Two Novel Heterozygous Mutations of *EVC2* Cause a Mild Phenotype of Ellis–van Creveld Syndrome in a Chinese Family

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Ellis–van Creveld syndrome (EvC, chondroectodermal dysplasia; OMIM 225500) is an autosomal recessive skeletal dysplasia with associated multisystem involvement. The syndrome is characterized by short limbs, short ribs, postaxial polydactyly, dysplastic nails, and abnormal teeth. Congenital heart defects occur in 50–60% of cases. In this study, we report EvC in a 6-year-old Chinese girl with hypodontia and polydactyly, mild short stature, and abnormalities of the knee joints. No signs of short ribs, narrow thorax, or congenital heart defects were found in this patient. The EvC phenotype shares some similarity with Weyers acrofacial dysostosis (Weyer; OMIM 193530), an autosomal dominant disorder clinically characterized by mild short stature, postaxial polydactyly, nail dystrophy, and dysplastic teeth. Mutations in *EVC* or *EVC2* are associated with both EvC syndrome and Weyers acrodental dysostosis, but the two conditions differ in the severity of the phenotype and their pattern of inheritance. In this study, two novel heterozygous *EVC2* mutations, IVS5-2A > G and c.2653C > T (Arg885X), were identified in the patient. The IVS5-2A > G mutation was inherited from the patient's mother and the c.2653C > T from her father. Her parents have no phenotypic symptoms similar to those of the patient. These findings extend the mutation spectrum of this malformation syndrome and provide the possibility of prenatal diagnosis for future offspring in this family.
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Key words: Ellis–van Creveld syndrome; Weyers acrofacial dysostosis; *EVC*; *EVC2*; mutation

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INTRODUCTION

Ellis–van Creveld syndrome (EvC, or chondroectodermal dysplasia; OMIM 225500) is an autosomal recessive skeletal dysplasia with associated multisystem involvement. The syndrome is characterized by short limbs, short ribs, postaxial polydactyly, and dysplastic nails and abnormal teeth. Congenital heart defects occur in 50–60% of cases, with atrial septal defects particularly prevalent [Digilio et al., 1999]. EvC is a rare genetic disorder which has been most commonly observed in the Amish population. The prevalence is 7 per 1 million births [Stoll et al., 1989]. EvC syndrome is attributed to mutations in two adjacent genes on chromosome 4p16, *EVC*, and *EVC2* [Polymeropoulos et al., 1996; Howard et al., 1997; Ruiz-Perez et al., 2000; Galdzicka et al., 2002; Ruiz-Perez et al., 2003].

Heterozygous mutations in the *EVC* or *EVC2* genes also cause Weyers acrofacial dysostosis (OMIM 193530), an allelic disorder with autosomal dominant inheritance. It is clinically characterized by mild short stature, postaxial polydactyly, nail dystrophy, and dysplastic teeth. Despite its strong similarities to EvC, Weyers acrofacial dysostosis can be distinguished by its mode of inheritance and degree of phenotypic severity [Howard et al., 1997].

The *EVC* and *EVC2* genes are separated by 2,624 bp in humans and 1,647 bp in the mouse. They are arranged in a head-to-head configuration that is conserved from fish to man [Ruiz-Perez et al., 2003]. Recent analysis indicates that such head-to-head configurations may be a common feature of the human genome, and instances of coregulation by a single promoter with bidirectional activity have been found [Shimada and Lin, 1989; Shimada et al., 1989; Platzer et al., 1997]. Similarly, a single promoter could coordinate the expression of *EVC* and *EVC2*. Several potential transcription factor binding sites, including *Sp1*, *AP-2*, *myogenin*, and *C/EBP* have been identified for this promoter region [Galdzicka et al., 2002]. This organization of adjacent genes suggests that they may be transcribed in a coordinated fashion and are therefore functionally related [Sund et al., 2009].

In this study, we sequenced *EVC* and *EVC2* in a 6-year-old Chinese girl with mild short stature, postaxial polydactyly, dysplastic nails, abnormal teeth, and genus valgum, but without short ribs, narrow thorax, or cardiac defects. We identified two novel heterozygous mutations in *EVC2*, which were inherited from both parents, respectively.

MATERIALS AND METHODS
Patient and Control Samples

The patient was brought to the stomatology clinic because of absent and fused teeth, short arms and legs, postaxial hexadactyly of both hands, and toe syndactyly. Control DNA samples from Han Chinese individuals (n = 100) were also genotyped. All samples were taken with informed consent, and blood samples or oral swabs were coded to maintain confidentiality.

DNA Isolation

Genomic DNA was extracted from peripheral blood lymphocytes using a standard high-salt method or from buccal epithelial cells with a salt/ethanol precipitation method. The extracted DNA samples were stored at −20°C until analysis.

Polymerase Chain Reaction and DNA Sequencing

Screening for sequence in *EVC* and *EVC2* was by direct sequencing of the PCR products resulting from amplification of all coding exons and at least 100 bp of the flanking introns. The resulting sequencing chromatograms were aligned and compared with the reference nucleotide sequence of *EVC* and *EVC2* transcripts (NM_153717.2 and NM_147127.4). CCDS3383.1 and CCDS3382.2 were used as the reference protein sequence of EVC and EVC2. For numbering the mutations we considered the A of the ATG translation initiation codon of *EVC* and *EVC2* as nucleotide +1.

CLINICAL REPORT

The patient was a 6-year-old Han girl, the firstborn child of non-consanguineous parents. Her parents and relatives were healthy with no similar features. Physical examination showed multiple manifestations including mildly short stature, with a height of 99 cm (average height for girls of this age is 115 cm). Intraoral examination documented five missing deciduous teeth (Fig. 1 A–C), with no medical history of tooth extraction. Panoramic radiography showed congenital absence of eight permanent teeth, excluding the wisdom teeth (Fig. 1 B and C). Fused right-side maxillary and left-side mandibular incisors and canines in the deciduous dentition were found. There were no extra frenula
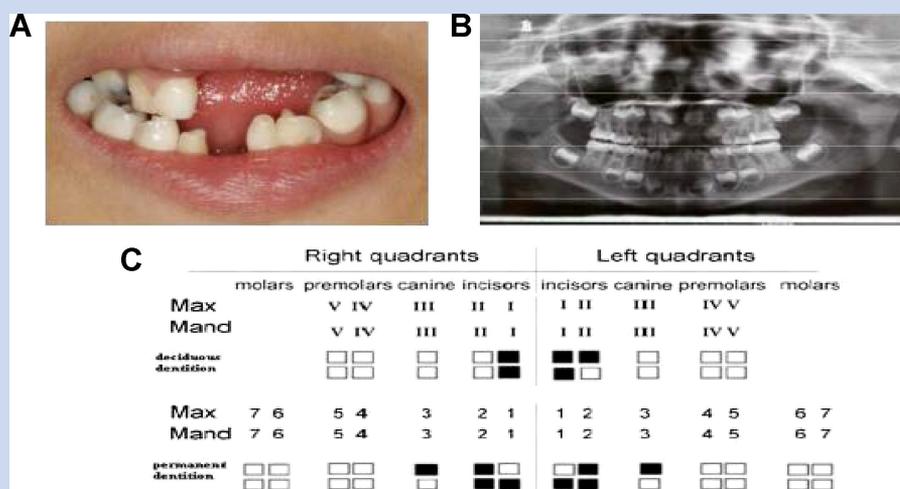
FIG. 1. Dentition of the proposita. A: Intraoral photograph of deciduous dentition showing hypodontia of both maxillary and mandibular incisors, and the left lateral incisor; fused teeth at the right maxillary and left lateral mandibular incisors and canines. B: Orthopantomography reveals agenesis of the central incisors and maxillary and mandibular lateral incisors in the permanent dentition. C: Missing teeth are represented by a filled square (Max, maxillary; Mand, mandibular).

FIG. 2. Clinical features of the patient. A: Postaxial polydactyly of both hands. B: Syndactyly of the toes and nail dysplasia (marked by the black arrow). C: Abnormalities of the knee joints.

(Fig. 1A). The patient also had distal limb shortening; postaxial polydactyly of hands; bilateral syndactyly of toes 2 and 3; nail dystrophy of the feet; and abnormalities of knee joints (Figs. 2 A–C and 3B). Chest X-ray showed abnormal morphology of the clavicle without narrow chest or short ribs (Fig. 3A). Patient has normal hair. No cardiovascular defects were found. Table I summarizes the phenotypic features in the patient.

RESULTS

Mutation Analysis of EVC and EVC2

Two heterozygous mutations (IVS5-2A > G and c.2653C > T) were detected in *EVC2* (Fig. 4). The IVS5-2A > G mutation was inherited from the patient's mother and the c.2653C > T originated from her father. Both mutations were deduced to be disease-related as they were not present in 200 normal chromosomes sequenced in this study. No mutation was identified in *EVC*.

DISCUSSION

A variety of clinical presentations are observed in patients with EvC. The skeletal features of EvC include disproportionate short stature,

distal shortening of limbs, short ribs, and postaxial polydactyly of hands and feet. Tooth abnormalities, multiple oral frenulae, and hypoplastic nails are consistent findings in EvC, and congenital heart defects are present in 40–50% of patients, typically an atrial or atrioventricular septal defect. Mild mental retardation is occasionally found [da Silva et al., 1980; Arya et al., 2001; Cağdaş et al., 2008; Temtamy et al., 2008].

Some elements of the phenotype of EvC are similar to Weyers acrofacial dysostosis (OMIM 193530), which, like EvC, is associated with mutations in *EVC* or *EVC2* (Table I). It is possible that Weyers acrofacial dysostosis is a heterozygous expression of a mutation that in homozygous form causes the autosomal recessive disorder EvC. In contrast to EvC, in which a large number of case reports followed the first description, relatively few families affected by Weyers have been described.


FIG. 3. Radiographs of the patient. A: The length of ribs, chest, and morphology of cardiac silhouette were not abnormal. The clavicular morphological abnormality is marked by an arrow. B: Distal limb shortening and genu valgum (especially of the left knee joint).

TABLE I. Clinical Features of Ellis–van Creveld Syndrome (EvC), Weyers Acrofacial Dysostosis and the Patient Reported in This Study

Feature	EvC	Weyers	Patient
Postaxial polydactyly	+++	++	+
Cardiac anomalies	++	−	−
Narrow chest	++	−	−
Short stature	+++	+	+
Distal limb shortening	++	−	+
Dysplastic nails	+++	+	+
Excess frenula	++	+	−
Hypodontia	++	+	+
Tooth abnormalities	++	++	+
Syndactyly	++	+	+
Hair changes	+	−	−
Genu valgum	+	−	+
Abnormalities of other organs	+	−	abnormal clavicles

In the "EvC" and "Weyers" columns, the symbol +++ indicates a nearly invariant finding, ++ indicates a frequent finding, + indicates an occasional finding, and − indicates a finding that is not considered part of that syndrome. In the columns describing the patient, + indicates a feature is present and − indicates a feature is absent.
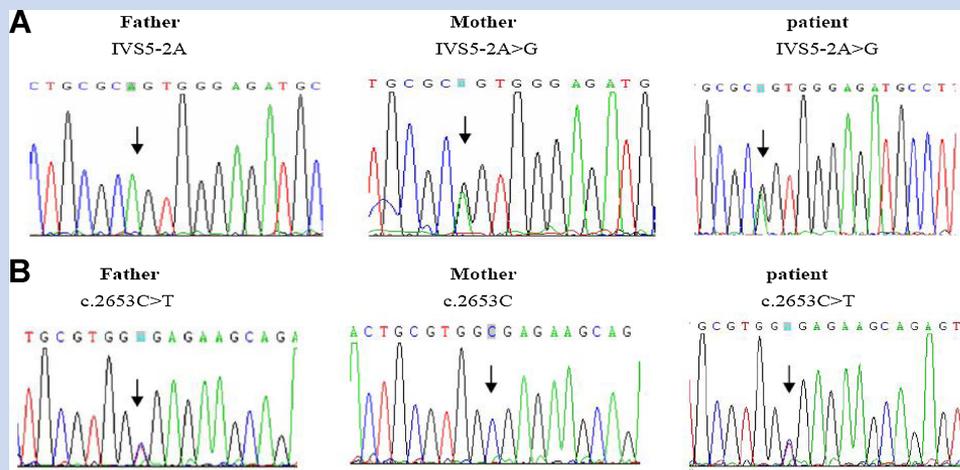
FIG. 4. The sequence of two novel heterozygous mutations. A: The patient and her mother had a novel heterozygous A to G mutation in the 3′ end of intron 5 (IVS5-2A > G) in *EVC2*. This sequence was normal in her father. B: Sequence analysis of exon 15 in *EVC2* showed a heterozygous mutation, c.2653C > T (Arg884X), in both the patient and her father. Her mother was normal.

Three Weyers-linked mutations have been identified and are only associated with the last exon of *EVC2* [Ye et al., 2006; Valencia et al., 2009]. This exclusive association with exon 22 of *EVC2* suggests specific residues encoded by this exon are a key part of the protein, and the Weyer variants may be related to a negative effect on Hedgehog signaling. Protein stability could also play a role in determining the pattern of inheritance of *EVC2* exon 22 mutations in vivo. As the final exon mutations escape nonsense-mediated mRNA decay (NMD), the more stable EVC2 Weyer proteins could cause the dominant phenotype [Valencia et al., 2009].

The patient we describe in this report has a mild EvC phenotype due to double heterozygous mutations in *EVC2* (IVS5-2A > G and c.2653C > T). Neither mutation was in exon 22. We confirmed that the compound heterozygous mutations of the patient were inherited from her non-affected parents respectively, consistent with recessive inheritance. As shown in Table I, the clinical features of the patient (postaxial polydactyly, nail dysplasia, tooth abnormalities, and mild short stature with no congenital heart defects or narrow thorax) demonstrate a mild EvC phenotype.

Mutations in *EVC* or *EVC2* are associated with EvC syndrome. The majority of mutations have been nonsense mutations or frameshift mutations that introduce a nonsense codon and, for these, it is likely that transcripts undergo nonsense-mediated decay [Tompson et al., 2007; Ruiz-Perez and Goodship, 2009]. We identified the IVS5-2A > G mutation as a conserved mutational splice site. Other mutations of the splice site have been detected, for example, IVS1 + 1G > A, IVS3 + 5_6GA > AC, and IVS5-1G > C in *EVC*, and IVS4 + 2T > C in *EVC2*. Tompson et al. [2007] confirmed that the previously reported mutation in *EVC* (IVS13 + 5G > T) produced three alternative splicings in affected individuals.

In our study, the proposita with EvC had an A to G heterozygous mutation in the 3′ end of intron 5 (IVS5) and the -2 position of exon 6, which is within the conserved AG splice acceptor site at the intron–exon boundary in the wild-type. We hypothesize that this may produce four alternative splicings (Fig. 5). Exon skipping is the most common alternative splicing and accounts for conserved alternative splicing events. Intron retention is the rarest alternative splicing event in human and mouse genomes [Sugnet et al., 2004]. In our patient, exon 6 skipping [Tompson et al., 2007; Valencia et al., 2009] would cause a missense mutation and produce a truncated protein of 302 amino acids. The predicted splice site may be at a position upstream [Kleppa et al., 2007] or downstream of the splice site [Tompson et al., 2007]. The score for acceptor site predictions was 0.63 and 0.77, respectively (www.fruitfly.org/seq_tools/splice.html) [Reese et al., 1997; Valencia et al., 2009], but
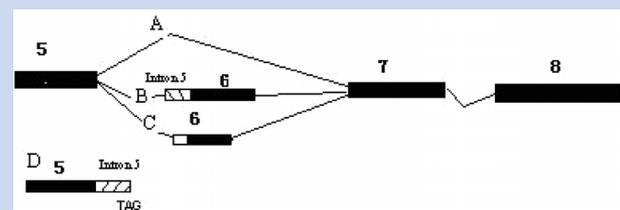
FIG. 5. The novel heterozygous mutation (IVS5-2A > G) that was located in the acceptor splice site may produce four alternative splicings. A: Exon 6 skipping alternative splicing will cause a missense mutation and produce a truncated protein containing 302 amino acids. B: Predicted splice site may be located upstream of the mutational site. C: The predicted splice site may be located downstream of the mutational site. D: Intron 5 of *EVC2* may not be spliced. The first transcription termination position (TGA) in intron 5 was only 21 nucleotides from the 3′ end of exon 5, which would produce a protein of 241 amino acids. (Black boxes represent normal exons; boxes with slashes represent part of intron 5; the white box represents the deleted fragment of exon 6.)

these low scores may mean the two types do not exist in this instance. The last alternative splicing could lead to intron retention, producing a truncated protein containing 241 amino acids. Given these possibilities, exon 6 skipping appears to be the most likely form of alternative splicing.

A considerable number of EvC cases have already been screened for mutations, including the systematic screening of 65 EvC cases in which all coding exons of both genes were sequenced [Tompson et al., 2007]. With the exception of two families reported by Temtamy et al. [2008], all EvC patients screened so far have been shown to have mutations in either *EVC* or *EVC2*, when mutations could be found, and so the patient in our study, with two mutations in *EVC2*, falls within this pattern. The exception reported by Temtamy et al. had a contiguous gene deletion involving both genes. Temtamy described a 520-kb homozygous deletion that comprised *EVC*, *EVC2*, *C4orf6*, and *STK32B*, and patients with homozygous deletions were deficient in *EVC* and *EVC2* with no increase in the severity of the typical features of EvC. The observation that none of the carriers of this deletion had phenotypic features of EvC, formally excluded *EVC/EVC2* digenic inheritance as a mechanism for EvC [Temtamy et al., 2008]. Despite sequencing the coding exons of both genes, the study by Tompson et al. [2007] did not identify mutations in 20 of the 65 (31%) patients who met clinical criteria for the diagnosis of EvC. This suggests further genetic heterogeneity may underlie EvC.

The growth plate of *Evc*−/− mice shows delayed bone collar formation and premature chondrocyte differentiation. Indian hedgehog (Ihh) is expressed normally in the growth plates of *Evc*−/− mice, but expression of the Ihh downstream genes *Ptch1* and *Gli1* was markedly decreased [Ruiz-Perez et al., 2007]. Sund et al. [2009] carried out in situ hybridization and immunofluorescence and identified co-localization of Evc and Lbn mRNA and protein. Due to the locus heterogeneity of human mutations predicted to result in a loss of protein function, a bidirectional genomic organization, and overlapping expression patterns, it is postulated that these proteins function coordinately [Ruiz-Perez et al., 2007; Sund et al., 2009]. However, the precise role of these two proteins in hedgehog signal transduction remains to be elucidated.

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