Copy number variation in the ATP-binding cassette transporter ABCC6 gene and ABCC6 pseudogenes in patients with pseudoxanthoma elasticum

Marianne K. Kringen1, Camilla Stormo2, Jens Petter Berg2,3, Sharon F. Terry4, Christine M. Vocke4, Samar Rizvi4, Doris Hendig5 & Armin P. Piehler6

1Department of Pharmacology, Oslo University Hospital, Ullevål, Oslo, Norway
2Department of Medical Biochemistry, Oslo University Hospital, Ullevål, Oslo, Norway
3Department of Medical Biochemistry, Institute of Clinical Medicine, University of Oslo, Oslo, Norway
4PXE International, Genetic Alliance, Washington, DC
5Herz- und Diabeteszentrum NRW, Institut f€ur Laboratoriums- und Transfusionsmedizin, Universit€atsklinik der Ruhr-Universit€at Bochum, Bad Oeynhausen, Germany
6Furst Medical Laboratory, Oslo, Norway

Abstract

Single mutations in the ATP-binding cassette transporter (ABCC6) gene (OMIM 603234) are known to cause the rare autosomal recessive disease pseudoxanthoma elasticum (PXE). Recently, we have found that copy number variations (CNVs) in pseudogenes of the ABCC6 gene are quite common. The aim of this study was to investigate the frequency and possible contribution of CNV in ABCC6 and its pseudogenes in PXE. Genomic DNA from 212 PXE individuals were examined for copy number by pyrosequencing and quantitative polymerase chain reaction (PCR) and compared with healthy individuals. The frequency of PXE individuals with any CNV was higher than in healthy individuals. The majority of variation comprised known and possibly new deletions in the ABCC6 gene and duplications of the ABCC6P1 and ABCC6P2 genes. ABCC6 deletions and ABCC6P2 duplications were not observed in 142 healthy individuals. In conclusion, by pyrosequencing and quantitative PCR, we were able to detect known and possibly new deletions in the ABCC6 gene that may have caused the PXE phenotype. Pyrosequencing may be used in PXE patients who have obtained incomplete genotype from conventional techniques. The frequency of ABCC6P2 pseudogene duplication was more common in PXE patients than healthy individuals and may affect the PXE phenotype.

Introduction

The ATP-binding cassette transporter ABCC6 belongs to a large family of membrane proteins (ABC transporters) that are highly conserved and present in all organisms from bacteria to man (Higgins 1992). The ABCC6 gene is located on the short arm of chromosome 16 between two shorter, almost identical (>99% sequence identity) pseudogenes, ABCC6P1 and ABCC6P2 (Pullkinnen et al. 2001). Pseudogenes are generally defined as nonfunctional, meaning that they cannot produce a functional protein (Mighell et al. 2000; Balakirev and Ayala 2003). However, recently, we could show that a significant fraction of the ABC-transporter pseudogenes are transcribed. Moreover, we found evidence for a regulatory interdependency between ABCC6 and its pseudogene ABCC6P1 (Piehler et al. 2008).

Single mutations in ABCC6 are known to cause the rare (prevalence between 1:25,000 and 1:100,000), autosomal recessive disease pseudoxanthoma elasticum (PXE), a metabolic disorder characterized by ectopic mineralization of soft connective tissues (Li et al. 2009; Plomp et al. 2010; Uitto et al. 2010). Patients with PXE typically present with pathological findings in the
skin (yellowish papules in the flexural areas), the eye (angioid streaks and choroidal neovascularization), and the cardiovascular system (atherosclerosis). In most cases, PXE is associated with considerable morbidity and, in rare cases, mortality due to cardiovascular complications. Generally, the phenotype of PXE is highly variable showing significant inter- and intrafamilial heterogeneity (Plomp et al. 2010; Uitto et al. 2010). More than 388 mutations have been described to cause PXE (Leiden Open Variation Database at NCBI). Although an association between mutation site and severity of the disease has been postulated, no correlation has been observed (Pfendner et al. 2007; Costrop et al. 2010; Koblos et al. 2010). The wide range of the PXE phenotype severity has also led to the initiation of studies seeking modifier genes for this disease (Hendig et al. 2007; Hovnanian 2010).

In a recent study, it could be shown that larger deletions including the ABCC6 gene significantly contribute to PXE (Costrop et al. 2010). These findings were not surprising, though, as ABCC6 is located on chromosome 16, a known hotspot of chromosomal instability showing several genomic duplications and deletions (generally called copy number variations [CNVs]) (Sharp et al. 2006). On the basis of this information, we recently determined CNVs of ABCC6 and ABCC6 pseudogenes in different healthy populations, and found that CNVs of the ABCC6 pseudogenes are quite common (Kringen et al. 2012). Having less or more copies of ABCC6 pseudogenes is likely to influence the expression level of these pseudogenes, and therefore, may have an impact on ABCC6 including the mRNA level, protein level and function, and the PXE phenotype. The aim of this study was to gain insight into the frequency and contribution of CNV in ABCC6 and its pseudogenes in PXE.

Methods

PXE patients

DNA from patients diagnosed with PXE was obtained from the PXE International Registry and BioBank. The patients included 212 individuals and were mainly of European descent (White = 185, Hispanic = 2, Asian = 1, African American = 1, Unknown = 23). Of these, 148 (70%) were female and 64 (30%) were male. PXE phenotypes were categorized according to the five organ systems (skin, eye, gastrointestinal, vascular, and cardiac) and severity (Phenodex™, PXE International, Washington, DC) (Pfendner et al. 2007). In addition, information about diagnosis of high cholesterol or other lipid disorder was available, however, the information was self-reported and not available for all patients. ABCC6 mutation information was available for most patients (Table S1). Written informed consent was obtained from all subjects before blood samples were taken. The study was approved by the Norwegian Regional Ethics Committees.

Controls

Genomic DNA from healthy individuals from the National Institute of General Medical Science (NIGMS) was purchased from the Coriell Cell Repositories (Camden, NJ). The populations were Caucasians (n = 50), Chinese (n = 24), Middle East (n = 20), Mexicans (n = 24), and Africans (n = 24).

CNV analysis

The CNV was analyzed in short specific regions involving exon 2, intron 7, and intron 11 of ABCC6 (NM_001171.5; chr16: 16,243,422–16,317,328; GRCh37/hg19 Assembly). For absolute copy number determination of ABCC6, a TaqMan® Copy Number Assay targeting ABCC6 specifically in intron 11 was used (Hs03952142_cn; Applied Biosystems, Foster City, CA). Two pyrosequencing assays were used to determine the relative copy number of ABCC6P1 (NR_003569.1; chr16: 18,582,570–18,609,607; GRCh37/hg19 Assembly) versus ABCC6 (targeting intron 7 of both genes) and the relative copy number of ABCC6P2 (NR_023387.1; chr16: 14,916,289-14,918,559; GRCh37/hg19 Assembly) versus ABCC6 and ABCC6P1 (targeting exon 2 of all three genes). The analyses were performed as described previously (Kringen et al. 2012). The absolute copy number for each gene (ABCC6, ABCC6P1, and ABCC6P2) was finally deduced from the TaqMan® Copy Number Assay and the two pyrosequencing assays. Calculation of the absolute copy numbers of ABCC6 pseudogenes was based on relative quantities (ratios) of the ABCC6 gene compared to the ABCC6 pseudogene(s). When one or more assay(s) deviated with respect to absolute copy numbers (e.g., for smaller duplications/deletions that were detectable in exon 2, but not in intron 7 or 11), the absolute copy numbers of the three genes were interpreted as the most reasonable.

The limitation of this method is that CNV is analyzed in short specific regions of ABCC6 and ABCC6 pseudogenes, and deletions/insertions in other parts of the genes, or other chromosomal reorganization events, may not be identified.

Statistical methods

The Fisher’s two-tailed exact test was used for testing categorical variables between patients and controls. Ps < 0.05 were taken as statistical significance.
Results and Discussion

Results of CNV in ABCC6 and ABCC6 pseudogenes were obtained from 207 of the 212 PXE individuals (161 singletons, 17 families × 2, and 4 families × 3). Five patients were excluded from further analysis because of inconclusive copy number results. Both deletions and duplications were found for ABCC6, ABCC6P1, and ABCC6P2 in the PXE patients. The frequency of individuals with any CNV was higher than in a healthy population of Caucasian (controls) (19% and 6%, respectively; Ρ = 0.02, Fisher’s exact test) (PXE patients with only one member from each family compared to healthy controls; 18% and 6%, respectively; Ρ = 0.045, Fisher’s exact test) (Table 1). The majority of variation comprised duplications of the ABCC6P1 gene, which also have been found to be rather common in different healthy populations (Kringen et al. 2012). Duplication of the ABCC6P2 gene was another common event that was observed in 10 PXE patients. ABCC6P2 duplication was not observed in any of the healthy populations studied previously (Kringen et al. 2012). There are a variety of repeat elements, for example, Alu repeats, in ABCC6 and ABCC6 pseudogenes. Such repeats may mediate chromosomal rearrangements and have been suggested to be responsible for the existence of the ABCC6 pseudogenes and also to have contributed to deletions in ABCC6 causing PXE (Ringpfeil et al. 2001). The high frequency of CNV in PXE patients is in agreement with the liability of crossover events in this chromosomal area.

In this study, we demonstrated that pyrosequencing is a fast and convenient method for the detection of CNV involving deletions of the entire or part of the ABCC6 gene. In patients with incomplete ABCC6 genotypes (not detected or not applicable), we identified a deletion of ABCC6 in 10/169 (~6%) of the uncharacterized alleles (Table S1). For 10 of the 11 analyzed PXE patients with ABCC6 deletions, none or one mutant allele only had previously been identified. Table 2 describes the predicted sizes of the observed PXE deletions: three patients had deletions in both ABCC6 and ABCC6P2 (#1) and three patients had deletions in ABCC6 only (#2). Five patients had smaller deletions (#3 and #4) in ABCC6 that were observed in either one or two of the analyzed regions (Exon 2 and/or intron 7) (Table 2). These predicted deletions may represent new unidentified deletions causing PXE. One known deletion, c.179_195del is located within the sequencing region of the exon 2 – assay. This deletion was, however, not identified in our patients. The number of deletions in ABCC6 comprised 6% of the PXE patients investigated in this study and was not observed in healthy controls (Caucasians) (Table 1). Deletions in ABCC6 comprise ~6.5% of the genetic variation causing PXE (Costrop et al. 2010) which is in accordance with our results. Many of the deletions and duplications in ABCC6 and ABCC6 pseudogenes were smaller in size than found in healthy individuals. In three cases, a micro deletion (~30 kb) in ABCC6 was accompanied by a duplication of ABCC6P1. This duplicated segment of ABCC6P1 may in fact have contributed to the ABCC6 deletion by gene conversion. Mendelian transmission of ABCC6 pseudogene CNV could be demonstrated for a few families. In one family (three siblings) with three copies of ABCC6P2, the PXE manifestation was present in individuals with and

Table 1. Copy number variation in ABCC6, ABCC6P1, and ABCC6P2 in PXE patients and healthy controls.

| Genes   | Copies | PXE patients (N = 207), N (%) | Nonrelated PXE patients1 (N = 182), N (%) | Caucasians (Kringen et al. 2012) (N = 50), N (%) | Assorted populations (Kringen et al. 2012) (N = 142), N (%) |
|---------|--------|-----------------------------|------------------------------------------|---------------------------------------------|----------------------------------------------------------|
| ABCC6   | 1      | 11 (5)                      | 11 (6)                                   | 0 (0)                                       | 0 (0)                                                    |
|         | 2      | 192 (93)                    | 168 (92)                                 | 50 (100)                                   | 140 (99)                                                 |
|         | 3      | 4 (2)                       | 3 (2)                                    | 0 (0)                                      | 2 (1)                                                    |
| ABCC6P1 | 1      | 7 (4)                       | 4 (2)                                    | 0 (0)                                      | 5 (3)                                                    |
|         | 2      | 186 (90)                    | 167 (92)                                 | 47 (94)                                    | 126 (89)                                                 |
|         | 3      | 14 (7)                      | 11 (6)                                   | 3 (6)                                      | 11 (8)                                                   |
| ABCC6P2 | 1      | 6 (3)                       | 6 (3)                                    | 0 (0)                                      | 1 (1)                                                    |
|         | 2      | 191 (92)                    | 167 (92)                                 | 50 (100)                                   | 141 (99)                                                 |
|         | 3      | 9 (4.5)                     | 8 (4.5)                                  | 0 (0)                                      | 0 (0)                                                    |
|         | 4      | 1 (0.5)                     | 1 (0.5)                                  | 0 (0)                                      | 0 (0)                                                    |
| Total number of individuals with any CNVs | 40 (19) | 33 (18) | 3 (6) | 19 (13) |

Some PXE patients are relatives, and some patients vary in more than one gene, therefore the total number of CNVs (deviations from the normal copy of 2) exceeds the total number of individuals with CNVs. PXE, pseudoxanthoma elasticum; CNV, copy number variation.

1Only one individual (randomly picked) from each family and singletons were included in the analysis.
without this duplication, however, eye, skin, and gastrointestinal symptoms were more severe for the two individuals with three copies of ABCC6P2 (data not shown).

For the four patients with duplication in ABCC6, mutational information was limited (Table S1). The functional consequence of having three copies of ABCC6 is unknown. Previously, by in vitro studies, we found that reduced mRNA expression of ABCC6P2 could influence the mRNA expression of ABCC6 (Piehler et al. 2008). Therefore, having three copies of ABCC6 may similarly have an effect on ABCC6 pseudogenes which again may affect the ABCC6 gene expression. However, in our previous study of ABCC6 CNVs, the expression of ABCC6 in lymphoblastoid cell lines with two or three copies of ABCC6 was too low to be reliably detected by RT-qPCR or pyrosequencing (Kringen et al. 2012).

When correlating CNV of ABCC6 and ABCC6 pseudogenes to clinical outcome (Phenodex™ and cholesterol diagnosis), we observed a higher frequency of patients with gastrointestinal bleeding (G1 or G2 according to Phenodex™) in patients with more than two copies of ABCC6P2 (3/6) compared to patients with two or less copies of ABCC6P2 (11/178) (P = 0.02, Fisher’s exact test). It would be interesting to investigate this association further as the pathophysiological cause of gastrointestinal bleeding in PXE is unknown. No significant correlation was observed for other clinical phenotypes, cholesterol diagnosis, and CNV of ABCC6 and/or ABCC6 pseudogenes.

In summary, by pyrosequencing, we were able to detect known and possibly new deletions in the ABCC6 gene that may have caused the PXE phenotype. This method may be used in combination with quantitative PCR in PXE patients who have obtained incomplete genotype from conventional techniques. The frequency of ABCC6P2 pseudogene duplication was more common in PXE patients than healthy individuals and therefore may affect the PXE phenotype.

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Copy numbers of ABCC6, ABCC6P1, and ABCC6P2 mutations in PXE patients.