal, ocular and cardiovascular tissues [10]. Generally, cutaneous manifestations are the first and most prevalent sign of PXE. The skin shows yellowish papules or plaques with laxity and loss of elasticity, mainly within flexural areas particularly in the neck, axilla, antecubital fossa and groin [11]. The diagnosis of PXE is confirmed histologically through the demonstration of fragmented calcified elastic fibers by applying the von Kossa stain to lesional skin. Characteristic ocular findings consist of peau d'orange or mottled hyperpigmentation of the retina, and angioid streaks, which are breaks in the calcified elastic lamina of Bruch's membrane. Choroidal neovascularization and hemorrhages in the macula can lead to severe loss of visual acuity [12]. The involvement of the vascular system can be manifested by hypertension, intermittent claudication, gastrointestinal bleeding and myocardial infarcts at a relatively early age [13, 14]. Furthermore, recent studies report on collagen and elastic fiber alterations in the majority of all vessel structures, for example the descending aorta, ascending aorta, iliac artery and vena cava, as well as all small and medium-sized vessels [15]. A comparable degradation of elastic and collagenous vessel wall components is also a characteristic for AAA formation.

PXE was first described as a sporadic disorder, but in a large number of families, autosomal recessive inheritance has been observed [16, 17]. In some cases an autosomal dominant fashion has been reported [10, 18]. Recently, the PXE candidate gene, ATP-binding cassette transporter subfamily C member 6 (*ABCC6*), was identified, and up to now more than 70 *ABCC6* mutations were found to be associated with PXE, including nonsense, missense, insertions and deletions, as well as putative splice site mutations [17, 19–26]. The *ABCC6* gene contains 31 exons and encodes a 165-kDa transmembrane

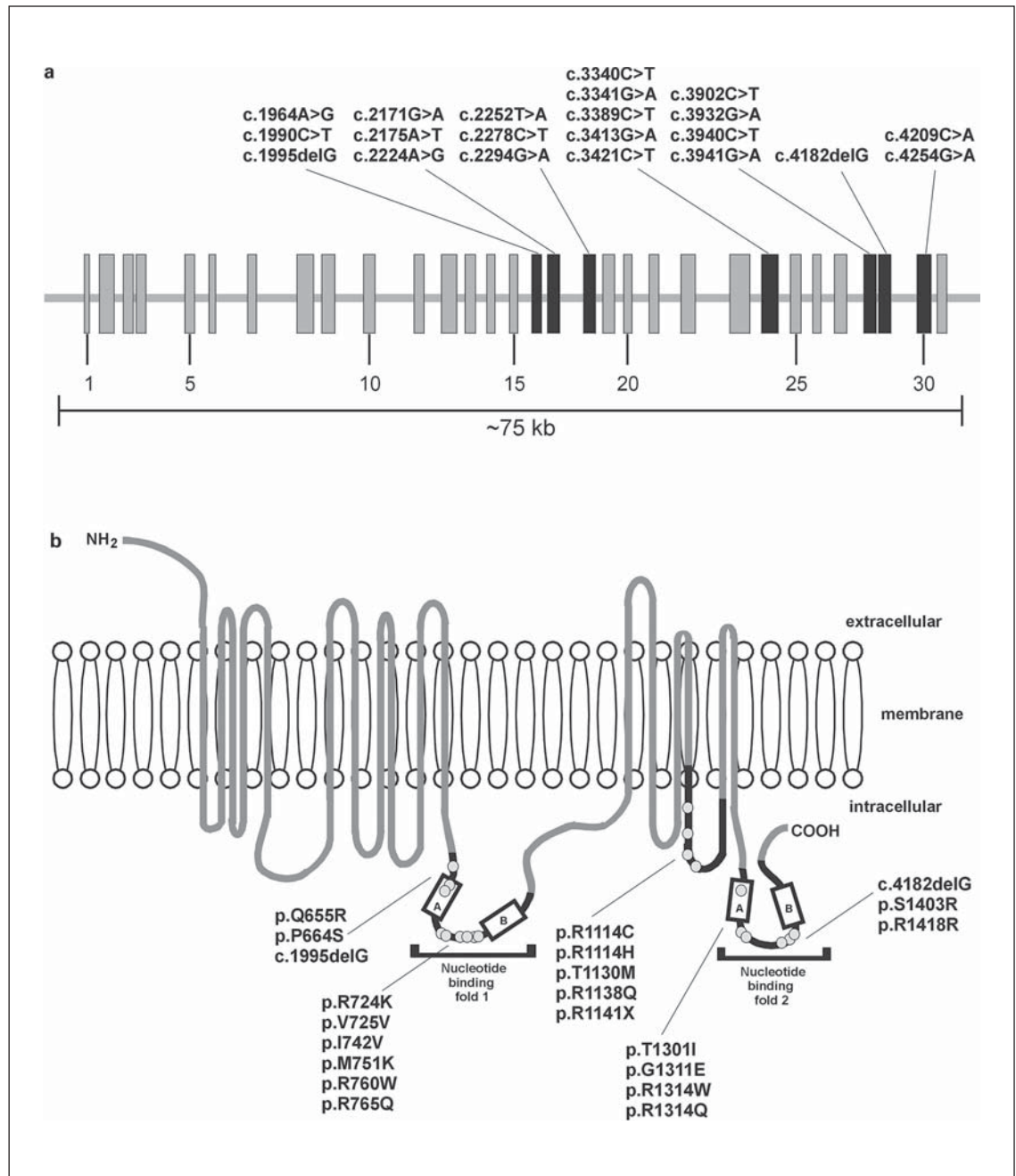
protein of 1,503 amino acids termed multidrug-resistance-associated protein 6 (MRP6). MRP6 is predominantly expressed in the liver and the kidneys, and to a lesser extent in tissues frequently affected with PXE [19, 27]. Different *in vitro* studies suggest an involvement of MRP6 in transmembrane transport of glutathione conjugates [28, 29], but the physiological function of MRP6 and, consequently, the pathobiochemical mechanisms of PXE remain to be elucidated.

Based on histopathological similarities observed in PXE and AAA, we consider *ABCC6* to play a role in AAA formation. Therefore, the aim of this study was to determine the frequencies of polymorphisms and mutations in the *ABCC6* gene of AAA and PXE patients and to find out whether *ABCC6* sequence variations are associated with AAA. The mutational analysis was restricted to the *ABCC6* exons 16–18, 24 and 28–30, since, according to other PXE studies [16, 30], these exons encode functionally important MRP6 regions and harbor the majority of PXE-causing mutations. Employing this strategy, we detected several different *ABCC6* sequence alterations, which are presented and discussed in this study (fig. 1).

## Materials and Methods

### *Patients and Controls*

Venous blood samples for DNA isolation were obtained from 133 Caucasian patients with infrarenal atherosclerotic abdominal aortic aneurysm (AAA). Patients with inflammatory aortic aneurysms [31] were excluded based on computed tomography findings in order to maintain a homogenous patient sample. An aneurysm was defined as a permanent dilatation of the aorta, with a diameter at least 50% greater than that of the proximal neck [32]. The diagnosis was established using color-coded duplex sonography and computed tomography. In the patient group, coronary and peripheral artery disease were confirmed or excluded by angiographic examination of the coronary and peripheral arteries. Diabetes mellitus was defined as fasting blood glucose levels >110 mg/dl measured on three occasions, pathologic oral glucose tolerance tests and HbA1c >6.5%. Hyperlipidemia was defined as fasting total serum cholesterol >200 mg/dl, LDL cholesterol >130 mg/dl or serum triglycerides >180 mg/dl and treatment with lipid-lowering medication. Arterial hypertension was diagnosed according to the WHO criteria. Patients who smoked more than three cigarettes per day were regarded as current smokers. Clinical history and physical examination were evaluated with special attention to atherogenic risk factors and cardiovascular comorbidities. The presence of the Marfan and Turner syndrome was excluded by clinical and genetic methods. Gender, age, smoking habits, hyperlipidemia, hypertension, diabetes mellitus, coronary and peripheral artery disease and a history of cerebrovascular events were recorded at initial presentation. The characteristics of the AAA patients are listed in table 1. Unfortunately, additional information about a familial occurrence of aneurysms was not available. The study was approved by the



**Fig. 1. a** Exon-intron structure of the *ABCC6* gene, spanning approximately a 75-kb genomic region. Grey lines represent introns and boxes describe exons. The number of every fifth exon is indicated. Black boxes represent the *ABCC6* exons 16–18, 24 and 28–30, which were analyzed in this study and encode the nucleotide binding folds and the cytoplasmic eighth loop of the MRP6 protein. The localization of the DNA variations found in these selected *ABCC6* exons in our cohorts of AAA patients, PXE patients, their family members and German blood donors is indicated. **b** Predicted structure of the MRP6 protein with its potential nucleotide binding folds and the Walker motifs A and B, which are represented by white boxes. Black lines show the MRP6 regions that are coded by the exons analyzed in this study. The positions of the identified mutations are indicated.

**Table 1.** Characteristics of the AAA and PXE patients and their relatives

Characteristics	AAA patients n = 133	PXE patients n = 54	PXE relatives n = 23
Male sex	97 (72.9) <sup>a</sup>	18 (33.3)	7 (30.4)
Age, years	67.2 ± 11.8 <sup>a</sup>	48.6 ± 13.9	45.1 ± 21.3
Arterial hypertension	112 (84.2)	18 (33.3)	1 (4.35)
Diabetes mellitus	18 (13.5)	3 (5.56)	0 (0)
Hyperlipidemia	76 (57.1)	1 (1.85)	0 (0)
Current smokers	45 (33.8)	11 (20.4)	8 (34.8)
Chronic renal failure	18 (13.5)	ND	ND
Marfan syndrome	0 (0)	ND	ND
Turner syndrome	0 (0)	ND	ND
PXE-specific organ involvement			
Eyes	ND	47 (87.0)	0 (0)
Skin	ND	48 (88.9)	0 (0)
Gastrointestinal tract	ND	5 (9.26)	0 (0)
Kidney	ND	7 (13.0)	0 (0)

ND = Not determined.  
<sup>a</sup> The values shown are either means ± SD (age), or total numbers (%).

Ethics Committee of the University of Vienna, and all AAA patients gave written informed consent.

EDTA-anti-coagulated whole blood samples were obtained from 54 Caucasian PXE patients (aged 16–78 years) and from 23 unaffected or not yet affected relatives (14–80 years old). In all patients, the diagnosis of PXE was consistent with the reported consensus criteria [33, 34]. The status of the PXE patients was determined by the presence of ocular findings and dermal lesions, and was histologically confirmed by the observation of calcification in the elastic fibers in skin biopsies following von Kossa staining. The biopsy samples were taken from lesional skin. All participants of the study were thoroughly questioned by medical specialists about personal diseases, organ involvement and their family history. The characteristics of the PXE patients are listed in table 1. The study cohort comprised 54 patients with PXE and 23 direct relatives from a total of 49 non-consanguineous families with an apparently autosomal recessive or sporadic mode of inheritance of the PXE phenotype. For control purposes, EDTA-anti-coagulated whole blood samples were obtained from 910 healthy Caucasian blood donors (407 males and 503 females), aged 18–68 (mean age ± SD, 39.7 ± 19.3 years). The study was approved by the Institutional Review Board, and patients provided informed consent.

#### *DNA Extraction and PCR Amplification of the ABCC6 Exons*

Genomic DNA was extracted from whole blood using the QIAamp blood kit (Qiagen, Hilden, Germany) or the Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minn., USA) according to the manufacturer's instructions. To amplify the exons 1–31 of the *ABCC6* gene, *ABCC6*-specific primers were designed in the flanking introns to amplify DNA fragments encompassing the exon/intron boundaries and the exon. The PCR reactions were performed as described previously [35] with fragment-specific annealing temperatures. The primer sequences, the annealing tem-

peratures and the sizes of the PCR products of the DHPLC-analyzed *ABCC6* exons (16–18, 24 and 28–30) are given in table 2.

#### *DHPLC and DNA Sequencing for Mutational Analysis*

DHPLC analysis was carried out on an automated HPLC device equipped with a DNA separation column (Wave System, Transgenomic, Omaha, Nebr., USA). DNA heteroduplexes for DHPLC analysis were created by mixing the PCR product of a healthy subject, whose wild-type sequence was confirmed by double-stranded sequencing with the corresponding amplicon of an AAA or PXE patient or family member in a ratio of 1:1. The reaction mixture was denatured for 5 min at 94°C and then gradually reannealed by decreasing the sample temperature from 95 to 25°C over a period of 40 min. The DNA duplexes were then separated at a flow rate of 0.9 ml/min by means of a linear acetonitrile gradient. The mobile phase of the column consisted of a mixture of 0.1 M triethylamine acetate (pH 7.0) with (buffer B) or without (buffer A) 25% acetonitrile. The gradient parameters for each amplicon were determined using the Transgenomic Wavemaker software and the Stanford melt program (<http://www.insertion.stanford.edu/melt.html>). For the successful resolution of heteroduplexes from homoduplexes, these programs also revealed an optimal analysis temperature for each melting domain of the amplicon. If recommended by the software, different temperatures for the DHPLC analysis of the DNA fragment were used. The PCR products showing heteroduplexes by DHPLC analysis were bidirectionally sequenced using the Big Dye Ready Reaction sequencing kit and an ABI Prism 310 capillary sequencer (Applied Biosystems, Foster City, Calif., USA). In order to avoid genotype misinterpretation due to amplification errors, these specimens were PCR amplified and again bidirectionally sequenced.

**Table 2.** Primer sequences used for the amplification of the DHPLC-analyzed *ABCC6* exons 16–18, 24 and 28–30

Exon	Upper primer sequence (5' to 3')	Lower primer sequence (5' to 3')	Annealing temperature, °C	Product size, bp
16	E16U: AGCGAGGAAGTGGGAC	E16L: AGAGGCGGGCTGAAC	58	241
17	E17U: CCAAATGACTCCCAACT	E17L: CTCCTGTGACCAAAGTAA	55	264
18	E18U: GGTTAGGACTGGATGCT	E18L: TTTCACCCTGTAGATGCT	51	289
24	E24Ua: CTCCCATCCATCCTTCT	E24La: CCTCGCTACCATACAATATGA	56	307
	E24U: TGACCTCGCTACCATACAAT	E24L: CCTGTACTTGGGGCTCTCT	55	377
28	E28Ua: CCCCTCCACCAGCCTCA	E28La: CCTTTCCTCCCAACCCCG	66	201
	E28U: CCGCAGAGAGCCAGGG	E28L: GCCTTTCCTCCCAACCC	64	291
29	E29Ua: CCTCGCCTCGGTCAGCAC	E29La: TCCTGTTCCCTGGCTCTC	59	153
	E29U: GCCATCCCCTCCTCTCC	E29L: ATCAGCATCATCCCCAG	61	316
30	E30U: TGCTCCGCCTCCTTC	E30L: CGTCCCAGCTAATTGTCCC	61	292

#### Mutation Genotyping through PCR and RFLP/DHPLC Analysis

Different RFLP screening assays were developed for verification of the *ABCC6* mutations c.3340C>T, c.3341G>A, c.3389C>T and c.3932G>A in PXE patients and for the demonstration of their absence in healthy controls. The restriction enzyme digestions of the PCR products E24a and E28a were performed according to the manufacturer's instructions. The restriction enzyme *Esp3I* (MBI Fermentas, St. Leon-Rot, Germany) was used for the detection of the mutations c.3340C>T, c.3341G>A and c.3389C>T and *AluI* (Gibco BRL, Gaithersburg, Md., USA) for the allele-specific genotyping of the mutation c.3932G>A. Digested and undigested products were subjected to agarose gel electrophoresis and visualized after ethidium bromide staining. The c.3421C>T, c.2175A>T, c.2224A>G, c.4209C>A and Ex23\_Ex29del PCR and RFLP genotyping was performed as described previously [35–37]. DHPLC analysis of the PCR products E16, E17, E18, E29a and E30 was used to demonstrate the presence or absence of DNA variations in healthy controls.

#### Statistical Analysis

The allelic frequencies were estimated by gene counting and scoring. Significant difference in the alleles observed between the groups was tested using Fisher's exact test. All statistical tests were performed using GraphPad Prism software. *p* values of 0.05 or less were considered significant.

## Results

#### *ABCC6* Sequence Variations in AAA Patients

Mutational analysis of the *ABCC6* exons 16–18, 24 and 28–30 from 133 AAA patients, 54 PXE patients, 23 PXE relatives and a cohort of healthy control subjects revealed 22 different sequence variations in the coding *ABCC6* regions and six DNA alterations in the flanking introns (table 3). Among the exon variations, five were found in patients with AAA, namely DNA alterations

c.1964A>G, c.2171G>A, c.2175A>T, c.2224A>G and c.4254G>A in the exons 16, 17 and 30 of the *ABCC6* gene. These sequence variations led to the missense and silent amino acid variants p.Q655R, p.R724K, p.V725V, p.I742V and p.R1418R. The p.Q655R mutation occurred in a heterozygous state in one AAA patient and was absent in our PXE cohort and in 143 control subjects. DNA sequencing of all 31 *ABCC6* exons showed that the AAA patient had no further *ABCC6* mutations. The exon 17 variations p.R724K, p.V725V and p.I742V were identified in a compound heterozygous form in 3 AAA patients and were also found in this state in 1 PXE patient and his 79-year-old unaffected mother, as well as in 2 healthy controls. The p.R1418R variant occurred in a heterozygous state in 6 AAA patients and 2 control subjects, but was not observed in our PXE cohort. However, the differences in the c.1964A>G, c.2171G>A, c.2175A>T, c.2224A>G and c.4254G>A frequencies between the alleles of AAA patients and controls were statistically not significant. The frequent PXE mutation Ex23\_Ex29del was absent in our AAA cohort.

#### *ABCC6* Screening in the PXE Cohort

We found 19 different sequence variations in the exons 16–18, 24 and 28–30 of the *ABCC6* gene in our group of PXE patients and their relatives (table 3). The most common PXE mutation was the nonsense mutation p.R1141X which was observed in a homozygous form in 4 (7.4%) and in a heterozygous state in 20 (37.0%) PXE patients. The genotype frequencies of the 23 unaffected or not yet affected PXE family members were 60.9, 39.1 and 0 for wild-type, heterozygous and homozygous forms, respectively. Another frequent PXE mutation was the deletion Ex23\_Ex29del, which occurred in a heterozygous

**Table 3.** Allelic frequencies of the *ABCC6* sequence variations in AAA patients (266 alleles), PXE patients (108 alleles), their family members (46 alleles) and unrelated blood donors

Exon <sup>a</sup>	Sequence variation	Allele frequency			
		AAA patients	PXE patients	PXE relatives	blood donors
16	c.1964A>G (p.Q655R)	1	0	0	0/286
16	c.1990C>T (p.P664S)	0	0	0	1/286
16	c.1995delG (frameshift)	0	3	0	0/286
17	c.2171G>A (p.R724K)	3	1	1	2/254
17	c.2175A>T (p.V725V)	3	1	1	2/254
17	c.2224A>G (p.I742V)	3	1	1	2/254
i-17	IVS17+22T>G	1	0	0	0/254
18	c.2252T>A (p.M751K)	0	2	0	0/204
18	c.2278C>T (p.R760W)	0	1	0	0/204
18	c.2294G>A (p.R765Q)	0	3	0	0/204
24	c.3340C>T (p.R1114C)	0	1	0	0/400
24	c.3341G>A (p.R1114H)	0	1	0	0/400
24	c.3389C>T (p.T1130M)	0	2	0	0/400
24	c.3413G>A (p.R1138Q)	0	2	0	ND
24	c.3421C>T (p.R1141X)	0	28	9	1/1,820 <sup>b</sup>
i-24	IVS24+15G>A	1	0	0	ND
28	c.3902C>T (p.T1301I)	0	1	0	ND
28	c.3932G>A (p.G1311E)	0	1	0	0/400
28	c.3940C>T (p.R1314W)	0	1	0	ND
28	c.3941G>A (p.R1314Q)	0	1	1	ND
i-28	IVS28+49C>T	59	ND	ND	ND
i-28	IVS28-30C>T	48	ND	ND	ND
29	c.4182delG (frameshift)	0	3	0	0/400
i-29	IVS29+9G>A	5	ND	ND	ND
30	c.4209C>A (p.S1403R)	0	1	0	0/244
30	c.4254G>A (p.R1418R)	6	0	0	2/244
i-30	IVS30+11C>G	0	2	0	0/244
23-29	Ex23_Ex29del	0	5	3	ND

i = intron; ND = not determined.  $p > 0.05$ , AAA patients vs. blood donors.

<sup>a</sup> The exon that contains the sequence variation.

<sup>b</sup> Data taken from Götting et al. [35].

form in 5 (9.3%) PXE patients and 3 (13.0%) relatives. In addition, we detected a silent variation (p.V725V) and 14 missense mutations (p.R724K, p.I742V, p.M751K, p.R760W, p.R765Q, p.R1114C, p.R1114H, p.T1130M, p.R1138Q, p.T1301I, p.G1311E, p.R1314Q, p.R1314W and p.S1403R) in their heterozygous, compound heterozygous and homozygous forms in 17 PXE patients. Apart from these DNA alterations, we found two deletions, c.1995delG and c.4182delG, which are predicted to result in a premature termination of the MRP6 protein. The deletions occurred in a heterozygous and homozygous form in 5 PXE patients. With the exception of the variants p.R724K, p.V725V and p.I742V, none of the 19 *ABCC6* sequence variations identified in our PXE cohort occurred in the group of AAA patients.

#### Novel *ABCC6* Sequence Alterations

Among the 22 *ABCC6* sequence variations found in this study, 17 had been previously described as PXE-causing mutations and *ABCC6* polymorphisms [16, 19, 22-24, 30, 35, 36], and 5 were novel DNA alterations resulting in the missense and silent variants p.Q655R, p.P664S, p.R1114C, p.G1311E and p.R1418R. An alignment using the ClustalW program showed that the missense mutations alter amino acid residues conserved in the human, mouse and rat MRP6 proteins. In addition, the mutation p.R1114C changes an amino acid conserved in the human MRP proteins. The variations p.R1114C and p.G1311E occurred in a heterozygous form in 2 PXE patients, and RFLP or DHPLC analysis revealed that they were not present in our groups of healthy controls

and AAA patients. DNA sequencing of all 31 *ABCC6* exons showed that the first PXE patient was compound heterozygous for p.R1114C and the known PXE mutation c.1995delG in exon 16, whereas the second carried the new variant p.G1311E and the PXE-causing exon 24 mutation p.R1141X in a compound heterozygous state. The p.Q655R and p.P664S mutations were identified in a heterozygous state in only 1 AAA patient and 1 control subject, respectively.

## Discussion

Abdominal aortic aneurysms result from several different pathogenic mechanisms, including environmental, biochemical and genetic factors [3, 4]. At present, sequence variations in at least three genes have been shown to be associated with AAA formation. Polymorphisms in the genes matrix metalloproteinase-9 [38], methylenetetrahydrofolate reductase [4] and heme oxygenase-1 [39] belong to these DNA alterations. Most certainly, the aneurysmal process is multifactorial and will not be determined by the above-mentioned gene polymorphisms alone. Consequently, further genes that possibly affect the development of AAAs should be characterized by mutational analysis. The pathogenesis of aneurysms is accompanied by a degradation of extracellular matrix components, such as elastic and collagenous fibers [2, 8, 9]. Elastin is the major structural component of the aortic wall, and its degradation may be considered as the initial event in the formation of aneurysms [40]. Fragmentation of elastic fibers is also a typical hallmark of the connective tissue disorder PXE [10], which is caused by mutations in the *ABCC6* gene [19–24]. This disease mainly leads to alterations in dermal, ocular and all vascular tissues, and, in some cases, aneurysmatic dilatations were observed [41, 42]. In order to determine if the occurrence of sequence variations in the *ABCC6* gene is associated with the presence of AAAs and to compare the frequencies of *ABCC6* polymorphisms and mutations in AAA and PXE patients, we screened the *ABCC6* exons 16–18, 24 and 28–30 from 133 unrelated patients with AAA, 54 PXE patients and 23 PXE relatives. We selected these particular *ABCC6* exons for mutational screening, because the majority of the PXE-causing mutations had been observed in these regions [16, 30]. The exon 24 codes for a cytoplasmic loop between the 15th and 16th transmembrane region of the MRP6 protein, whereas the exons 16–18 and 28–30 encode the two nucleotide binding folds [16]. The high frequency of PXE-causing mutations in

these regions suggests that these domains are critical for a normal function of the MRP6 protein, although its exact physiological function is still unknown. In our study, we identified 19 different *ABCC6* sequence variations in our cohort of PXE patients. Among these, 3 variants (p.R724K, p.V725V and p.I742V) had already been described as *ABCC6* polymorphisms [36]. 14 DNA alterations had been shown to be PXE-causing mutations [16, 19, 22–24, 30, 35, 36] and, consequently, have not been further examined in this study. Two *ABCC6* sequence alterations were novel, namely the alterations p.R1114C and p.G1311E. The new mutations were considered to be PXE-causing because on the one hand none of these variations was found among healthy controls and on the other hand they are located in the MRP6 domains which are considered to be critical for the function of the protein. In addition, both mutations alter amino acid residues, which are conserved in the human, mouse and rat MRP6 proteins and, in the case of p.R1114C, also in the human MRP protein family. The glycine residue at position 1311 of the MRP6 protein is not conserved in the human MRP proteins, but there is always an uncharged residue at this position. Therefore, it is possible that the observed change in an uncharged amino acid to a charged residue can result in an incorrect folding of the protein and consequently lead to a dysfunctional MRP6 protein. However, the assumption that the novel mutations are disease-causing is confirmed by the fact that those PXE patients who were identified to be heterozygous carriers of the p.R1114C and p.G1311E mutations were found to be compound heterozygous for only one other PXE-causing *ABCC6* mutation.

Mutational analysis of the 133 AAA patients revealed a total of five *ABCC6* sequence alterations, which occurred in a heterozygous or compound heterozygous state in 10 patients. The mutation p.Q655R was identified in a heterozygous form in 1 AAA patient who was found to bear no other mutation in the coding region of the *ABCC6* gene, including the frequent PXE mutation Ex23\_Ex29del. Unfortunately, DNA samples from direct relatives were not available. Consequently, the potential cosegregation of p.Q655R with AAA formation could not be analyzed in the present study and supply information about the pathogenicity of this mutation. Among healthy controls, p.Q655R was absent, but the difference in the allelic frequencies in AAA patients and controls was not statistically significant. Therefore, we suppose the p.Q655R mutation not to be pathogenic, although there is still the possibility that the heterozygous p.Q655R variant may play a role in AAA development. The variants

p.R724K, p.V725V, p.I742V and p.R1418R were also assumed not to be a risk factor for AAA formation, because they occurred in healthy controls as well as in our PXE cohort, and their allelic frequencies were not significantly different between AAA patients and healthy subjects. In addition, several intronic changes were identified. The influence of these variations on mRNA splicing is difficult to predict because the rules for splicing are complex and multiple degenerate motifs are involved [43]. Based on their location within the introns, these alterations were not supposed to have an effect on mRNA splicing [43, 44], and therefore, pathogenetic consequences of these variations are unlikely. For this reason, they have not been further examined in this study, although a pathological relevance can not be completely excluded.

The statistical significance of our results is limited due to the relatively small study cohort. Large multicenter studies are required to evaluate our initial findings. A further limitation of our study is that the mutational analysis was restricted to those seven *ABCC6* exons which encode the functionally important MRP6 regions and harbor the majority of mutations in PXE patients. Therefore, the remaining *ABCC6* exons may contain DNA alterations which could be associated with the development of AAA. In addition, each method which is currently used for the de novo screening for DNA variations bears a risk of false-negative results. The DHPLC technique has been described to have a very high detection rate of sequence variations similar to that of direct DNA sequencing and to be superior to other screening methods like SSCP or TGGE [45, 46]. Large deletions within the *ABCC6* gene have been reported to occur in PXE patients, but with the

exception of the Ex23\_Ex29del mutation these deletions are very rare [16, 37]. Therefore, we excluded the occurrence of the Ex23\_Ex29del mutation in our AAA patients and anticipated other large deletions covering the whole *ABCC6* gene to be absent in our cohort.

In summary, we found five sequence variations in particular exons of the *ABCC6* gene in our cohort of AAA patients. Since the differences in the frequencies of these alterations between the alleles of AAA patients and healthy controls were not statistically significant, we suggest that *ABCC6* is not a risk factor for AAA. Undoubtedly, other genetic risk factors may play an important role for the susceptibility to AAA, and, therefore, future studies should focus on the mutational analysis of further genes which may affect the pathogenesis of AAA. In addition, the mutational screening of our AAA and PXE cohorts revealed several new polymorphisms and PXE-causing mutations in the *ABCC6* gene. The identification of novel PXE mutations is a further step towards detecting the whole number of *ABCC6* mutations which lead to PXE, which is a prerequisite for clarifying the molecular pathology underlying this disease.

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