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Novel deletions causing pseudoxanthoma elasticum underscore the genomic instability of the *ABCC6* region

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ABSTRACT

Mutations in *ABCC6* cause pseudoxanthoma elasticum (PXE), a heritable disease that affects elastic fibers. Thus far, more than 200 mutations have been characterized by various PCR-based techniques (primarily direct sequencing), identifying up to 90% of PXE causing alleles. This study wanted to assess the importance of deletions and insertions in the *ABCC6* genomic region, which is known to have a high recombinational potential.

To detect *ABCC6* deletions/insertions, which can be missed by direct sequencing, multiplex ligation-dependent probe amplification (MLPA) was applied in PXE patients with an incomplete genotype. MLPA was performed in 35 PXE patients with at least one unidentified mutant allele after sequencing of exon/intron boundaries and exclusion of the recurrent exon 23-29 deletion. Six multi-exon deletions and four single-exon deletions were detected.

Using MLPA in addition to sequencing, we expanded the *ABCC6* mutation spectrum with 9 novel deletions and characterized 25% of unidentified disease alleles. Our results further illustrate the instability of the *ABCC6* genomic region and stress the importance of screening for deletions in the molecular diagnosis of PXE.

INTRODUCTION

Pseudoxanthoma elasticum (PXE; OMIM#264800) is an autosomal recessive disorder, affecting the skin (yellowish papules, increased laxity in flexural areas), the eyes (peau d'orange, angioid streaks, retinal hemorrhage and vision loss) and the cardiovascular system (occlusive artery disease, gastro-intestinal bleeding) (Hu *et al.* 2003; Vanakker *et al.* 2008). The disease results from abnormal calcification and fragmentation of elastic fibers in the middermis, in the Bruch's membrane of the retina and in the elastic laminae of blood vessels (Neldner 1988).

PXE is caused by mutations in *ABCC6* (OMIM#603234), a gene spanning 74 kb on chromosome 16p13.1. *ABCC6* encodes an adenosine triphosphate (ATP)-binding cassette transporter (subfamily C), previously referred to as multidrug resistance protein 6. This transmembrane protein is expressed mainly in liver and kidney and to a much lower extent in the tissues affected in PXE. The substrate(s) transported by ABCC6 is unknown and PXE is now considered a metabolic disease (Jiang *et al.* 2009; Le Saux *et al.* 2006).

At present, more than 200 different *ABCC6* mutations have been identified. These are primarily located at the 3' end of *ABCC6* between exons 24 and 30. Two of these mutations are particularly prevalent, a deletion of exon 23-29 (del23-29) and p.R1141X (c.3421C>T) (Chassaing *et al.* 2005; Miksch *et al.* 2005; Pfendner *et al.* 2007). To date, only 16 different large *ABCC6* deletions (entire exons, whole gene deletions) have been identified in PXE patients (Bergen *et al.* 2000; Chassaing *et al.* 2007; Katona *et al.* 2005; Le Saux *et al.* 2001; Meloni *et al.* 2001; Miksch *et al.* 2005; Ringpfeil *et al.* 2001). Nevertheless, *ABCC6* is extremely prone to genomic rearrangements due to the high content of repetitive elements in all introns and in the genomic sequences surrounding the gene (Ringpfeil *et al.* 2001).

We hypothesized that, due to the documented instability of the *ABCC6* genomic region, the unidentified mutant alleles remaining after direct sequencing and screening for the recurrent exon 23-29 deletion, may consist of deletions and/or insertions. In this study we aimed to screen for the presence of such deletions and/or insertions using the multiplex ligation-dependent probe amplification (MLPA) technique. Our cohort consisted out of 35 patients with a clear-cut diagnosis of PXE but with only one or no *ABCC6* mutations identified by current PCR based techniques (Schouten *et al.* 2002). Recently, a PXE-like disease (OMIM#610842) that shares a significant phenotypical overlap with PXE was described. Therefore, patients whose genotypes remained incompletely ascertained after MLPA analysis were screened for the possible presence of mutations in *GGCX* (OMIM#137167), the gene responsible for the PXE-like syndrome (Vanakker *et al*. 2007).

PATIENTS AND METHODS

Patients and samples

In a cohort of 331 clinical and biopsy-proven PXE patients of Belgian,-French or -Italian ancestry, an extended analysis of *ABCC6* coding regions and exon/intron boundaries was performed by the traditional approach described previously (Hu *et al.* 2004). After this analysis, we failed to identify *ABCC6* mutations in one or both alleles in 35 patients (9 male, 26 female). In 29 of these patients, only a single *ABCC6* mutation was found while we could not detect any *ABCC6* mutation in 6 affected subjects, representing a total of 41 unidentified alleles.

The PXE diagnosis was made by experienced clinicians (ADP, OMV, LM). The clinical diagnosis was based on the presence of ophthalmological manifestations (including retinal peau d'orange and/or angioid streaks) and skin involvement (macroscopic skin lesions including yellowish papules and/or plaques in the neck and other flexural areas and microscopic skin lesions on full thickness skin biopsy) (Lebwohl *et al.* 1994). All patients had a positive skin biopsy with calcification and fragmentation of elastic fibers.

This study was reviewed and approved by the Ethics Committee of the Ghent University Hospital and informed consent was obtained from all participating patients.

MLPA analysis

Genomic DNA was isolated from peripheral blood or fibroblasts using a Puregene kit or QIA-amp DNA extraction respectively.

For MLPA analysis, the commercially available SALSA reagent set p092B kit was used (MRC-Holland, Amsterdam, The Netherlands, www.mrc-holland.com). This kit contained 23 probes corresponding to *ABCC6* exons 2, 4, 5, 7-15, 17, 18, 21-28 and 30 and 12 control probes for quality control. The p092B kit lacked probes for *ABCC6* exons 1, 3, 6, 16, 19, 20, 29 and 31. Because *ABCC1* is in close proximity to *ABCC6* (6.5 kb telomeric), an *ABCC1* probe was also included. The construction of the kit precludes generation of signals from the *ABCC6* pseudogenes (Schouten *et al.* 2002).

MLPA analysis was performed according to the manufacturer's recommendations (www.mlpa.com), using 100 ng of DNA in a 5 µl reaction. MLPA generated fragments were detected using an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, California, USA, www3.appliedbiosystems.com) with ROX500 (Applied Biosystems, Foster City, California, USA, www3.appliedbiosystems.com) as an internal size standard. The genemapper software (Applied Biosystems, Foster City, California, USA, www3.appliedbiosystems.com) was used to calculate fragment size and concentration, whereas the quantification analysis was performed using Coffalyzer (MRC Holland, Amsterdam, the Netherlands, www.mrc-holland.com). All samples were tested in duplicate.

Confirmation by probe complementarity experiments

During an MLPA experiment, exon specific probes hybridize to the DNA sample. The presence of deletions and/or duplications can be detected by a decrease/increase in the annealing of probes. However, a decrease in probe/DNA binding can also be the result of differences in nucleotide sequence between MLPA probes and their target sequences due to genomic variations such as Single Nucleotide Polymorphisms (SNPs). This may give rise to false positive results. To ascertain the presence of SNPs, we sequenced the region covered by the probes using an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, California, USA, www3.appliedbiosystems.com). Primers and methods were used as described in Hu *et al.* 2004. The nucleotide sequence of the probes was obtained from MRC-Holland, Amsterdam, The Netherlands.

Confirmation by long-range PCR experiments

Long-range PCR was performed using an Iproof high fidelity polymerase (Biorad, Nazareth, Belgium, www3.bio-rad.com). In a first step, primer sets flanking the deleted region were designed and optimized. PCR conditions for long-range amplification were as follows [98°C 30'', (98°C 10'', annealing temperature 30'',

72°C 3')x32, 72°C 10']. After amplification, DNA fragments were visualized on a 0.8% agarose gel. Deletion break points were characterized by excision of shorter DNA fragments and direct sequencing. The sequences of the PCR products were compared to the *ABCC6* genomic reference sequence (RefSeq NM_001171.5).

Confirmation by array comparative genomic hybridization (aCGH)

To confirm whole *ABCC6* gene deletions, a Whole Human Genome Oligo Microarray 4x44K (Agilent technologies, Santa Clara, California, USA, www.agilent.com) was used with particular focus on chromosome 16. For smaller multi-exon deletions, an array specific to chromosome 16 with a high probe density for the chr16:15687873-17130000 region was designed. Probes were selected from the high-density comparative genomic hybridization database (HD CGH, Agilent Technology eArray; https://earray.chem.agilent.com/earray/). After submitting the selected probes to the UCSC genome browser (http://genome.ucsc.edu/), the probe density around all *ABCC6* exons was checked. The presence of the *ABCC6* pseudogenes rendered probe design more difficult for exons 1 through 9, with little or no probes being available in the HD CGH Database. In each hybridization experiment, patient and control DNA were labelled with CY3 (green) and CY5 (red) respectively.

GGCX sequencing

In those patients with an incomplete genotype following MLPA (n=28), the coding sequence of *GGCX* (RefSeq NM_000821.4) was verified using primers and PCR conditions as described previously (Li *et al*, 2008). Patients harbouring homozygous or compound heterozygous mutations in specific regions of *GGCX* develop the PXE – like syndrome, which associates skin and eye features of PXE with a generalized cutis laxa and vitamin K-dependent clotting deficiency (Vanakker *et al*, 2007). The disease potential of identified variants was estimated by using the Polyphen and SIFT tools (http://genetics.bwh.harvard.edu/pph/, http://blocks.fhcrc.org/sift/SIFT.html).

RESULTS

MLPA results

To identify deletions and insertions in *ABCC6* we performed MLPA in PXE patients with unidentified mutant alleles. Of the 41 unknown disease alleles from 35 patients, 11 alleles could be further identified by MLPA. These included one intragenic multi-exon deletion, four whole gene deletions and six single-exon deletions. (Figure 1) The multi-exon deletion in patient 3 encompassed exons 24 to 27. The four whole gene deletions in patients 13, 20, 26 and 34 expanded beyond *ABCC6* as indicated by the 0.5 ratio for the *ABCC1* control probe. The single-exon deletions in patients 4, 6, 7, 16, 28 and 32 involved exons 30, 14, 2, 9, 24 and 30 respectively. All these exons encode an intracellular domain of ABCC6, except for exon 2, which corresponds to a transmembrane segment as predicted by the human protein reference database.

Confirmation of the intragenic multi-exon deletion and the whole gene deletions

The multi-exon and whole gene deletions were confirmed by aCGH in all patients except patient 20 for whom no appropriate DNA for array analysis was available. The aCGH analysis enabled simultaneously breakpoint determination for patients 3, 13, 26 and 34. (Figure 2A) The minimum/maximum length of the deletions were

5.5-10.5kb, 784-1642kb, 1257-1955kb and 2756-2958kb respectively. (Table I) The exact breakpoints of patient 3 could be determined by long-range PCR and measured 8118 bp (c.3307-1006_3735+1582del). (Table II)

Confirmation of the single-exon deletions

To exclude false positive MLPA results, sequence similarities between the MLPA probes and patient's DNA were compared in the case of single exon deletions. The sequences were identical for patient 4, 16 and 32, validating our results. (Table II) Patient 7 exhibited a homozygous nucleotide variant in the probe-binding site. However, we verified that this nucleotide change had no influence on probe binding efficacy with an MLPA experiment using a control sample containing the same variant (data not shown). In contrast, a false positive MLPA result was obtained as a consequence of the presence of a known heterozygous point mutation in the probe annealing region in patient 6 (c.1798C>T; p.R600C). The presence of a heterozygous variant in the same region demonstrates that the patient is not hemizygous for this region. (Table II)

In patient 32, an aCGH experiment could be performed, demonstrating that the deletion comprises exon 30 and 31 and is 5-6kb in length. (Figure 2B) For the other patients no fresh DNA was available for aCGH analysis.

To characterize the deletion in patient 28, long-range PCR was carried out to amplify across the deleted region and the resulting PCR product was sequenced. The deletion measured 1754 bp (c.3307-904_3506+660del). (Table II) Unfortunately, our various attempts to determine the breakpoints for patients 4, 7 and 16 using many different primer pairs were unsuccessful.

Finally, we performed MLPA analysis in patients with a previously identified apparently homozygous p.R1141X mutation (exon 24) to verify whether the subjects were indeed homozygotes or whether this variant was paired with a deletion of the exon 24 region. Surprisingly, we identified deletions removing the region of exon 24 indicating that a non-negligible proportion of patients with R1141X were in fact compound heterozygous.

Overall, out of 35 patients with incomplete genotypes, MLPA analysis allowed us to obtain the complete genotype for 7 patients (20%) and a partial characterization for 25 affected individuals, while the disease-causing alleles remained undetected in only 3 subjects with biopsy-proven diagnosis.

Subsequently, the DNA samples of the 28 patients remaining with an incomplete genotype after MLPA were screened for possible nucleotide changes in GGCX that could account for the missing causative alleles. Because no MLPA probes were available to search for GGCX deletions we used direct sequencing of exonic regions only. No mutation was detected, though a heterozygous variant was found in exon 10 (p.S452T) for one patient. This nucleotide variant was not present in 100 control samples (200 alleles) but was predicted to be benign by *in silico* analysis.

DISCUSSION

PXE, a heritable disorder affecting the skin, eyes and the cardiovascular system, is caused by mutations in the *ABCC6* gene (Bergen *et al*. 2000; Le Saux *et al.* 2000; Neldner 1988; Ringpfeil *et al.* 2000). Of the various techniques applied to mutation analysis of *ABCC6*, the currently prevailing and most efficient technique is direct sequencing with a mutation detection rate of about 90%. Various deletions involving parts or the whole *ABCC6* gene have been previously reported (Bergen *et al.* 2000; Chassaing *et al.* 2007; Katona *et al.* 2005; Le Saux *et al.*

2001; Meloni *et al.* 2001; Miksch *et al.* 2005; Ringpfeil *et al.* 2001). Small or large deletions are expected to make up for the bulk of the unidentified alleles because heterozygous middle-sized deletions are notoriously difficult to detect with traditional PCR-based assays. Furthermore, the presence of numerous repetitive elements makes the *ABCC6* region subject to genomic rearrangements.

Deletions often originate from homologous recombination between identical sequences of repeated DNA. Several types of repeats (long and short interspersed nuclear elements, Alu repeats,…) are abundantly present in the intra –and extragenic region of *ABCC6* (UCSC genome browser, http://genome.ucsc.edu/) (Chassaing *et al.* 2007, Symmons *et al.* 2008). Some of them are strongly suspected to have caused the recurring del23-29 deletion and the unique exon 15 deletion (Le Saux et al 2001; Ringpfeil et al 2001). Similarly, such rearrangements may have been responsible for the existence of the two *ABCC6* pseudogenes, which further illustrates the recombinational potential of this region of chromosome 16. Indeed, Cai et al. have shown that the *ABCC6* pseudogenes contribute to mutations in the parent gene (Cai *et al.* 2001; Symmons *et al.* 2008). Until now, a total of 16 different large deletions (exons, whole gene deletions) have been described (Bergen *et al.* 2000; Chassaing *et al.* 2007; Katona *et al.* 2005; Le Saux *et al.* 2001; Meloni *et al.* 2001; Miksch *et al.* 2005; Ringpfeil *et al.* 2001). Because of the wide variety of repeat elements in the *ABCC6* region, we anticipated that several of the unidentified mutant alleles in our PXE cohort would consist of deletions and insertions.

In our patients with incomplete genotypes, we identified a deletion in approximately 25% of the uncharacterized alleles. Nine of the deletions were novel, hereby increasing the spectrum of known large *ABCC6* deletions from 16 to 25.

Of the 10 patients in whom a deletion was detected, four carried a deletion removing the whole *ABCC6* gene. In these 4 patients, the absence of *ABCC1* was also observed indicating that these deletions were not restricted to a single gene. *ABCC1* is located 6.5kb telomeric to *ABCC6* and encodes a plasma membrane drug-efflux pump (ABCC1) closely related to ABCC6. Array CGH revealed that the whole gene deletions were of variable length (784-1642kb, 1257-1955kb and 2756-2958kb). Moreover, aCGH showed the absence of several other genes as summarized in table I, indicating that the deletions extend far beyond *ABCC1-ABCC6.*

Besides expanding the *ABCC6* mutation spectrum, our results have several practical implications. First, by increasing the mutation detection rate, familial screening and genetic counselling can be improved. Second, the identification of deletions is of relevance for the interpretation of direct sequencing results. Every patient diagnosed with a homozygous *ABCC6* mutation could in fact be compound heterozygous for that particular mutation and a deletion of the corresponding region. In the molecular analysis of *ABCC6*, verification of the multi-exon deletion 23-29 and analysis of other recurrent mutations followed by sequencing of the whole coding region remain the first essential steps (Vanakker *et al.* 2008). If after this approach, one or more mutant alleles remain undetected or in the case of a homozygous mutation (and unavailable parents), MLPA can be applied to search for deletions or duplications.

Because of the phenotypic overlap between PXE and the PXE-like syndrome, we analysed the *GGCX* gene in the 28 patients remaining with an incomplete genotype after MLPA but did not detect mutations nor functional polymorphisms in this gene.

One may note that the MLPA kit we used in this study covered only 23 of the 31 exons of *ABCC6*. Nevertheless, the mutational screening we applied was quite effective since we identified 10 deletions, 9 of them being novel.

By adding the missing eight *ABCC6* exon probes (exons 1, 3, 6, 16, 19, 20, 29, 31) the deletion detection will be further improved. Especially the addition of an exon 29 probe would be of interest as many mutations have been described in this functionally important protein region (Vanakker *et al*. 2008).

In conclusion, our approach has increased the *ABCC6* mutation detection rate, the mutation spectrum and has also confirmed that a second gene locus for PXE is unlikely. In addition our results underline the importance of genomic instability in the *ABCC6* region. Furthermore, we have demonstrated the heterogeneous nature of the *ABCC6* whole gene deletions and have shown that most of these expand far beyond the *ABCC6* genomic region. Our results clearly showed that deletions of various sizes constitute a sizable proportion of *ABCC6* mutations accounting for some of the previously undetectable alleles. Furthermore, we showed that in certain cases, the mutation status of patients may erroneously be interpreted as homozygous due to the technical limitation of direct sequencing. As it is now evident that small deletions are more common than previously thought, we propose MLPA as an efficient complementary technique to *ABCC6* molecular diagnosis.

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Competing interests: none declared.

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Table I. Overview of aCGH results

Pt.	Min.deleted area	Max.deleted area	Genes deleted (min.area)	Genes possibly deleted
3	16,164,551-16,159,020	16,156,390-16,166,980	Not applicable	Not applicable
13	15,399,818-16,183,616	15,164,187-16,807,118	MPV17L, KIAA04, MIRN48, NDE1, MYH11, ABCC1, ABCC6	NOMO 3, PKD1P1, PKD1P2
26	14,956,252-16,213,237	14,852,061-16,807,177	PDXDC, NTAN1, RRN3, PKD1P6, MPV17I, KIAA04, MIRN48, NDE1, MYH11, ABCC1, ABCC6	NOMO1, PKD1P3,NPIP, NOMO 3, PKD1P1, PKD1P2
32	16,151,286-16,155,931	16,150,211-16,155,985	Not applicable	Not applicable
34	15,164,187-17,919,962	15,062,188-18,020,277	MPV17L, KIAA04, MIRN48, NDE1, MYH11, ABCC1, ABCC6, NOMO3,PKD1P1, PKD1P2, XYLT1	RRN3

Minimal en maximal multi-exon deletion sizes as predicted by aCGH. According to this size range, a prediction was made on the presence or absence of other surrounding genes for patients with a whole *ABCC6* gene deletion.

Table II. Summary of MLPA results and subsequent confirmation strategy for deletions detected by MLPA.

Pt.	Allele 1	Allele 2	Probe/DNA similarity	Long Range +Breakpoint determination	aCGH	Remark
3	p.Arg391Gly c.1171A>G	**24-27 del.**	Identical	c.3307-1006_3735+1582del	Yes	*, Min.(16157574-16164330) Max.(16157202-16164346)
4	p.Arg1221Cys c.3661C>T	**exon 30 del.**	Identical	c.4209-?_4403+?del	/	*, True positive
7	p.Arg518Stop c.1552C>T	**exon 2 del.**	Not identical	c.37-?_219+?del	/	*
13	**WGD**	Not found	/	c.(?_-37)_(*568_?)del	Yes	Min.(15399818-16183616) Max.(15164187-16807118)
16	IVS21+1G>T	**Exon 9 del.**	Identical	c.999-?_1176+?del	/	*, True positive
20	p.Arg518Stop c.1552C>T	**WGD**	/	c.(?_-37)_(*568_?)del	/	
26	**WGD**	Not found	/	c.(?_-37)_(*568_?)del	Yes	***,** Min.(14956252-16213237) Max. (14852061-16807177)
28	p.Arg518Gln c.1553G>A	**Exon 24 del.**	/	c.3307-904_3506+660del	/	
32	c.3144_3145delTT	**Exon 30-31 del.**	Identical	c.4209-?_4512+?del	Yes	*, True positive Min. (16151286-16155931) Max.(16150211-16155985)
34	**WGD**	Not found	/	c.(?_-37)_(*568_?)del	Yes	*, Min. (15164187-17919962) Max. (15062188-18020277)
6	p.Arg600Cys c.1798C>T	**Exon 14 del.**	Heterozygous mutation	c.1780-?_1867+?del	Yes	False positive

For 11 out of 35 patients a mutation was found using MLPA. Mutations in grey were previously identified, mutations in black were identified by MLPA analysis. Confirmation studies revealed the presence of a false positive result for patient 6. For patients 7 and 16, probe and DNA sequence are not 100% identical, but the homozygous SNP present does not influence probe binding, as verified for a control sample with the same variant. For multi-exon deletions it is less likely that all consecutive exons have a SNP or mutation that influences probe binding, which makes confirmation of these larger deletions unnecessary. pt: patient; WGD: Whole gene deletion. * = SNPs in the deleted region are verified and in a homozygous state, which is an argument for a true deletion. ArrayCGH was only performed when fresh DNA could be isolated from

available fibroblasts (“yes”). c.(?_-37)_(*568_?)del denotes a deletion of the entire *ABCC6* gene (sequence running from –37 (cap site) to *568 (polyA-addition site). RefSeqNM_001171.5.

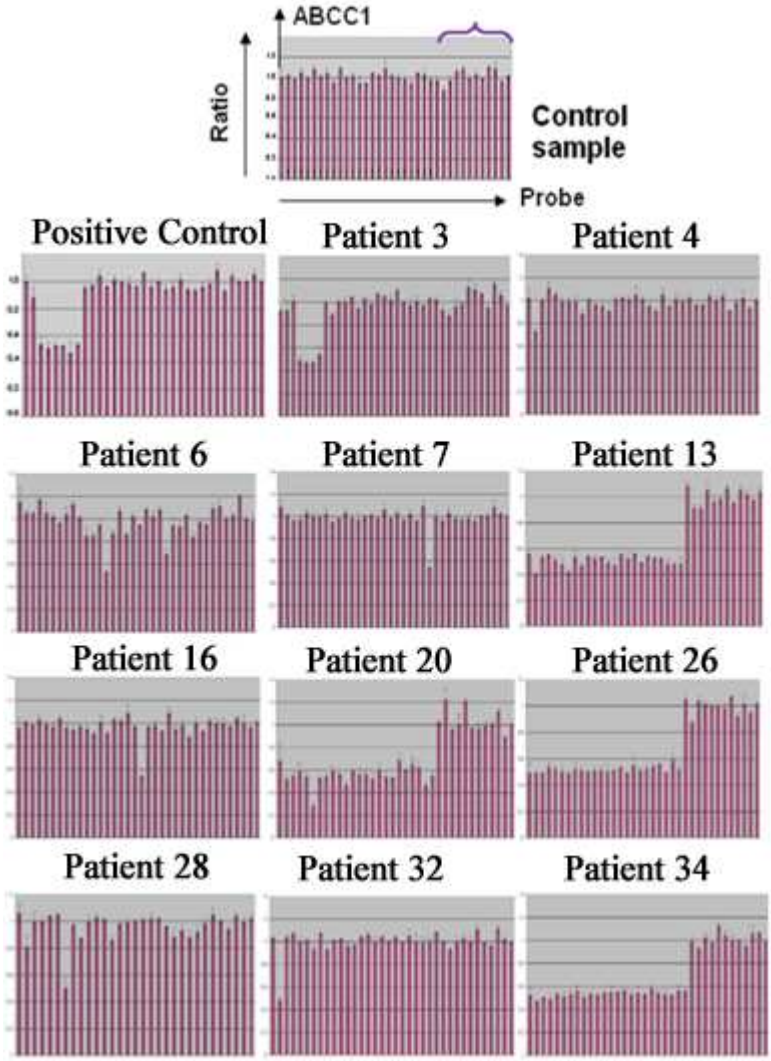
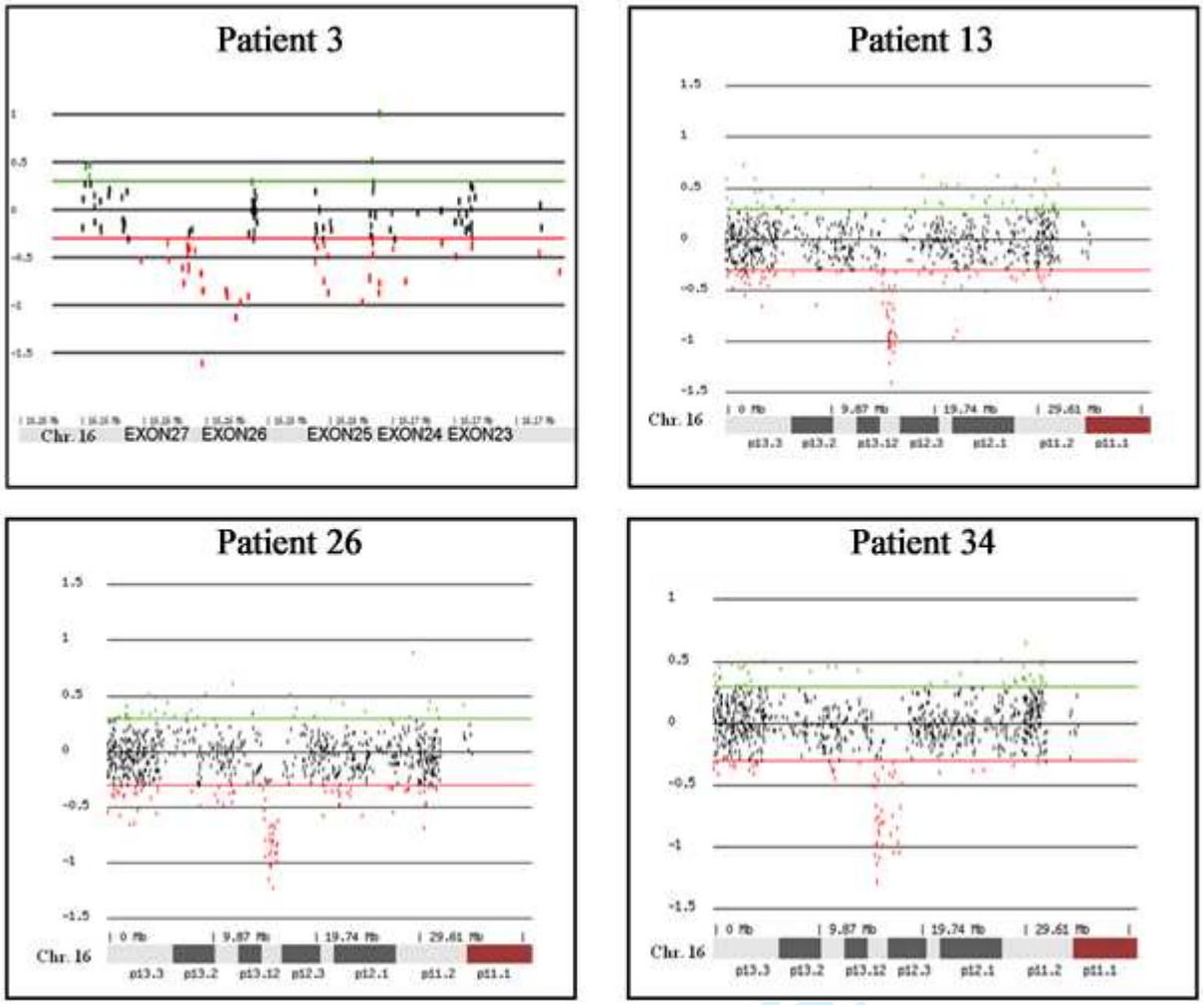


Figure 1. Overview of MLPA results. Each patient with a positive MLPA result, a patient without deletions/duplications (negative control) and a positive control (deletion exon 23-29) are shown. Every bar is the ratio result of 1 probe pair PCR product. From left to right: ratio for the *ABCC1* probe, *ABCC6* exon 30, exon 28-21, exon 18-17, exon 15-7, exon 5, exon 3, exon 2, followed by 12 bars representing the 12 control probes (purple bracket). All ratios for the negative control sample are approximately 1, indicating that this individual does not show deletions/duplications. As expected, a 0.5 ratio is observed for exon 23, 24, 25, 26, 27 and 28 for the positive control sample. Exon 29 is not incorporated in the kit. A ratio of 1.0 for the *ABCC1* control probe is observed for the positive control sample.

A



B

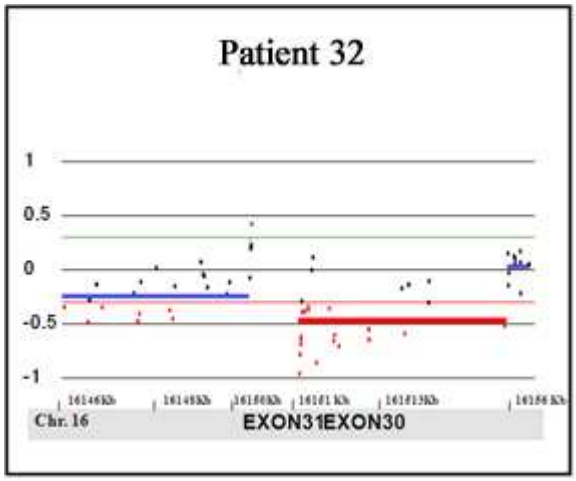


Figure 2. Array CGH based results. Red and green lines represent cut-off values and the chromosomal region is indicated underneath each panel. (A) Results for patients 3, 13, 26 and 34 with a multi-exon deletion. Patient 3: deletion exon 24-27. Region chr.16: 16,156,390- 16,166,980, where *ABCC6* exons 24-27 are located, exhibit decreased DNA hybridisation for

patients' 3 DNA (ratio -1). Patients 13, 26, 34: whole *ABCC6* gene deletion. A -1 ratio is observed for region chr16:15,399,818-16,183,616, chr16: 14,956,252-16,213,237 and chr16: 15,164,187-17,919,962 respectively, suggesting these 3 patients have a deletion of several genes surrounding the *ABCC6* gene, as further detailed in table 1. (B) Result for patient 32: deletion exon 30-31. A -1 ratio is observed for region chr.16: 16150211-16155985.