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ARTICLE

High frequency of *COH1* intragenic deletions and duplications detected by MLPA in patients with Cohen syndrome

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Cohen syndrome is a rare, clinically variable autosomal recessive disorder characterized by mental retardation, postnatal microcephaly, facial dysmorphisms, ocular abnormalities and intermittent neutropenia. Mutations in the *COH1* gene have been found in patients from different ethnic origins. However, a high percentage of patients have only one or no mutated allele. To investigate whether *COH1* copy number changes account for missed mutations, we used multiplex ligation-dependent probe amplification (MLPA) to test a group of 14 patients with Cohen syndrome. This analysis has allowed us to identify multi-exonic deletions in 11 alleles and duplications in 4 alleles. Considering our previous study, *COH1* copy number variations represent 42% of total mutated alleles. To our knowledge, *COH1* intragenic duplications have never been reported in Cohen syndrome. The three duplications encompassed exons 4–13, 20–30 and 57–60, respectively. Interestingly, four deletions showed the same exon coverage (exons 6–16) with respect to a deletion recently reported in a large Greek consanguineous family. Haplotype analysis suggested a possible founder effect in the Mediterranean basin. The use of MLPA was therefore crucial in identifying mutated alleles undetected by traditional techniques and in defining the extent of the deletions/duplications. Given the high percentage of identified copy number variations, we suggest that this technique could be used as the initial screening method for molecular diagnosis of Cohen syndrome.
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INTRODUCTION

Cohen syndrome (OMIM 216550) is an autosomal recessive disorder first described in 1973 by Cohen *et al.*[1] It is characterized by non-progressive mental retardation, characteristic facial features, hypotonia, pigmentary retinopathy, myopia and intermittent neutropenia.[1–3] The peculiar craniofacial features of Cohen syndrome include microcephaly, downslanting and wave-shaped palpebral fissures, short philtrum and prominent upper central incisors.[1–3]

In 2003, mutations in the *COH1* gene were identified as causative of Cohen syndrome in the Finnish population.[4] The *COH1* gene maps to chromosome 8q22 and consists of 62 exons encoding for a potential transmembrane protein presumably involved in vesicle-mediated sorting and intracellular protein transport.[4,5]

The phenotypic spectrum in Finnish patients is highly homogeneous and molecular analysis revealed a founder effect with a common ancestral mutation causative of the majority of cases.[4] On the other hand, Cohen syndrome was found to be associated with mutations in the *COH1* gene in different populations with a broader clinical spectrum than the Finnish subtype.[4,6–10] About 100 mutations in the *COH1* gene have been identified so far.[9] Most of them are truncating mutations resulting in a null allele, whereas missense mutations and in-frame deletions are less frequent.[9]

Methods for the detection of point mutations in the *COH1* gene are well established in our laboratory and consist of denaturing high performance liquid chromatography (DHPLC) followed by automatic sequencing.[10] Until now, we used real-time quantitative PCR (qPCR) for the detection of large *COH1* deletions/duplications.[10] However, as *COH1* is a large gene, spanning 846 kb of genomic DNA and composed by 62 exons, qPCR assays designed on a limited number of target regions are prone to miss a high fraction of intragenic rearrangements and do not allow the characterization of the extent of the deletions/duplications. Very recently, a targeted oligonucleotide

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array was designed, enabling the detection of *COH1* copy number changes with higher resolution.[11] The authors analyzed 35 patients (from 26 families) with unexplained Cohen syndrome and identified deletions in 9 patients from 7 families, showing that large deletions are an important cause of Cohen syndrome.[11]

To detect *COH1* copy number variations, we used multiple ligation-dependent probe amplification (MLPA), a technique that has greatly improved mutation screening allowing the relative quantification of up to 40 different nucleic acid sequences in a single reaction tube at a relatively low cost.[12] By the use of two MLPA assays designed to screen copy number changes in almost all coding exons (60 out of 62) of *COH1*, we analyzed a group of patients with a clinical diagnosis of Cohen syndrome in which traditional tests failed to identify mutations in both alleles.

MATERIALS AND METHODS

Patients

Clinical geneticists from Italy, France, Holland and the United States assessed patients and diagnosed Cohen syndrome on the bases of published criteria.[13] Patients were considered as having Cohen syndrome when six of the following eight criteria were fulfilled: developmental delay, microcephaly, typical facial features, truncal obesity with slender extremities, sociable behavior, joint hypermobility, retinopathy or myopia, and intermittent neutropenia. Our series includes three children younger than 5 years (Table 1). As chorioretinal dystrophy does not manifest in young patients, the diagnosis of Cohen syndrome in children is considered when learning disabilities are associated with two of the following features: typical facial gestalt, pigmentary retinopathy or neutropenia.[14]

Overall, we collected 14 patients from 11 families, ranging in age from 18 months to 52 years. This group included four patients (1, 8, 9A, 9B) originally described by Katzaki *et al*[10] and 10 newly ascertained cases. The main clinical features are summarized in Table 1. Enrolled cases included one consanguineous family with an affected child (8) and 10 non-consanguineous families: 7 with one affected child (1, 2, 3, 4, 5, 6, 7), one with two affected sisters (9A, 9B), one with two affected brothers (10A, 10B) and one with an affected brother (11A) and sister (11B). A distinct phenotype was present in the two affected brothers (10A and 10B), presenting five of eight diagnostic criteria (Table 1); these patients were classified as Cohen-like.[13]

COH1 molecular analysis

Genomic DNA was isolated using QIAamp DNA blood maxi kit, according to the manufacturer's protocol (Qiagen, Hilden, Germany). PCR amplification of the 62 exons was carried out using published primers.[4,10] Mutation analysis was performed by DHPLC using the Transgenomic WAVE (Transgenomic, San Jose, CA, USA).[10] Quantitative PCR was also performed in one familiar case (9A, 9B) and one sporadic case (8) with a Custom TaqMan Assay designed on exon 16 (Applied Biosystems, Foster City, CA, USA).[10]

MLPA analysis was performed using two distinct SALSA MLPA kits (P321-A1/P322-A1) designed by MRC-Holland (Amsterdam, The Netherlands). The two assays include 69 *COH1* probes to screen copy number changes in almost all coding gene exons (60 out of 62) and 16 control probes. No probe was present for exons 6 and 14. For exons 3, 16, 17, 24, 31, 34, 35 and 36, two distinct probes were designed. The analysis was carried out as previously described.[12] Briefly, 100 ng of genomic DNA was diluted with TE buffer to 5 μl, denatured at 98°C for 5 min and hybridized with SALSA Probe-mix at 60°C overnight. Ligase-65 mix was then added and ligation was performed at 54°C for 15 min. The ligase was successively inactivated by heating the samples at 98°C for 5 min. PCR reaction was performed in a 50 μl volume. Primers, dNTPs and polymerase were added and amplification was carried out for 35 cycles (30 s at 95°C, 30 s at 60°C and 60 s at 72°C). The amplification products were separated on an ABI Prism 310 automatic sequencer and analyzed using the GenScan software ver.3.1 (Applied Biosystems). For data analysis, the values of peak sizes and areas were exported to an Excel table and compared with a normal control (MRC-Holland). Dosage alterations were considered significant if sample values deviated more than 30% from the control.

For exons 6 and 14, we designed two specific qPCR assays (Supplementary Table 1). In addition, MLPA results were confirmed by qPCR using probes located in exons 16, 24, 34, 42, 48 and 58 (Supplementary Table 1).[10] Reactions were performed in a 96-well optical plate with a final reaction volume of 50 μl using an ABI prism 7000 (Applied Biosystems). A total of 100 ng of DNA (10 μl) was dispensed in each of the four sample wells for quadruplicate reactions. Thermal cycling conditions included a pre-run of 2 min at 50°C and 10 min at 95°C. Cycle conditions were 40 cycles at 95°C for 15 s and 60°C

Table 1 Summary of the clinical features in Cohen patients

Case	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9A*	*9B*	*10A*	*10B*	*11A*	*11B*
Patients ID	C8[a]	C91	C104	C145	C155	C167	C185	R111[a]	C42[a]	C43[a]	C160	C161	C164	C268
Sex	M	F	F	F	F	F	F	M	F	F	M	M	M	F
Consanguineous parents	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No
Age at assessment	5 years	20 years	10 years 6 months	19 years	17 years	19 years	3 years 6 months	6 years 3 months	52 years	51 years	45 years	40 years	4 years 6 months	2 years 4 months
Mental retardation (degree)	Yes	Severe	Mild-moderate	Moderate	Moderate	Mild-moderate	Moderate	Moderate	Yes	Yes	Moderate	Moderate	Moderate	Moderate
Microcephaly	+	+	+	+	3° cnt	+	+	+	+	+	–	–	+	+
Typical facial gestalt	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Truncal obesity	–	–	+	+	+	+	–	+	+	+	–	–	–	–
Narrow H/F; slender/ tapering fingers	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Retinopathy	+	+	+	+	+	+	+	+	+	+	+	+	–	–
Myopia (diaptres)	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Neutropenia	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Joints hyperlaxity	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Sociable behavior	–	NR	+	+	NR	+	+	+	+	+	NR	NR	+	+
Other	Pes varus	Mild mitral insufficiency	Leg asymmetry	IUGR hip asymmetry			Neonatal hypotonia	Syndactyly (II–III toes)	Breast cancer, bilateral cataract	Breast cancer, bilateral cataract	Mitralic insuffi-ciency			

NR, not reported.
[a]Patients already reported.[10]

for 1 min, according to the TaqMan Universal PCR Protocol (PE Applied Biosystems). The TaqMan Universal PCR Master Mix and Microamp reaction tubes were supplied by Applied Biosystems. The starting copy number of the unknown samples was determined using the comparative C_t method, as previously described.[15]

In case 11, long-range PCR was performed with the Expand Long Template PCR kit as specified by the manufacturer (Roche Diagnostics, Basel, Switzerland), using a forward primer located in intron 59 (ggatggctctgaacagatga) and a reverse primer located in intron 56 (agaagcaattggcaagaggt). These primers are divergent in the normal genome and they do not amplify the control's DNA. PCR conditions were as follows: 300 nM of each primer, 350 μM of dNTPs, 2.0 mM $MgCl_2$, 0.75 μl of enzyme mix and 1× Buffer II, and the following cycling parameters: 94°C 5 min; 94°C 10 s, 59°C 30 s, 68°C 5 min, 10 cycles; 94°C 15 s, 59°C 30 s, 68°C 5 min +20 s/cycle, 25 cycles; final extension 68°C 30 min.

Haplotype analysis

A set of 10 markers covering a region of about 4 Mb encompassing the *COH1* gene were used for haplotype analysis (Supplementary Table 2) in three of our cases with the 6–16 deletion (case 5, 8 and 9A) and one member of the large Greek consanguineous family reported by Bugiani *et al*[16] harboring the 6–16 deletion in homozygous state. Haplotype analysis was also performed in all available family members of the 6–16 deleted patients and in 50 Italian control individuals. The forward primers were fluorescently labeled with FAM. Markers were amplified by polymerase chain reaction. Conditions were optimized for individual primer pairs in a 9600 thermocycler (Applied Biosystems). The programs used were 95°C for 12 min, followed by 30 cycles of melting at 94°C for 15 s, annealing at the optimal temperature for 15 s, and then extension at 72°C for 30 s. A final extension was performed at 72°C for 10 min. PCR products were run on an ABI 3130 sequencer (Applied Biosystems) and analyzed with GeneMapper v.4.0. The size of the PCR products of the microsatellite markers were compared among the families carrying the recurrent deletion 6–16 in heterozygous or homozygous state, in order to define the haplotype co-segregating with the deletion.

RESULTS

Phenotype

All 14 patients displayed the typical Cohen facial gestalt, narrow extremities and truncal adiposity even if not all cases were obese (7 out of 14) (Table 1, Figure 1).[10] Microcephaly was present in the majority of patients (9 out of 14) (Table 1).[10] The retinopathy was absent in one family with two affected children younger than 5 years (11A, 11B) (Table 1). Neutropenia was absent in one patient (3) and one case did not show joint hyperextensibility (8). Among the 14 patients, two brothers (10A, 10B) presented an atypical phenotype, lacking microcephaly and truncal obesity. However, the diagnosis of Cohen syndrome was suggested based on the association of retinopathy, neutropenia and facial appearance (Figure 1).

COH1 molecular analysis

The 14 patients (11 families) with a clinical diagnosis of Cohen syndrome were analyzed for the presence of *COH1* point mutations by DHPLC followed by sequencing of the samples with an abnormal elution profile.[10] This analysis led to the detection of 12 different mutations, including six frame-shift, three splice site, two nonsense and one complex rearrangement (Table 2). Moreover, in one family (9A and 9B) and in one sporadic patient (8) a partial heterozygous *COH1* gene deletion was already detected by qPCR using a TaqMan probe designed on exon 16.[10] To identify missed mutated alleles and to characterize the extent of the deletions/duplications, we used two MLPA assays (P321-A1/P322-A1) designed to detect *COH1* copy number changes in 60 out of 62 exons of the gene. This method led us to identify 5 different multi-exonic deletions in 11 alleles and 3 different duplications in 4 alleles (Table 2). In particular, MLPA characterized heterozygous copy number variations in nine patients (seven families) displaying a point mutation previously identified by

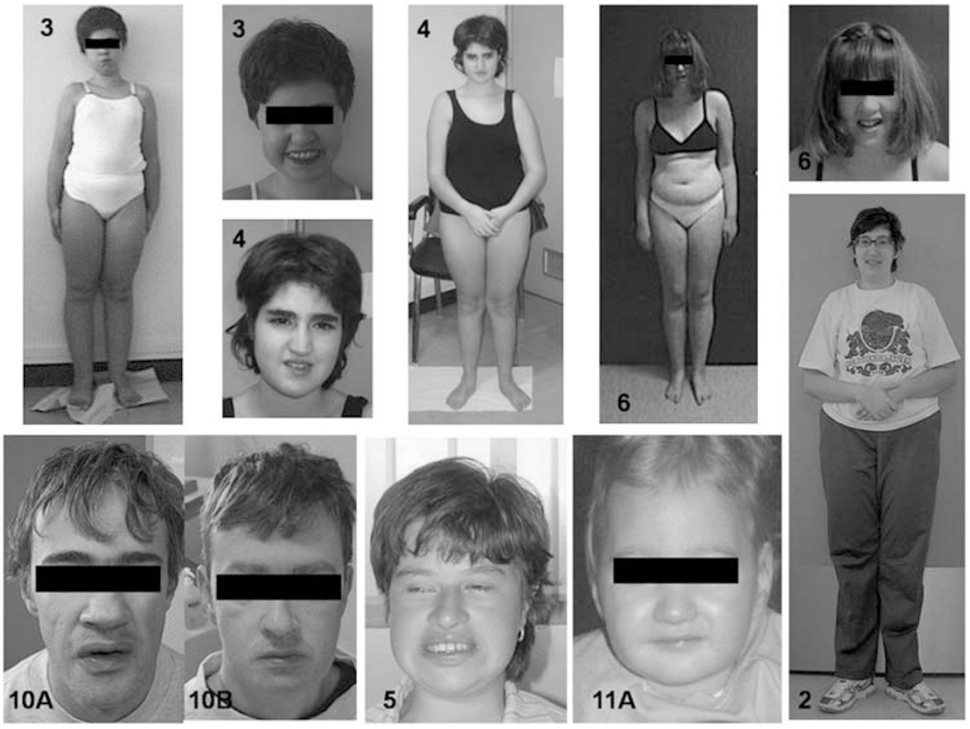

Figure 1 Clinical features of Cohen syndrome patients. Note the typical facial gestalt of patients 3, 4, 5, 6, 10A, 10B and 11A. Frontal views of patients 2, 3, 4 and 6, showing truncal obesity.

Table 2 *COH1* point mutations and large deletions/duplications identified in the study

Case	*Patient ID*	*Nucleotide change*	*Amino-acid change*	*Copy number change*	*Inheritance*
1	C8[a]	c.3427C>T	p.R1143X	DelEX32-35	M
					P
2	C91	c.11695delAGTG	p.S3899fsX42	DupEX4-13	*De novo*
					P
3	C104	c.11556insT;	p.V3853fsX32		M
		IVS24+2T>C			P
4	C145	c.402insT	p.L135fsX10	DupEX20-30	M
					P
5	C155			DelEX6-16[b]	NA
6	C167	c.4474delA;	p.I1492fsX42		P
		IVS14-2^{A}>G			M
7	C185	c.219_20delACinsT	p.K73fsX20	DelEX40-43	M
					P
8	R111[a]	c.11564delA	p.Y3855fsX22	DelEX6-16	P
					M
9A/B	C42/C43			DelEX6-16	NA
				DelEX46-50	NA
10A/B	C160/161	IVS4-2A>G		DelEX4-16	M
					P
11A/B	C164/268	c.5331insT;	p.D1778X	DupEX57-60	M
		c.10880insTTdelCTGCGA	p.T3627_H3633delinsI		P
		GGCAGCTTGTGCAC			

NA, not available for testing; P, paternal; M, maternal.
[a]Patients previously described.[10]
[b]In homozygous state (The reference sequence of *COH1* gene is according to UCSC Genome Browser, http://genome.ucsc.edu, on Human March 2006 Assembly, hg18; NM_017890).

DHPLC on the other allele (1, 2, 4, 7, 8, 10A, 10B, 11A and 11B), two different compound heterozygous deletions in two affected sisters (9A and 9B) and one homozygous deletion in one sporadic patient (case 5) (Table 2, Figure 2, Supplementary Figures 1 and 2).

In four patients, MLPA showed the presence of a deletion spanning from exons 7 to 16 (Figure 2, Table 2). As the MLPA assays contain 69 probes not including exon 6, we designed a targeted qPCR probe assay for this exon (Supplementary Table 1). This analysis showed that the four deletions spanned indeed from exons 6 to 16 (Figure 3, Table 2).

In two sporadic patients (cases 2 and 4), MLPA detected a significant increase in the fluorescent signals corresponding to exons 4–13 and 20–30, indicating the presence of two differently sized duplications (Table 2, Supplementary Figure 1). In case 2, a specific qPCR assay indicated that exon 14 is not included in the duplication (data not shown). In a familial case (11A, 11B) in which DHPLC followed by sequencing had already detected a complex rearrangement in exon 56 (c.1088insTTdelCTGCGAGGCAGCTT GTGCAC; p.T3627_H3633delinsI), MLPA also disclosed a significant increase in peak heights 57–60, suggesting the presence of a heterozygous duplication (Table 2, Figure 4). Analysis of the parental DNA indicated that the rearrangement p.T3627_H3633delinsI was in *cis* with the duplication detected by MLPA (Table 2).

To better characterize the 57–60 duplication, we performed long-range PCR using a forward primer in intron 59 and a reverse primer in intron 56 (Figure 5a). We obtained a product of ~1 kb in the two affected sibs and in the carrier father. Automatic sequencing of the PCR product permitted us to characterize the junction sequence of the duplicated segment (Figure 5), 95 bp downstream with respect to the rearrangement. The duplicated segment, starting within intron 56, is inserted within exon 61 in position g.100953994 (NM_017890) (Figure 5). According to prediction software, this insertion interrupts the protein product creating a premature stop codon after 10 new amino acids.

Not all parental DNAs were available for testing (Table 2). For patients 9A and 9B, the DNAs of two healthy sibs have been analyzed to determine whether the two rearrangements were in *cis* or *trans*. MLPA revealed that the brother and the sister were carriers of the deletions spanning exons 6–16 and exons 46–50, respectively, confirming that the rearrangements were in compound heterozygosity. In the cases where parental DNAs have been tested, all mutations were inherited except in one patient (case 2) harboring a *de novo* point mutation (c.11695delAGTG; p.S3899fsX42) (Table 2).

All copy number changes identified by MLPA were confirmed by qPCR using specific probes for exons 16, 24, 34, 42, 48 and 58 (data not shown).

Haplotype analysis

To investigate a founder effect for the recurrent deletion of exons 6–16, we performed haplotype analysis in three of our cases and one additional case belonging to a large Greek consanguineous family reported by Bugiani *et al*.[16] A founder effect is expected to result in sharing of allelic sequence polymorphisms in the vicinity of the deletion. We examined 10 microsatellite markers within a region of about 4 Mb encompassing the *COH1* gene (Supplementary Table 2, Table 3). For heterozygous markers, the phase was assigned by genotyping other family members: parents in case 8 (a carrier mother and noncarrier father) and sibs in case 9 (one carrier and one noncarrier sister) (data not shown).

To determine how frequently alleles of the same size can be obtained by chance in a general population, we genotyped DNA from 50 Italian control samples using primers for the same 8 microsatellite markers

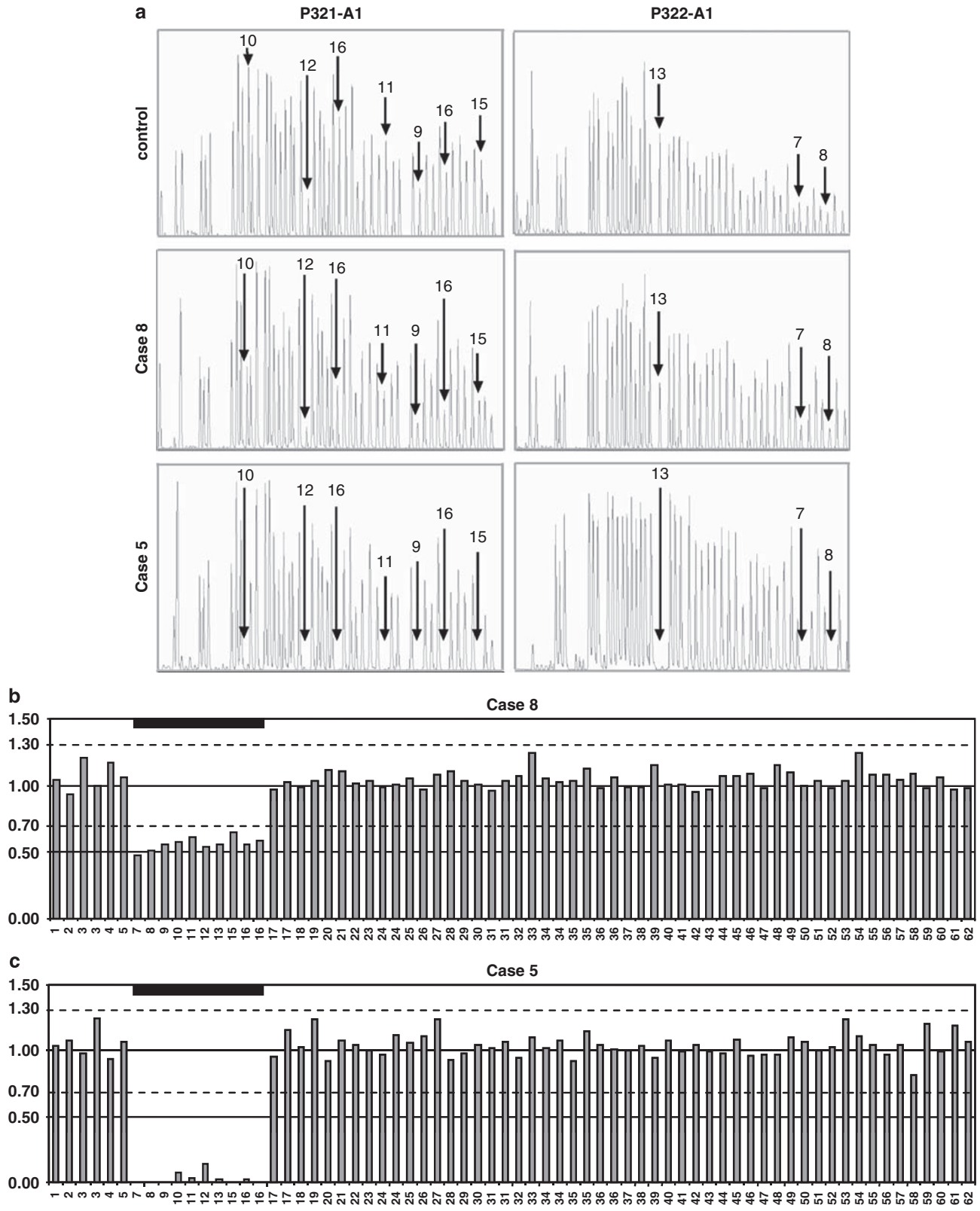

Figure 2 MLPA analysis results showing the recurrent deletion in heterozygous (Case 8) and homozygous (Case 5) states. (**a**) Electropherograms obtained with P321-A1 kit (on the left) and P322-A1 kit (on the right) for a normal control sample, patient 8 and patient 5. Numbers and arrows indicate the exon probes with reduced fluorescence signals with respect to the control sample. In patient 8, the signal is half-reduced for probes 7–16, whereas in patient 5 there is no signal for the same probes. (**b**, **c**) Peak area histograms for patients 8 (**a**) and 5 (**b**) normalized with the control sample. Exon dosage is reported on the *y* axis (normal values spanning from 0.7 to 1.3 are indicated with broken lines). MLPA analysis shows reduced peak area for exons 7–16, compatible with a heterozygous deletion in patient 8 and a homozygous deletion in patient 5. Deletions are indicated with a heavy black line.

(minimal common haplotype, Table 3). None of the healthy controls and none of the noncarrier family members showed the minimal common haplotype (data not shown).

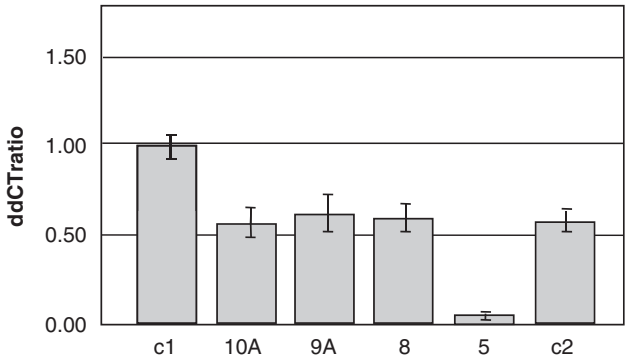

Figure 3 Quantitative PCR results for exon 6 of *COH1*. ddCT ratios and standard deviations of a normal control sample (c1), a deleted control sample (c2) and patients 5, 8, 9A and 10A. Compared with controls, patients 8, 9A and 10A show ddCT ratio values of about 0.5, indicating a deletion in the heterozygous state, whereas patient 5 shows ddCT ratio values of about 0.0, indicating a deletion in the homozygous state.

DISCUSSION

In this study, we report the first application of the MLPA technique to screen for *COH1* large deletions and duplications. In a group of 14 patients (11 families) with a clinical diagnosis of Cohen syndrome, MLPA allowed us to obtain rapid and high quality results disclosing 11 deleted and 4 duplicated *COH1* alleles. The use of MLPA led us to identify all *COH1* mutations undetected by conventional screening, suggesting that this technique is an important tool for the molecular characterization of Cohen syndrome.

Our series included 12 patients with true Cohen syndrome and two brothers with an atypical phenotype, lacking microcephaly and truncal obesity. However, the association of retinopathy, neutropenia and facial appearance addressed the clinical diagnosis. Their facial features, although not typical, were not in disagreement with the diagnosis of Cohen syndrome consisting of long face, heavy eyebrows, mildly down-slanting palpebral fissures, prominent root of the nose, normal philtrum and prognatism (Figure 1). Three patients from two families were children aged less than 5 years. They presented the typical facial features of younger patients, including round face with full lower lip, not excessively short philtrum, slightly downward-slanting eyes with wave-shaped eyelids and less prominent nasal bridge (Figure 1).[10]

Copy number changes in *COH1* have been previously investigated in patients with Cohen syndrome by qPCR using probes designed on a

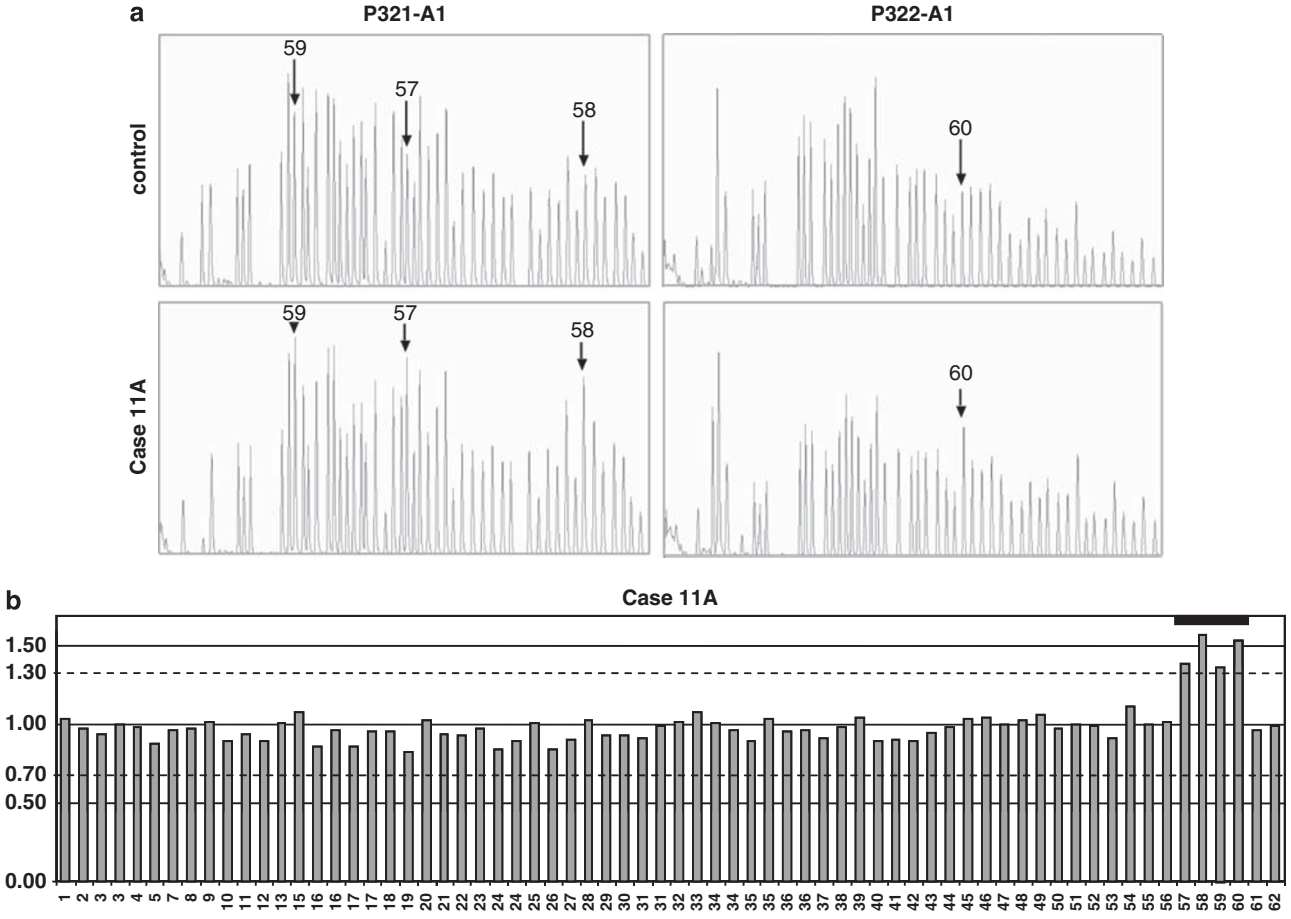

Figure 4 MLPA analysis results showing the duplication spanning exons 57–60 in the familial case with an affected brother (11A) and sister (11B). (**a**) Electropherograms obtained with P321-A1 kit (on the left) and P322-A1 kit (on the right) for a normal control sample and patient 11A. Numbers and arrows indicate the exon probes with increased fluorescence signals with respect to the control sample. (**b**) Peak area histograms for patient 11A normalized with the control sample. The exon dosage is reported on the *y* axis (normal values spanning from 0.7 to 1.3 are indicated with broken lines). The consistent increase in the peak area for exons 57–60 is compatible with a duplication of these exons (indicated with a heavy black line).

limited number of exons.[10,16] Only recently, a targeted oligonucleotide array with a median resolution of 200 bp was designed within the gene, which considerably increased the mutation detection rate.[11] Using this technique, the authors identified *COH1* large deletions in nine patients from seven families, showing that they represent an important cause of Cohen syndrome.[11] The present results and our previous study on a group of 18 patients disclosed a total of 21 alleles with point mutations (58%) and 15 with copy number variations (42%), confirming that deletions and duplications account for a significant percentage of *COH1* mutations.[10]

In four patients from three families, MLPA identified a *COH1* large deletion sharing the same extent with one previously reported in an isolated Greek Island population, spanning from exons 6 to 16.[16] In our patients, the deletion was heterozygous in two families and homozygous in an apparently non-consanguineous family.[10] Interestingly, this latter patient displays the same constellation of facial features reported in Greek patients with homozygous deletion including thick hair with low hairline, strabism, lack of nasofrontal angle, short upturned philtrum and prominent maxillary central incisors (patient 5, Figure 1).[16] Moreover, they show milder microcephaly and more severe visual impairment than the original phenotype described in the Finnish population.[4,16]

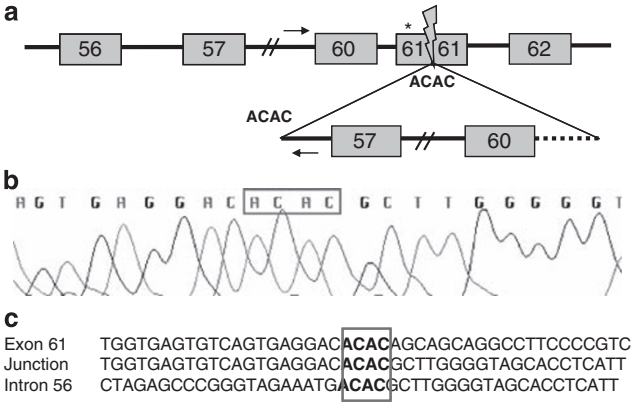

Figure 5 Characterization of duplication 57–60 in familial case 11. (**a**) Schematic drawing of the duplicated region. The star indicates the position of the MLPA probe in exon 61, whereas the thunder represents the insertion point of the duplicated segment. Arrows indicate the primers located within introns 59 and 56 used in the long-range PCR experiment. (**b**) Sequence analysis showing the junction between intron 56 and exon 61. (**c**) Aligned exon 61 and intron 56 sequences at the duplication junction. Region of homology across the duplication junction is boxed.

Our three families with the same deletion encompassing exons 6–16 come from different Italian regions, two in Central Italy and one in Southern Italy. The results obtained by haplotype analysis in these families, in one member of the large Greek consanguineous family previously reported by Bugiani *et al*[16] harboring the 6–16 deletion in homozygous state and in 50 healthy Italian controls, suggest that the recurrent deletion is due to an ancestral founder effect in the Mediterranean area (Table 3).

In this study, we also identified two deletions spanning exons 4–16 and 40–43, sharing the same exon coverage with two deletions already reported in the Northern European population.[11] Also, in these cases we cannot exclude a founder effect for the deleted alleles. Alternatively, these could be independent mutations favored by the presence of repeated elements located at the break points. Accordingly, RepeatMasker software analysis of the genomic region containing *COH1* revealed a higher frequency of LINEs, SINEs and DNA repeat elements in comparison with the average for autosomal sequences.[11] In a previous study, it was suggested that the most likely mechanism for genomic rearrangements in the *COH1* gene is the non-homologous end joining, leading to non-recurrent deletions.[11] Considering our latest results, the non-allelic homologous recombination mechanism cannot be ruled out.

In four patients from three families, MLPA identified three different size duplications spanning exons 4–13, 20–30 and 57–60, respectively. To our knowledge, *COH1* intragenic duplications have never been reported in Cohen syndrome.

In one family with two affected sibs (cases 11A/B), we identified a complex rearrangement (p.T3627_H3633delinsI) in *cis* with the downstream duplication detected by MLPA. We initially hypothesized that this rearrangement could be located at the break point of the duplication within exon 56. However, sequencing analysis of the long PCR product using a forward primer in intron 59 and a reverse primer in intron 56 indicated that the duplication effectively starts in intron 56, 95 bp after the rearrangement (Figure 5). This sequence is joined to exon 61 in position g.100953994 (NM_152564) (Figure 5). As the MLPA probe of exon 61 is located upstream of the junction point (Figure 5a) and its signal does not increase, we can suppose that the duplication is not in tandem. The insertion of the duplicated segments within exon 61 creates a premature stop codon after 10 new amino acids of the protein product. Even if detailed mapping of the extent of all the duplications has not yet been undertaken, these rearrangements

Table 3 Haplotype analysis in patients harboring the recurrent exons 6–16 deletion

Marker	*Position (Mb)*	*C37 (Greek)*		*Case 5 (Italian)*		*Case 9A (Italian)*		*Case 8 (Italian)*	
D8S1018	97 598	315	319	315	315	319	323	319	315
D8S257	99 451	109	109	109	109	109	—	109	113
8-23TC	99 924	214	214	214	214	214	218	214	204
8-25GT	100 056	353	353	353	353	353	379	353	351
8-20TG	100 601	169	169	169	169	169	173	169	173
VPS13B	—	*del6_16*	*del6_16*	*del6_16*	*del6_16*	*del6_16*	*del46_49*	*del6_16*	*Y3855fsX22*
D8S1789[a]	100 738	255	255	255	255	255	255	255	255
D8S470[a]	100 743	226	226	226	226	226	226	226	226
D8S300	100 987	485	485	485	485	485	499	485	499
8-18AC	101 066	95	95	95	95	95	97	95	97
D8S398	101 588	141	141	141	141	137	141	137	141

[a]Intragenic markers.
Gray columns: haplotype co-segregating with the deletion.
(The reference sequence of *COH1* gene is according to UCSC Genome Browser, http://genome.ucsc.edu, on Human March 2006 Assembly, hg18; NM_017890).

probably led to a frameshift and a premature truncation of the protein at different levels.

In conclusion, our study confirms that *COH1* copy number variations are a frequent cause of Cohen syndrome and consist of intragenic deletions as well as duplications. Therefore, incorporation of detection tools for *COH1* copy number variations is mandatory in the molecular diagnosis of Cohen syndrome.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Cohen Jr MM, Hall BD, Smith DW, Graham CB, Lampert KJ: A new syndrome with hypotonia, obesity, mental deficiency, and facial, oral, ocular, and limb anomalies. *J Pediatr* 1973; **83**: 280–284.
2 Carey JC, Hall BD: Confirmation of the Cohen syndrome. *J Pediatr* 1978; **93**: 239–244.
3 Norio R, Raitta C, Lindahl E: Further delineation of the Cohen syndrome; report on chorioretinal dystrophy, leukopenia and consanguinity. *Clin Genet* 1984; **25**: 1–14.
4 Kolehmainen J, Black GC, Saarinen A *et al*: Cohen syndrome is caused by mutations in a novel gene, COH1, encoding a transmembrane protein with a presumed role in vesicle-mediated sorting and intracellular protein transport. *Am J Hum Genet* 2003; **72**: 1359–1369.
5 Velayos-Baeza A, Vettori A, Copley RR, Dobson-Stone C, Monaco AP: Analysis of the human VPS13 gene family. *Genomics* 2004; **84**: 536–549.
6 Falk MJ, Feiler HS, Neilson DE *et al*: Cohen syndrome in the Ohio Amish. *Am J Med Genet A* 2004; **128**: 23–28.
7 Hennies HC, Rauch A, Seifert W *et al*: Allelic heterogeneity in the COH1 gene explains clinical variability in Cohen syndrome. *Am J Hum Genet* 2004; **75**: 138–145.
8 Mochida GH, Rajab A, Eyaid W *et al*: Broader geographical spectrum of Cohen syndrome due to COH1 mutations. *J Med Genet* 2004; **41**: e87.
9 Seifert W, Holder-Espinasse M, Spranger S *et al*: Mutational spectrum of COH1 and clinical heterogeneity in Cohen syndrome. *J Med Genet* 2006; **43**: e22.
10 Katzaki E, Pescucci C, Uliana V *et al*: Clinical and molecular characterization of Italian patients affected by Cohen syndrome. *J Hum Genet* 2007; **52**: 1011–1017.
11 Balikova I, Lehesjoki AE, de Ravel TJ *et al*: Deletions in the VPS13B (COH1) gene as a cause of Cohen syndrome. *Hum Mutat* 2009.
12 Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G: Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res* 2002; **30**: e57.
13 Kolehmainen J, Wilkinson R, Lehesjoki AE *et al*: Delineation of Cohen syndrome following a large-scale genotype-phenotype screen. *Am J Hum Genet* 2004; **75**: 122–127.
14 Chandler KE, Kidd A, Al-Gazali L *et al*: Diagnostic criteria, clinical characteristics, and natural history of Cohen syndrome. *J Med Genet* 2003; **40**: 233–241.
15 Ariani F, Mari F, Pescucci C *et al*: Real-time quantitative PCR as a routine method for screening large rearrangements in Rett syndrome: report of one case of MECP2 deletion and one case of MECP2 duplication. *Hum Mutat* 2004; **24**: 172–177.
16 Bugiani M, Gyftodimou Y, Tsimpouka P *et al*: Cohen syndrome resulting from a novel large intragenic COH1 deletion segregating in an isolated Greek island population. *Am J Med Genet A* 2008; **146A**: 2221–2226.

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