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Research paper

Revealing the function of a novel splice-site mutation of *CHD7* in CHARGE syndrome

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ABSTRACT

Most cases of CHARGE syndrome are sporadic and autosomal dominant. *CHD7* is a major causative gene of CHARGE syndrome. In this study, we screened *CHD7* in two Turkish patients demonstrating symptoms of CHARGE syndrome such as coloboma, heart defect, choanal atresia, retarded growth, genital abnomalities and ear anomalies. Two mutations of *CHD7* were identified including a novel splice-site mutation (c.2443-2A>G) and a previously known frameshift mutation (c.2504_2508delATCTT). We performed exon trapping analysis to determine the effect of the c.2443-2A>G mutation at the transcriptional level, and found that it caused a complete skip of exon 7 and splicing at a cryptic splice acceptor site. Our current study is the second study demonstrating an exon 7 deficit in *CHD7*. Results of previous studies suggest that the c.2443-2A>G mutation affects the formation of nasal tissues and the neural retina during early development, resulting in choanal atresia and coloboma, respectively. The findings of the present study will improve our understanding of the genetic causes of CHARGE syndrome.

1. Introduction

CHARGE syndrome occurs in approximately 1 in every 10,000 newborns worldwide, and almost all cases of CHARGE syndrome occur sporadically (Bergman et al., 2011). The recurrence rate is approximately 1% among sib-pairs, monozygotic twins, and 2-generation families (Jongmans et al., 2006; Lalani et al., 2006; Delahaye et al., 2007; Jongmans et al., 2008; Vuorela et al., 2008; Wincent et al., 2008; Pauli et al., 2009; Bergman et al., 2011). Familial CHARGE syndrome follows an autosomal dominant inheritance with variable penetrance (Bergman et al., 2011). In parent-to-child cases, the parents are generally asymptomatic or exhibit only mild phenotypes, whereas their children show more severe phenotypes (Zentner et al., 2010; Bergman et al., 2011).

CHD7, a gene located on chromosome 8q12.1 and encoding the chromodomain helicase DNA-binding (CHD) protein 7, is a major causative gene of CHARGE syndrome (Vissers et al., 2004). *CHD7* contains one non-coding and 37 coding exons, and encodes for the 2997 amino acids CHD7 protein belonging to a family of nine CHD proteins that can modify chromatin structure (Vissers et al., 2004). Approximately 60%–70% of patients with CHARGE syndrome have pathogenic mutations in *CHD7* (Zentner et al., 2010). While approximately 70% of these mutations are truncating mutations, such as nonsense or frameshift mutations (Zentner et al., 2010), the incidence of splice-site or missense mutations is low (Zentner et al., 2010). The most likely cause of CHARGE syndrome is the haploinsufficiency of *CHD7*. This finding is supported by mouse model studies in which homozygous *Chd7* mice with loss-of-function mutations showed embryonic lethality, while heterozygous *Chd7* mice showed phenotypic features similar to those associated with CHARGE syndrome (Bosman et al., 2005; Hurd et al., 2007).

Because many of the symptoms and features of CHARGE syndrome are common to other syndromes, genetic analysis of *CHD7* is necessary

Abbreviations: CHD, chromodomain helicase DNA-binding; PCR, polymerase chain reaction; ECHO, echocardiogram; ASD, atrial septal defect; CCA, common carotid artery; MRI, magnetic resonance imaging; 3D CT, three-dimensional computed tomography; ICA, internal carotid artery; RT-PCR, reverse transcription-PCR.

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for an accurate diagnosis of CHARGE syndrome (Corsten-Janssen et al., 2013). In the present study, we have performed DNA-sequencing analysis of *CHD7* in two Turkish patients with several clinical characteristics of CHARGE syndrome.

2. Materials and methods

2.1. Subjects and clinical examinations

This study included two Turkish patients who were diagnosed with CHARGE syndrome. Both of the patients were evaluated by clinical medical geneticist and detailed physical examinations were performed covering all the systems and required tests were performed to confirm diagnosis. The mutations identified in these patients were screened for in 48 other individuals who had no known genetic defects. Before their participation in the study, written informed consent was obtained from all participants, and guardians when necessary.

2.2. Genetic analysis

Blood samples were collected from both the patients and their family members, and genomic DNA was extracted using the EZ1 DNA blood kit (Qiagen, Hilden, Germany). The 38 exons and exon–intron boundaries of *CHD7* were amplified by polymerase chain reaction (PCR) with h-Taq polymerase (Solgent, Daejeon, Korea) and specific primers designed using Primer3 v0.4.0 (http://gmdd.shgmo.org/primer3). To eliminate unincorporated nucleotides and primers, the PCR products were digested using shrimp alkaline phosphatase (USB, Cleveland, OH, USA) and exonuclease I (USB). Sequences of the amplified products were identified by direct sequencing with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Subsequently, the amplified products were purified using ethanol precipitation, and the purified products were sequenced using the 3130xl genetic analyzer (Applied Biosystems). Sequencing data were analyzed using ABI sequencing analysis software v5.2 (Applied Biosystems) and Chromas pro v1.5 (Technelysium Pty Ltd., Tewantin, QLD, Australia). Sequencing data of the patients with CHARGE syndrome were compared with reference sequence of *CHD7* gene (NM_017780.3) obtained from the National Center for Biotechnology Information database (http://blast.ncbi.nlm.nih.gov).

2.3. Exon trapping analysis

An *in vitro* splicing assay was performed using the amplified exon 7 of *CHD7*, and an approximately 300 bp intronic region flanking the 5′ and 3′ ends of exon 7, from the genomic DNA of patient 1 and a normal control. The amplified products were digested using *Xho*I and *Bam*HI and were inserted in pSPL3 splicing vectors containing exons A and B. The hybrid vectors were transfected into HeLa cells by using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and the cells were cultured in Dulbecco's modified eagle's medium containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in 5% CO_2. The transfected cells were harvested after 24 h, and total RNA was extracted using the RNeasy mini kit (Qiagen). Next, 1 μg of total RNA was reverse transcribed using the high capacity cDNA reverse transcription kit (Applied Biosystems) to synthesize cDNA. This cDNA was amplified using the pSPL3 vector-specific primers SD6 (5′-TCTGAGTCACCTGGACAACC-3′) and SA2 (5′-ATCTCAGTGGTATTTGTGAGC-3′). The size of the amplified products was determined by electrophoresis in 2% agarose gel, and the products were purified using the DokDo-prep gel extraction kit (Elpis Biotech, Daejeon, Korea). Finally, the sequences of the PCR products were determined by direct sequencing.

3. Results

3.1. Clinical features

Patient 1 was the third child born to a 34-year-old, gravida 3, para 3 mother; her parents were both healthy and were third-degree cousins (Fig. 1A). Pregnancy was uncomplicated with a spontaneous vaginal delivery, at term, according to normal birth parameters. However, choanal atresia was discovered upon birth and an operation was performed immediately. The patient was followed-up in the neonatal intensive care unit for twenty days. She was hypotonic at birth, and at 4 months old, her family realized she was not able to control her head. During a physical examination, a systolic murmur was determined. An echocardiogram (ECHO) revealed a wide secundum atrial septal defect (ASD), increased right ventricular dimensions, right side located arcus aorta and both truncus brachiocephalicus and the left common carotid artery (CCA) originated from the same root. These cardiovascular abnormalities were accompanied by a vascular ring, which was detected during a three-dimensional computed tomography (3D CT) chest examination. To elucidate these major vessel anomalies, intracranial angiography and cervical magnetic resonance imaging (MRI) angiography were used. A total occlusion of the left internal carotid artery (ICA) from its origin was detected and accompanied by collateral circulation by the vertebral artery. There were no brain malformations observed in the cranial MRI. When she was referred to us at 5-year-old, her height, weight, and head circumference were 97 cm (<3rd percentile), 17 kg (<50–75th percentile) and 48.5 cm (3rd percentile), respectively. She had prominent eyes, a broad nasal root and bridge, a thin upper lip and low-set, protruding, and posteriorly rotated ears. Autistic behaviors were also identified. The patient was referred to an otolaryngologist for a hearing test and was diagnosed with hearing loss. Ophthalmologic assessment determined choroidal coloboma in the left eye, adjacent to the optic disc. When she was 4.5 years old, she was evaluated by the Denver Developmental Screening test with the results of severe mental-motor retardation. Her fine motor adaption, gross motor ability, personal social activities, and language were compatible with 20, 17, 10, and 10 months, respectively. The severe mental-motor retardation was accompanied by autism spectrum symptoms, including stereotypy, restricted and repetitive behavior, and self-injury. She was given a high dosage of Risperdal for these symptoms, to no effect. She was also unable to take solid foods due to tracheal compression. Clinical assessment of the patient was compatible with CHARGE syndrome, including three of the major diagnostic characteristic criteria of CHARGE syndrome including ocular coloboma, choanal atresia, and abnormal features of the outer ear. Four of the minor diagnostic characteristics of CHARGE syndrome, consisting of cardiovascular malformation, growth deficiency, developmental delay, and distinctive facial appearance, were present as well.

Patient 2 was born at 40 weeks gestation, with a birth weight of 3000 g. She was the second child born to a 24-year-old, gravida 2, para 2 mother. Her parents were both healthy and were third-degree cousins (Fig. 1C). In the postnatal period, she was not able to cry, was cyanotic and had dyspnea. The patient was hospitalized for twenty-five days for these symptoms. After hospitalization, the condition of the patient had stabilized and there were no symptoms except for a flow of transparent fluid from the nose and recurrent infections. During a routine examination, atypical appearance and severe growth retardation were identified when she was 7 months old. All growth parameters were under 3rd percentile. She had a square face, down-slanting palpebral fissures, microphthalmia, a broad nasal root, hypoplasia of alae nasi, and cup-shaped and low-set ears. An ECHO revealed some cardiovascular malformations, followed by diagnostic angiography which showed ventricular septal defect, secundum ASD, pulmonary valvular stenosis, and the continuation of the azygos vein. Balloon pulmonary valvuloplasty was performed for the pulmonary valvular stenosis. The patient was referred to an otolaryngologist due to transparent runny