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Original article

Exon copy number alterations of the *CHD7* gene are not a major cause of CHARGE and CHARGE-like syndrome

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Abstract

CHARGE syndrome is a multiple congenital anomaly syndrome caused by mutations in the *CHD7* gene. Mutations in this gene are found in 60−70% of patients suspected of having CHARGE syndrome. However, if only typical CHARGE patients are taken into account, mutations in the *CHD7* gene are found in over 90% of cases. The remaining 10% might be caused by hitherto undetected alterations of the *CHD7* gene, including whole exon duplications and deletions that are missed by the currently used diagnostic procedures. Therefore we looked for these kinds of alterations by multiplex ligation-dependent probe amplification in 54 patients suspected of having CHARGE syndrome without a *CHD7* mutation. In one patient a partial deletion of the *CHD7* gene (exons 13−38) was identified, while in the other patients no abnormalities were found. The frequency of exon deletions in our cohort was 1.9% (1/54) and 5.6% (1/18) in all patients and in typical CHARGE patients, respectively. We conclude that exon copy number alterations of the *CHD7* gene are not a major cause of CHARGE and CHARGE-like syndrome.
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Keywords: CHARGE syndrome; *CHD7*; MLPA; Whole exon deletion; Whole exon duplication

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1. Introduction

In 2004 the underlying gene defect for CHARGE syndrome (the *CHD7* gene, OMIM #214800) was identified on chromosome 8 (8q12.1) [16]. This multiple congenital anomaly syndrome was originally described as a combination of *c*oloboma, *h*eart defects, *a*tresia of choanae, *r*etardation of growth and/or development, *g*enital hypoplasia and *e*ar anomalies and/or deafness [12]. Later, additional congenital malformations were recognised, of which agenesis of the semicircular canals and arhinencephaly were found to be present in nearly all patients [1,2,13]. The prevalence is approximately 1 in 10,000. CHARGE syndrome is diagnosed on clinical grounds according to two different sets of criteria as proposed by Blake et al. [5] and Verloes [15], and/or analysis of the *CHD7* (*c*hromodomain *h*elicase *D*NA binding protein 7) gene. Three large, independent studies found heterozygous mutations in the *CHD7* gene in 60−70% of patients suspected of having CHARGE syndrome [3,8,10]. However, if the clinical criteria for CHARGE syndrome [5,15] are strictly applied, mutations in *CHD7* are present in over 90% of the typical CHARGE patients.

The two sets of criteria used for diagnosing CHARGE syndrome, consist of phenotypic features that do not fully overlap (Table 1). Blake et al. consider a patient as having typical CHARGE syndrome if four major, or three major and three minor, criteria are present [5]. Based on new diagnostic insights, Verloes refined the criteria and included abnormalities of the semicircular canals [15]. Besides, Verloes distinguished three categories of CHARGE syndrome patients: typical, partial and atypical. Verloes considered typical CHARGE patients to present with three major, or two major and two minor, criteria. Patients were considered to have partial CHARGE syndrome when two major and one minor criteria were present, while atypical CHARGE patients have two major and no minor criteria, or one major and two minor criteria.

Table 1
Clinical criteria for identifying CHARGE syndrome patients

	Major criteria	Minor criteria	Inclusion rule
Blake et al. [5]	1. Ocular coloboma or microphthalmia 2. Choanal atresia or stenosis 3. Characteristic external ear anomaly, or middle ear malformations or mixed deafness 4. Cranial nerve dysfunction	1. Congenital cardiovascular malformations 2. Tracheoesophageal defect 3. Genital hypoplasia or delayed pubertal development 4. Cleft lip and/or palate 5. Developmental delay 6. Growth retardation 7. Characteristic face	*Typical CHARGE*: 4 majors, OR 3 majors + 3 minors
Verloes [15]	1. Ocular coloboma 2. Choanal atresia or stenosis 3. Hypoplasia of semicircular canals	1. Heart or oesophagus malformation 2. Malformation of the inner or external ear 3. Rhombencephalic dysfunction including sensorineural deafness 4. Hypothalamo-hypophyseal dysfunction 5. Mental retardation	*Typical CHARGE*: 3 majors, OR 2 majors + 2 minors *Partial CHARGE*: 2 majors + 1 minor *Atypical CHARGE*: 2 majors, but no minors, OR 1 major + 2 minors

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Most of the *CHD7* mutations that have been described in CHARGE syndrome are truncating (nonsense and frameshift), but missense mutations have also been found. Most mutations are unique, although some recurrent *de novo* mutations have been found. There seem to be no real mutation hot spots, but some exons (2, 3, 31 and 34) are more frequently mutated than others (own unpublished data). The higher mutation frequency of these exons appears to be related to their size, with the largest exon 2 most frequently mutated. Until now, only one intragenic deletion has been reported in a CHARGE patient [14]. Whole gene deletions of *CHD7*, although present in the two patients who contributed to the discovery of the *CHD7* gene [16], were not found in two large cohorts of CHARGE patients [8,10]. However, single exon deletion or duplication would have been missed with the techniques used in these studies. Since the underlying defect in the remaining 10% of typical CHARGE patients has not yet been discovered, we hypothesised that exon copy number alterations of the *CHD7* gene might contribute to CHARGE syndrome. We identified a cohort of 54 patients (suspected of) having CHARGE syndrome who did not have a *CHD7* mutation. We screened for single exon deletion or duplication in the *CHD7* gene using multiplex ligation-dependent probe amplification (MLPA).

2. Materials and methods

Fifty-four patients were selected from a group of patients who were referred for mutation analysis of *CHD7* because of clinical features suggestive of CHARGE syndrome. Mutation screening performed by polymerase chain reaction (PCR) followed by direct sequencing had not revealed any *CHD7* alterations in these patients [8]. The DNA samples were subsequently screened for exon deletions and/or duplications of the *CHD7* gene by MLPA analysis. Half of the patients (see Table 2) were analysed using home-designed synthetic oligonucleotides, covering all the coding exons of the *CHD7* gene in four probe sets as described before [9]. The other patients were screened with a commercially available set of probes, the SALSA P201 kit (MRC-Holland, Amsterdam, The Netherlands; http://www.mrc-holland.com). This set includes the usual control probes located on different chromosomes, together with probes for most exons of the *CHD7* gene (27 of 38 exons). When exons were located closely together, one representative exon was chosen for the region. Because probes for both the first non-coding exon (exon 1) and the last exon (exon 38, containing the stop codon) were included, both kits would also have detected whole gene deletions.

The MLPA analysis as well as the statistical analysis was performed as described previously [9]. A patient with a known heterozygous deletion of the whole gene was used as a positive control, and we included two negative controls in each analysis.

Clinical information on the 54 index patients was obtained from our investigations at the outpatient clinic for children with CHARGE syndrome or through the referring clinicians, by means of a written questionnaire submitted prior to DNA analysis. Additional information was requested when necessary. We scored patients for CHARGE features according to the two sets of criteria (Blake et al. [5] and Verloes [15]) as summarised in Table 1. Patients were classified as typical CHARGE syndrome when they fulfilled the criteria for typical CHARGE of at least one of the two scoring sets.

3. Results

In our cohort of 54 patients we found a deletion of exons 13–38 in patient no. 1 (Fig. 1). In all other patients no exon copy number changes in the *CHD7* gene were found.

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Table 2
Clinical features of 54 patients suspected of having CHARGE syndrome but with no mutation in the *CHD7* gene

Case	Sex[a]	Age[b] (yr)	Diagnostic criteria		Major criteria					Minor criteria[h]
			Blake	Verloes[c]	Eye anomaly[d]	Atresia of choanae[e]	Cranial nerve dysfunction[f]	Ear anomaly	SSC[g]	
1	**M**	**12**	**−**	**+**	**C**	**–**	**VII, VIII, IX, X**	**+**	**+**	**AN, B, CF, GH**
2	M	2	+	+	C, Mi	– (CLP)	VIII	+	+	MR, S
3*	F	10	+	+	C	S	VIII	–	–	H, MR, RG, S
4*	F	21	+	+	C	A	VII, VIII	–	+	B, H, HHD, GH, MR, R
5*	M	2 d	+	+	C, Mi	– (CLP)	U	+	U	CF, H, S, TE
6	F	3	+	+	C	S	VIII	+	U	MR
7	F	1	+	+	–	– (CP)	VIII	+	+	CF, H, RG
8	M	10	+	a	C	–	VIII	+	U	B, CF, GH, MR
9	F	7	+	a	Mi	A	VIII	+	U	B, CF, MR, S
10*	F	14	+	a	–	A	VIII	+	U	H, MR, RG, S
11*	M	3	+	a	–	A (CP)	VIII	+	U	B, H, MR
12*	F	4	+	a	C	–	VI, VII, VIII, IX, XII	+	U	CF, GH, H, MR
13*	F	29	–	+	C, Mi	A	–	+	U	MR
14*	F	6	–	+	C	–	–	+	+	H, MR, R, S
15	M	1	–	+	C	A	U	+	U	H
16	F	28	–	+	C, Mi	A	VIII	U	U	H, MR, S
17	F	1/2	–	+	C, Mi	– (CP)	U	+	U	TE
18*	M	3	–	+	C, Mi	– (CP)	VIII	–	U	GH, MR
19	M	7	–	p	C, Mi	– (CP)	–	+	U	
20	F	4	–	p	Mi	S	VIII	–	+	B
21	F	16	–	p	C, Mi	– (CP)	U	U	U	H, RG
22	F	13	–	p	C, Mi	A	U	U	U	MR, RG
23*	M	46	–	a	C	–	VIII	+	U	MR
24*	M	1	–	a	–	A	–	+	U	B, GH, H, R, S
25*	F	3	–	a	–	A	VIII	+	U	B, CF, MR
26*	M	1/2	–	a	–	A	VIII	+	U	H, S
27*	M	2	–	a	C	–	–	+	U	H, MR, RG
28*	M	36	–	a	C	–	VIII	–	U	B, GH, H, HHD
29*	F	11	–	a	C	–	–	–	U	H, MR, R, S
30	M	39	–	a	C, Mi	–	–	–	U	H, MR, RG, S
31	M	11 d	–	a	–	A	–	+	U	B, CF, H
32*	F	2	–	a	Mi	– (CLP)	–	+	U	B, H

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33*	F	12	–	a	C, Mi	–	–	+	U	B, MR, RG
34*	M	1/4	–	a	–	A	–	+	U	B, H, R
35*	F	29	–	a	–	A	VII, VIII	–	–	S, TE
36	F	4	–	a	–	A	–	+	U	B, MR, RG
37*	M	2	–	a	C	–	–	–	–	H, MR
38*	F	3	–	a	–	A	–	–	–	H, MR
39	M	4	–	a	–	A	–	+	U	H, MR, RG
40	M	3	–	a	C, Mi	–	VIII	–	U	B, CF, H, R, RG
41	F	5	–	a	C, Mi	–	VIII	–	U	GH, H, MR, S
42	F	1	–	a	C	–	VIII	–	U	H, MR, S
43	F	11	–	a	C, Mi	–	–	+	U	GH, H, R, RG
44	F	8	–	a	C	–	–	–	U	H, MR
45	F	3	–	a	–	A	VIII	+	–	H, MR, S
46	F	4	–	a	–	A (CP)	–	+	U	CF, H, MR, S
47	F	11	–	a	C	–	–	+	U	MR, RG
48	F	3	–	a	–	- (CP)	–	+	U	H
49*	F	22	–	–	–	–	VIII	+	U	H, HHD, S
50*	F	6	–	–	–	–	VII, VIII	+	–	B, H, MR, TE
51*	M	8	–	–	C, Mi	–	–	–	U	H, R, RG
52*	M	9	–	–	–	A	–	–	U	H
53	F	10	–	–	–	–	VIII	+	U	CF, MR, R, S, TE
54	M	4	–	–	–	– (CLP)	–	–	U	H, R

The patient with the partial *CHD7* deletion is depicted in bold.

The patients that were screened with home-designed synthetic oligonucleotides are indicated with an asterisk. The other patients were analysed with the SALSA P201 MLPA kit.

[a] F: female; M: male.

[b] d: day; yr: year.

[c] a: atypical; p: partial.

[d] C: coloboma; Mi: microphthalmia.

[e] A: atresia of choanae; CLP: cleft lip and palate; CP: cleft palate; S: stenosis of choanae. Presence of CLP or CP was counted as a major criterion, because this rarely occurs together with atresia of choanae [5].

[f] U: unknown.

[g] SSC: semicircular canal hypoplasia or vestibular dysfunction.

[h] AN: anosmia; B: brain abnormalities; CF: characteristic face; H: heart defect; GH: genital hypoplasia; HHD: hypothalamo-hypophyseal dysfunction; MR: mental retardation; R: renal abnormality; RG: retarded growth; S: skeletal abnormality; and TE: tracheoesophageal abnormalities.

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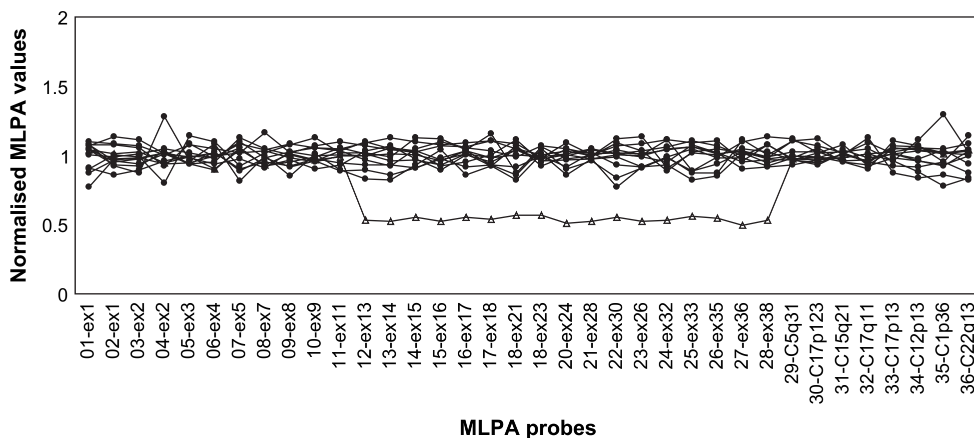

Fig. 1. Example of an MLPA analysis (SALSA P201 kit) with 13 different DNA samples from (suspected) CHARGE patients. On the *X*-axis the probes of the different exons are named (01- till 28-, representing exon 1 till 38 of the *CHD7* gene, and 29- till 36-, control probes that have two genomic copies in the normal population). On the *Y*-axis are the normalised values (see Koolen et al. for details [9]), 1 means that two copies are present, 0.5 means that only one copy is present. The sample marked with triangles is patient no. 1 with the partial deletion of the *CHD7* gene involving exon 13 till 38.

An overview of the clinical features of patient no. 1 is given in Table 2 (see also Fig. 2). The patient was born at 42 weeks of gestation with a birth weight of 3.685 kg. At birth right facial nerve palsy and dysmorphic ears were noted. In addition he had partial palsy of cranial nerves IX and X, for which he required tube feeding for 1 year. Choroid colobomas, hypoplasia of the

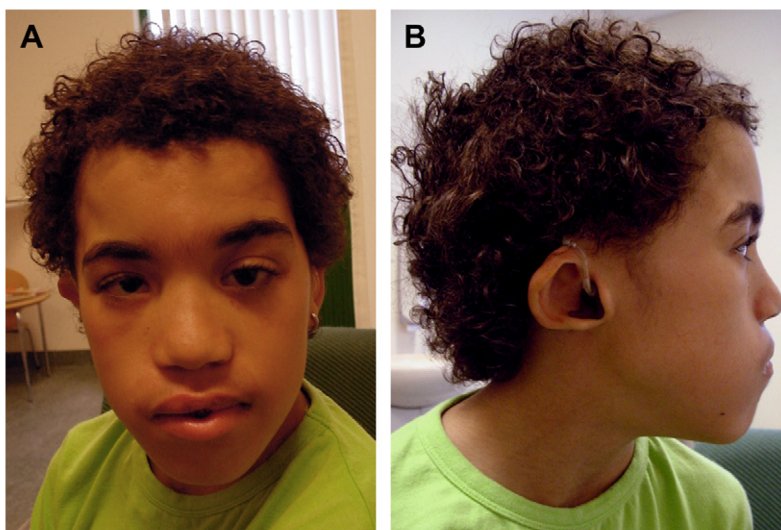

Fig. 2. Photographs of patient no. 1 with partial deletion of the *CHD7* gene at 12 years and 3 months of age. Note square face with facial palsy and microcornea (both right-sided), dysplastic ear with typical triangular concha and absence of the ear lobe, and a flat midface.

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pons and vermis of the cerebellum, severe bilateral mixed hearing loss, undescended testes and micropenis were present as well. At age 12 years, his height was 141.5 cm (−2 SD). He had a square and asymmetric face and proved to be anosmic by the University of Pennsylvania Smell Identification Test [6]. Unfortunately, imaging of the semicircular canals was not performed, but vestibular dysfunction was noted on physical examination.

Our cohort proved to be phenotypically heterogeneous, varying from typical CHARGE patients to partial and atypical patients and patients only suspected of having CHARGE syndrome. Table 2 gives an overview of their clinical features and scores according to Blake's et al. and Verloes' criteria. Eighteen patients had typical CHARGE syndrome (according to Blake et al. ($n = 5$), Verloes ($n = 7$) or both ($n = 6$)). Four patients had partial CHARGE, 26 had atypical CHARGE syndrome according to Verloes and six were suspected of having CHARGE syndrome but did not satisfy either set of criteria.

4. Discussion

A defect in the *CHD7* gene is found in 60–70% of all patients suspected of having CHARGE syndrome and in over 90% of typical CHARGE patients [8]. In the remaining 10% of typical patients the cause of CHARGE syndrome remains elusive. Genetic heterogeneity could be present, but the only other gene that has been implicated in CHARGE syndrome, the *SEMA3E* gene, was found to be mutated in only one CHARGE patient [11]. So far, mutations in this gene have not been reported in other CHARGE patients. It seems therefore more plausible to assume that *CHD7* is the major causative gene, considering the small percentage of typical patients in whom no mutation is found.

CHARGE patients normally undergo only a routine sequence analysis, which would miss any mutations located deep in the introns or in the promoter region of the *CHD7* gene. In addition, whole exon deletions or duplications would not be detected. Hitherto, only three CHARGE patients have been described with a genomic rearrangement leading to a deletion of the whole *CHD7* gene [4,7,16]. Whole gene duplications are not likely to cause CHARGE syndrome. Based on the mutations found so far (predominantly leading to a truncated protein) and on the presumed function of *CHD7*, the identified mutations most likely have a loss-of-function effect. This, however, does not rule out single exon duplication, which would result in a distortion of the reading frame, as a cause of CHARGE syndrome.

So far no intragenic duplications and only one patient with an intragenic deletion of exons 8–12 has been reported [14]. The described intragenic deletion was detected by multiplex PCR/liquid chromatography assay and appeared to have arisen through an *Alu*-mediated replacement event. This patient was one of a cohort of 13 typical CHARGE patients studied by Udaka et al., which leads to an exon deletion frequency of 7.7% (1/13). Another study, by Vuorela et al., did not find *CHD7* deletions with quantitative real-time PCR and MLPA analysis in 44 *CHD7* mutation negative patients [17]. Unfortunately they did not supply the clinical characteristics of their patients and therefore it is not known how many typical CHARGE patients were included in their cohort.

In our cohort of 54 patients suspected of having CHARGE syndrome without *CHD7* alterations on routine sequencing, we found one patient with a deletion of exons 13–38. In the other patients no exon copy number changes of the *CHD7* gene were found by MLPA analysis. Our patient with the partial *CHD7* deletion could clinically not be distinguished from the patients with normal MLPA results. One of the used MLPA kits (SALSA P201) did not cover all exons of the *CHD7* gene directly. However, exons missing in this MLPA kit are located close to exons

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that are represented in the kit. Very small deletions or duplications (within exons) will be missed using MLPA analysis in general, but bigger copy number variations are detectable with both MLPA kits.

The frequency of exon deletions in our cohort was 1.9% (1/54) and 5.6% (1/18) in all patients and in typical CHARGE patients, respectively. The latter figure is in accordance with the finding of Udaka et al. However, it should be noted that the classification of our CHARGE patients is based on the available clinical information. Unfortunately in 42 patients imaging of the semicircular canals has not been performed. This means that the 25 patients who satisfied the Verloes' criteria for atypical or partial CHARGE could in theory have typical CHARGE in the presence of semicircular canal anomalies. If we take this precaution into account, the frequency of partial *CHD7* deletions in typical CHARGE patients without a mutation in *CHD7* is 2.3–5.6% (1/43–1/18) according to our study.

Since we did not detect any exon copy number alterations in the 36 non-typical patients, it seems that MLPA analysis of the *CHD7* gene does not significantly improve the mutation analysis for this group of patients. In typical CHARGE patients however, we do recommend MLPA analysis of the *CHD7* gene, even though the frequency of partial *CHD7* deletions is low. A thorough clinical work-up is essential (e.g. imaging of semicircular canals) in order to classify patients as typical or non-typical CHARGE patients.

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