Dual Diagnosis of Ellis-van Creveld Syndrome and Hearing Loss in a Consanguineous Family

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Keywords

COL11A2 · Dual molecular diagnosis · Ellis-van Creveld syndrome · EVC2 · Next-generation sequencing · Nonsyndromic hearing loss

Abstract

Multilocus analysis of rare or genetically heterogeneous diseases is a distinct advantage of next-generation sequencing (NGS) over conventional single-gene investigations. Recent studies have begun to uncover an under-recognized prevalence of dual molecular diagnoses in patients with a “blended” phenotype that is the result of 2 clinical diagnoses involving 2 separate genetic loci. This blended phenotype could be mistakenly interpreted as a novel clinical extension of a single-gene disorder. In this study, we ascertained a proband from a large consanguineous Iranian family who manifests postlingual, progressive, moderate hearing loss in addition to suspected Ellis-van Creveld syndrome phenotype. NGS with a customized skeletal dysplasia panel containing over 370 genes and subsequent bioinformatics analysis

disclosed 2 homozygous mutations in *EVC2* (c.2653C>T; p.Arg885*) and *COL11A2* (c.966dup; p.Thr323Hisfs*19), respectively. This study highlights a dual molecular diagnosis in a patient with a blending of 2 distinct phenotypes and illustrates the advantage and importance of this staple technology to facilitate rapid and comprehensive genetic dissection of a heterogeneous phenotype. The differentiation between phenotypic expansion of a genetic disorder and a blended phenotype that is due to more than one distinct genetic aberration is essential in order to reduce the diagnostic odyssey endured by patients.

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Ellis-van Creveld syndrome (OMIM 225500), or chondroectodermal dysplasia, is a rare autosomal recessive disorder caused by defects in the genes *EVC* (OMIM 604831) and *EVC2* (OMIM 607261), called EvC ciliary complex subunit 1 and 2, respectively. *EVC* and *EVC2* are oriented in a head-to-head configuration in chromosome 4p16 and encode single-pass type I transmembrane pro-

teins. Patients with mutations in these 2 genes are phenotypically indistinguishable with short stature, genu valgum, short ribs, polydactyly, dysplasia of the teeth and nails as well as normal hearing [Ruiz-Perez et al., 2003]. Congenital atrial septal defects are observed in 50–60% of the patients [Digilio et al., 1999]. Mutations in *EVC* and *EVC2* each account for approximately half of Ellis-van Creveld syndrome diagnoses, and it is assumed that any additional unidentified genes comprise a small proportion of the overall diagnostic rate [Ruiz-Perez and Goodship, 2009]. Ellis-van Creveld syndrome has a prevalence in the general population of approximately 7 per 1,000,000, with equal occurrence in males and females [Stoll et al., 1989]. Interestingly, Ellis-van Creveld syndrome is much more frequent in the Old Order Amish in Lancaster County, Pennsylvania, with a 1 in 200 incidence rate due to a founder mutation [Ide et al., 1996].

Mutations in *COL11A2* (OMIM 120290) in chromosome 6p21.32 are associated with a number of different conditions that include autosomal dominant (DFNA13, OMIM 601868) or recessive (DFNB53, OMIM 609706) nonsyndromic hearing loss as well as Stickler syndrome (OMIM 184840), otospondylmegaepiphyseal dysplasia (OMIM 215150), fibrochondrogenesis (OMIM 614524), and Weissenbacher-Zweymuller syndrome (OMIM 277610) that are characterized by significantly overlapping skeletal abnormalities, hearing loss, and distinctive facial features. Hereditary hearing loss is the most common sensorineural disorder with a prevalence of roughly 1–2 per 1,000 newborns [Morton and Nance, 2006]. *COL11A2* encodes collagen type XI that is one of 4 collagen proteins in the tectorial membrane. This acellular gelatinous sheet-like structure in the inner ear is important for frequency-specific mechanical transmission and sound wave propagation to the inner hair cells [Masaki et al., 2009].

Next-generation sequencing (NGS) panels provide informative molecular genetic data about genes associated with a disease of interest. Gene panels are particularly ad-

vantageous for permitting customized content, deeper sequencing coverage in GC-rich regions compared to whole-exome sequencing, and rapid clinical interpretation without risk of incidental or secondary findings [Saudi Mendeliome Group, 2015; Falardeau et al., 2016]. A customized skeletal dysplasia (SKD) gene panel containing several hundred genes can provide informative diagnostic results about other phenotypes on the basis that many of the genes underlying SKD are also allelic with other disorders that involve deficits in non-skeletal organ systems. Such a panel also holds the potential for a “dual diagnosis” in patients. This involves the identification of an underlying molecular genetic cause for 2 separate diseases. Patients with 2 distinct disease phenotypes typically appear with a blended phenotype which may present as a novel syndrome to a clinician [Yang et al., 2013]. Additionally, patients with a dual diagnosis may mistakenly be presumed as manifesting an expanded phenotype of an already characterized disorder [Posey et al., 2017].

In this study, NGS gene panel sequencing and analysis ensued in a proband with consanguineous parents of Iranian descent who presented with atypical suspected Ellis-van Creveld syndrome that included short stature, polydactyly, nail hypoplasia, and hearing loss. Molecular genetics analysis showed segregation of homozygous nonsense and frameshift mutations in the genes *EVC2* and *COL11A2*, respectively. This study provides an example of multilocus variations aggregating in a single proband and supports consideration of dual molecular diagnoses in a proband with atypical disease symptoms.

Clinical Report

The female proband (VI:4) was born after a normal gestation to healthy parents. She is one of 3 offspring from 2 first-cousin parents of Iranian descent (Fig. 1A). Her family has a history of both Ellis-van Creveld syndrome and nonsyndromic hearing loss.

Fig. 1. Pedigree with hand and foot appearance of the proband with dual *EVC2* and *COL11A2* molecular diagnoses. **A** A 6-generation pedigree of the proband (VI:4, arrow) shows individuals with Ellis-van Creveld syndrome and nonsyndromic hearing loss, who are represented by a top- and bottom-shaded symbol, respectively. Ellis-van Creveld syndrome was diagnosed in individuals II:1, III:1, V:7, and VI:5. Nonsyndromic hearing loss was reported in individuals III:2, V:8, and VI:1. The proband (VI:4), represented by a black circle, has both phenotypic features. **B** Sequence chromatograms showing the homozygous, heterozygous, and wild-

type images for both *COL11A2* and *EVC2* variant regions. A control individual has been included for visualization of the *COL11A2* wild-type allele. **C** The lower limbs of the proband showing asymmetry, short feet, pes planus, broad toes, bilateral metatarsal shortening (T2–T4), and micronychia. Right dorsum (**D**), left dorsum (**E**), and right plantar (**F**) images of the proband’s hands after surgical correction of the bilateral postaxial hexadactyly showing hypoplasia of the fingers (F2–F4), generalized brachytelephalangy, and generalized micronychia.

(For figure see next page.)

Fig. 2. Radiographs of the proband. **A** Plain film radiographs of the hand after surgical correction of postaxial hexadactyly showing an absence of metacarpals or phalanges fusion, as well as brachytelephalangy, and cone-shaped epiphyses of the middle and distal phalanges. Radiographic lower-limb findings including asymmetry, shortening of the tubular bones as well as classical wide and slant proximal tibia metaphyses (**B–D**). Left proximal short fibula was also present (**B, C**).

Her brother (VI:5) and maternal first cousin once removed (V:7) were diagnosed with Ellis-van Creveld syndrome and both died at the age of 3 years. In addition to this syndrome, her brother also presented with agenesis of the stomach. Individuals II:1 and III:1 were diagnosed with Ellis-van Creveld syndrome and individuals III:2 and VI:1 were diagnosed with nonsyndromic hearing loss. None of these individuals have a blended phenotype with overlapping features.

The proband was 23 years old when she was referred for genetic counseling (Fig. 1A). She showed a disproportionate short-limb dwarfism with distalward shortening of extremities. Her body measurement of 137 cm (Z-score of -4) was in the 50th percentile of the adapted growth tables for patients with Ellis-van Creveld syndrome [Verbeek et al., 2011]. She showed a congenital absence of mandibular central and lateral incisors, postaxial hexadactyly of fingers, hypoplasia of the F2–F4 digits, generalized brachytelephalangy and micronychia, lower limb asymmetry, generalized broad toes, bilateral metatarsal T2–T4 shortening, pes planus, and bilateral increased sole creases (Fig. 1C–F) [Biesecker et al., 2009]. No cardiac defects were identified. She experienced irregular menstrual cycles and secondary amenorrhea. Vision and intelligence were normal at examination.

Plain radiographs revealed postaxial hexadactyly without fusion of metacarpals or fingers (Fig. 2A), distalward shortening of the tubular bones with short broad middle phalanges and hypoplastic distal phalanges (Fig. 2B), cone-shaped epiphyses of the middle and distal phalanges, the classical wide and slanted proximal tibia metaphyses and the left proximal short fibula were also present (Fig. 2B–D). The shortening of the tubular bones and mild irregularities of the proximal femoral metaphyses were also evident [Spranger et al., 2002].

Bilateral sensorineural hearing loss was diagnosed postlingually and is described as progressive. Audiogram thresholds indicate a moderate hearing loss (data not shown).

Materials and Methods

Next-Generation Sequencing

Genomic DNA from individuals VI:4 (proband), VI:3 (unaffected sister), V:5 (mother), and V:6 (father) (Fig. 1A) was prepared from whole blood using a standard salt extraction method. The proband was analyzed using a customized SKD NGS panel, which consists of 373 genes that have been curated for SKD-related phenotypes (gene list is available upon request). Library preparation was performed using the SureSelect XT Custom kit (Agilent Technology, Santa Clara, CA, USA) and NEBNextUltra DNA Library Prep kit (New England BioLabs, Ipswich, MA, USA) according to manufacturer's protocols. 1 μ g of genomic DNA was fragmented and 150–500-bp size products were selected for subsequent adapter and barcode labeling. RNA-based baits were hybridized with labeled DNA, and target genomic regions were enriched using the Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA). Libraries were quality controlled on the Agilent 2200 Tape Station (Agilent Technology). 150-bp paired-end sequencing was performed on an Illumina MiSeq sequencer using MiSeq v2 chemistry (Illumina, San Diego, CA, USA).

Bioinformatics Analysis

Sequencing data was de-multiplexed and FASTQ files generated by CASAVA v1.8.2 (Illumina). Sequencing reads were assembled and aligned to the human reference sequence (GRCh37/hg19), and variants were called using an in-house modified GATK pipeline. The raw VCF file was imported to Cartagena Bench Lab NGS software (Agilent Technology) for variant filtering. Variants from the filter chain were analyzed using Alamut 2.7.2 software (Interactive Biosoftware, Rouen, France). Allele frequencies were acquired from ExAC and gnomAD [Lek et al., 2016] as well as the Greater Middle Eastern database [Scott et al., 2016]. The Deafness Variation Database was used to assess variants in *COL11A2* [Shearer et al., 2014].

Fig. 3. Flow chart of the next-generation sequence variant filter chain. Numbers in parentheses are variant counts after each step. Blue boxes represent filters, orange/green/red boxes represent different outcome categories after each filter is applied.

Sanger Sequencing

Bidirectional Sanger sequencing was performed to confirm positive findings in the proband and family members using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on a 3730xl DNA Analyzer (Applied Biosystems). *EVC2* (GenBank: NM_147127) exon 15 was amplified using standard PCR cycling conditions and a forward (5'-TTCTG-TAATCGGCCACTGACAGG-3') and reverse (5'-ATGGATAT-GAGGAATGAGCTTTGC-3'; 338 bp) primer pair. A 214-bp region of exon 8 in *COL11A2* (GenBank: NM_080680) (forward: 5'-ATAGGTGTAATCGTAGGGCCC-3' and reverse primer: 5'-AACCTTGGAATCCTTTGGGC-3') employed standard cycling conditions. Primers were designed using Primer3 [Untergasser et al., 2012]. Sanger sequencing data were analyzed by Sequencher 5.3 software (Gene Codes, Ann Arbor, MI, USA).

Results

Genetic Investigations

Conventional chromosome banding analysis of the proband revealed a normal 46,XX karyotype. NGS gene panel sequencing yielded high-quality data with an average of 479x coverage. A total of 2,276 variant calls were

made by the pipeline and 2,123 passed quality control. After excluding intergenic and deep intronic variants, 730 variants in the coding region or intron-exon boundaries were analyzed through a multi-tier filter chain (Fig. 3). Subsequently, 2 homozygous pathogenic variants remained: a cytosine insertion resulting in a frameshift and a premature stop codon (c.966dup; p.Thr323Hisfs*19) in exon 8 of the gene *COL11A2* as well as a nonsense (c.2653C>T; p.Arg885*) mutation in exon 15 of the gene *EVC2* that were both predicted to be disease causing by MutationTaster [Schwarz et al., 2010] (Table 1). The *COL11A2* c.966dup mutation is not archived in ClinVar [Landrum et al., 2016], HGMD [Stenson et al., 2017], or LOVD [Fokkema et al., 2011]; however, it has been recently identified in 2 Iranian families and is annotated in the Deafness Variation Database as a pathogenic autosomal recessive nonsyndromic hearing loss mutation (Table 2) [Shearer et al., 2014; Sloan-Heggen et al., 2015]. The *EVC2* c.2653C>T mutation is described as pathogenic in ClinVar and HGMD and is associated with chondroectodermal dysplasia and Ellis-van Creveld syndrome, respectively. No other deleterious homozygous or com-

Table 1. Mutation interpretation using in silico prediction and data base tools

Tool	<i>COL11A2</i> c.966dup, p.Thr323Hisfs*19	<i>EVC2</i> c.2653C>T, p.Arg885*	Database reference
<i>Mutation prediction and clinical interpretation databases</i>			
ClinVar	no entry	pathogenic, chondroectodermal dysplasia	Landrum et al., 2016
Deafness Variation Database	autosomal recessive hearing loss, pathogenic	not applicable	Shearer et al., 2014
HGMD	no entry	Ellis-van Creveld syndrome (accession: CM116608)	Stenson et al., 2017
LOVD	no entry	no entry	Fokkema et al., 2011
MutationTaster	disease causing (1)	disease causing (1)	Schwarz et al., 2010
<i>Population frequency databases</i>			
ExAC	allele count: 16 0 homozygotes allele frequency: 0.0001342	allele count: 1 0 homozygotes allele frequency: 8.26e-06	Lek et al., 2016
Greater Middle Eastern database	genotype count: 2 0 homozygotes allele frequency: 0.001089	no entry	Scott et al., 2016
gnomAD	allele count: 29 0 homozygotes allele frequency: 0.0001049	allele count: 2 0 homozygotes allele frequency: 0.000008124	Lek et al., 2016

Table 2. Mutations in *COL11A2* causing autosomal recessive nonsyndromic hearing loss

Ethnicity	c. position	p. position	Hearing loss onset	Hearing loss severity	Gly-X-Y triplet repeat motif disrupted	Reference
Tunisian	c.109G>T	p.A37S	prelingual	profound	no	Chakchouk et al., 2015
Iranian (Fars)	c.966dup	p.T323Hfs*19	prelingual	profound	no	Sloan-Heggen et al., 2015
Iranian (Turk)	c.966dup	p.T323Hfs*19	prelingual	profound	no	Sloan-Heggen et al., 2015
Iranian (Fars)	c.966dup	p.T323Hfs*19	postlingual	moderate	no	present study
Iranian	c.1861C>A	p.P621T	prelingual	profound	yes	Chen et al., 2005
Japanese	c.2002C>T	p.P668S	not described	not described	yes	Miyagawa et al., 2013
Turkish	c.2662C>T	p.P888S	prelingual	profound	yes	Chakchouk et al., 2015

pound heterozygous mutations were identified. Segregation analysis was performed in 4 family members and confirmed both homozygous mutations (Fig. 1B). Sanger sequencing showed that both parents (V:5 and V:6) were heterozygous carriers for both *EVC2* and *COL11A2* mutations, and the sister (VI:3) was a heterozygous carrier for the *COL11A2* mutation but wild type at the *EVC2* mutation position.

Discussion

Ellis-van Creveld syndrome is usually diagnosed by pathogenic biallelic mutations in either *EVC* or *EVC2*. The homozygous nonsense mutation (c.2653C>T; p.Arg885*) identified in *EVC2* provided a molecular diagnosis of the suspected Ellis-van Creveld syndrome, the

more prominent phenotype in the proband, but did not provide a molecular basis for hearing loss. The blending of 2 distinct phenotypes prompted an in-depth data analysis that led to a dual molecular diagnosis by identifying a homozygous *COL11A2* single nucleotide duplication that likely explained the 2 features present as isolated symptoms in various other family members (Fig. 1A).

Genotype-phenotype correlations have not been performed for *EVC2*; however, the c.2653C>T (p.Arg885*) mutation has been previously described in 3 patients (Table 3). Interestingly, the 2 patients with compound heterozygous mutations exhibited differing clinical severities. The initial publication identifying the c.2653C>T mutation described this in a compound heterozygous state with an IVS5-2A>G change in a female Chinese patient [Shen et al., 2011]. This patient was described with a mild syndrome that included postaxial polydac-

Table 3. Genotype-phenotype correlations for patients with *EVC2* c.2653C>T, p.Arg885* mutations

Patient descent	<i>EVC2</i> allele 1	<i>EVC2</i> allele 2	Phenotype	Cardiac deficits	Reference
Iranian	c.2653C>T, p.Arg885*	c.2653C>T, p.Arg885*	mild phenotype; disproportionate short-limb dwarfism, congenital absence of mandibular central and lateral incisors, postaxial hexadactyly of fingers, hypoplasia of the F2–F4 digits, generalized brachytelephalangy and micronychia, lower limb asymmetry, generalized broad toes, bilateral metatarsal T2–T4 shortening, pes planus and bilateral increased sole creases, postaxial hexadactyly	no cardiac phenotype	present study
Chinese	c.2653C>T, p.Arg885*	IVS5-2A>G	mild phenotype; hypodontia, bilateral postaxial polydactyly, bilateral syndactyly of toes 2 and 3, nail dysplasia, abnormalities of knee joints, mild short stature	no cardiac phenotype	Shen et al., 2011
Japanese	c.2653C>T, p.Arg885*	c.1814C>A, p.Ser605*	severe phenotype; severe respiratory failure due to thorax deformity, severe developmental delay, chronic intestinal malfunction, bilateral postaxial polydactyly, nail hypoplasia, oral frenula, tooth dysplasia, nail hypoplasia, severe growth failure at 18 months of age	partial atrial septal defect	Iwakura et al., 2016
Lebanese	c.2653C>T, p.Arg885*	c.2653C>T, p.Arg885*	mild phenotype; severe growth failure, shortening of long bones, slight elongation of the thorax with short ribs, early ossification of femoral heads, bilateral polydactyly with short fingers	atrial septal defect	Valencia et al., 2015

tyly, nail dysplasia, tooth abnormalities, and mild short stature. Cardiac defects and narrow thorax were absent. In contrast, a Japanese patient with compound heterozygous nonsense mutations (c.2653C>T; p.Arg885* and c.1814C>A; p.Ser605*) had a distinctly severe form of Ellis-van Creveld syndrome that included a partial atrio-ventricular septal defect, prominent developmental delay, and chronic intestinal malfunction requiring surgical resection [Iwakura et al., 2016]. A Lebanese patient with the same homozygous c.2653C>T (p.Arg885*) mutation as the proband we describe displayed a mild syndromic presentation [Valencia et al., 2015]. The phenotype in our patient is comparatively mild. It remains unknown whether the deaths in individuals V:7 and VI:5 can be directly attributed to the syndrome.

There are presently 67 mutations reported in *EVC2* that constitute 27 nonsense, 14 small deletions, 7 small insertions/duplications, 6 each for gross deletions, missense, and splicing mutations as well as 1 gross insertion/duplication (HGMD, version 2017.1). The majority of these mutations encode a loss of function, which is supported by an absence of homozygous loss-of-function

alleles in the ExAC, the Greater Middle Eastern, or gnomAD databases (Table 1).

Although nonsyndromic sensorineural hearing loss and a phenotypic spectrum of syndromes involving skeletal malformation and hearing loss that include Stickler and Weissenbacher-Zweymuller syndromes, otospondyl-megaepiphyseal dysplasia, and fibrochondrogenesis are caused by allelic mutations in *COL11A2* (Fig. 4A, B), the c.966dup mutation appears restricted to autosomal recessive nonsyndromic sensorineural hearing loss and was described in 2 Iranian families of Fars and Turk ethnicities with affected individuals indicating prelingual, profound autosomal recessive nonsyndromic hearing loss (Table 2) [Sloan-Heggen et al., 2015]. As the family we present is of Iranian descent, it is tempting to speculate a founder effect considering that hearing loss due to homozygosity of this allele has only been reported in Iranians to date. In the presented family, 3 individuals from the paternal lineage have isolated nonsyndromic hearing loss. However, we cannot exclude that the hearing loss in these family members is due to another locus since these individuals were unavailable for molecular genetic testing.

Fig. 4. Map of pathogenic and likely pathogenic mutations in *COL11A2*. The N- and C- terminal are from left to right, respectively. **A** *COL11A2* mutations in autosomal recessive (mutations written in red) and dominant (written in blue) forms of nonsyndromic hearing loss. The P668S mutation (black) has not been assigned as an autosomal dominant or recessive allele [Miyagawa et al., 2013]. The mutation described in the proband is in bold print and under-

lined. **B** *COL11A2* mutations causing otospondylomegaepiphyseal dysplasia (mutations written in black), Stickler syndrome (green), fibrochondrogenesis (orange), and Weissenbacher-Zweymuller syndrome (purple). Mutations were collected from HGMD (2017.1), ClinVar, and the Deafness Variation Database (version 8). OSMED, otospondylomegaepiphyseal dysplasia.

Six previously published families from Iran, Japan, Tunisia, and Turkey have been identified as segregating autosomal recessive *COL11A2* alleles causing nonsyndromic hearing loss [Chen et al., 2005; Miyagawa et al., 2013; Chakchouk et al., 2015; Sloan-Heggen et al., 2015]. Affected members in these families were described with congenital or prelingual, severe-to-profound sensorineural hearing loss (Table 2). Only one of the families showed short stature with no other orthopedic abnormalities present [Chakchouk et al., 2015]. We describe the first patient with moderate, progressive, postlingual hearing loss caused by a homozygous autosomal recessive *COL11A2* allele (Table 2). The limited number of patients described in the literature with autosomal recessive deafness manifest prelingual, profound hearing loss.

The Gly-X-Y triplet repeat motif, wherein X and Y are the ring amino acids proline and hydroxyproline, is required to stabilize the triple-helix repeat domain in

COL11A2 [Yoneda et al., 1999]. There are currently 5 mutations recognized as causing recessive nonsyndromic hearing loss (Table 2). Two of the 5 mutations (c.966dup; p.Thr323Hisfs*19 and c.109G>T; p.Ala37Ser) do not disrupt the Gly-X-Y motif, whereas 3 mutations (c.1861C>A; p.Pro621Thr, c.2002C>T; p.Pro668Ser, and c.2662C>T; p.Pro888Ser) reside in regions of the protein that alter the Gly-X-Y repeat, likely impacting triple-helix stability (Fig. 4A). Further reports are required for an in-depth genotype-phenotype correlation of protein domain disruption on hearing loss outcome, including the potential outcome of the N- and C-terminal, laminin G and fibrillar collagen domains, respectively. Interestingly, the c.966dup mutation is the only identified mutation with a premature protein truncation that is linked to nonsyndromic hearing loss to date, expanding the present understanding that insertions, deletions, nonsense, or Gly-X-Y triplet repeat motif altering mutations are linked to severe

multisystemic phenotypes, such as otospondylmega-epiphyseal dysplasia [Haller et al., 2016], and missense mutations are responsible for nonsyndromic deafness [Tokgöz-Yılmaz et al., 2011].

NGS panels with extensive gene content provide an opportunity to apply a genotype-first approach to identify a clinically atypical patient who may otherwise be regarded as manifesting a phenotypic expansion of a mendelian disorder. It is important to emphasize that the field is still witness to rapid gene identification, making the efficiency of molecular diagnosis of multiple disorders imperfect, especially within the context of gene panels that are limited to disease-specific gene content. However, several studies have provided promising insight with current knowledge and have shown that multilocus genomic variation occurs in approximately 4–5% of the patients referred for genetic testing using whole-exome sequencing [Balci et al., 2017; Posey et al., 2017]. The largest retrospective study to date included 7,374 diagnostic patients, including 2,076 (28.1%) patients achieving a diagnosis. Among those with a diagnosis, 97 patients had a molecular diagnosis involving 2 loci, 3 patients had a molecular diagnosis involving 3 loci, and 1 patient encountered 4 molecular diagnoses, accounting for a combined 101 patients with a diagnosis involving 2 or more loci (4.9%). Focusing on the nonsyndromic hearing loss genetic results from this study, 4 of the 97 patients (4.1%) receiving a dual diagnosis had variants in genes responsible for nonsyndromic hearing loss in combination with an additional gene harboring pathogenic variants. This included one patient with pathogenic mutations in both *ACTG1* and *WFS1* that underlie autosomal dominant and recessive forms of hearing loss, respectively [Posey et al., 2017].

Several studies have concluded that parental consanguinity and multisystem disease are 2 essential factors increasing the likelihood of a dual molecular diagnosis [Balci et al., 2017]. In parts of the world where consanguinity is prevalent, it is not uncommon to observe an increase in autosomal recessive disorders in offspring due to homozygous inheritance of parental carrier mutations that are identical by descent. With respect to nonsyndromic hearing loss, an extremely heterogeneous disorder, a previous study has estimated that nonconsanguineous normal hearing individuals carry an average of approximately 1.4 deleterious variants, with some individuals carrying as many as 3 deleterious variants [Vona et al., 2014]. Approximately 70% of nonsyndromic hearing loss is transmitted as an autosomal recessive disorder, making the inheritance of recessively inherited hearing loss in a proband from a consanguineous union not unforeseen.

An accurate molecular diagnosis is critical for genetic counseling in this family. A mixture of different phenotypes and coexistence of multiple disease-causing genotypes in the proband makes counseling even more challenging. Since clinical symptoms were segregating in different pedigree branches and complex consanguinity was observed, counseling for each of the at-risk family members has to consider both disease conditions based on their mutation status, their relationship to the proband, and their own consanguinity level. In addition, carrier status of additional autosomal recessive diseases has been identified in the proband through NGS (data not shown). These may not explain the disease etiology of the proband; however, they may bring an extra layer of complexity to genetic counseling because all intrafamily members will have an increased risk of having offspring with these recessive conditions due to consanguinity. At this point, comprehensive genetic counseling and additional genetic testing may be necessary for all family members for future family planning.

In light of the uncovered findings, there are 2 possible scenarios that merit discussion. Firstly, if investigators had solely noted the *COL11A2* variant, with its association with hearing loss and overlapping skeletal malformation, this may have prematurely resulted in a diagnosis if further analysis had not ensued that uncovered the *EVC2* nonsense mutations. An alternative sequence of events could have involved the discovery of the *EVC2* nonsense mutation without further analysis, which may lead investigators to believe that hearing loss is a novel feature of Ellis-van Creveld syndrome. Both of these outcomes would have led to an incomplete result.

In summary, patients with multiple diagnoses are under-recognized in molecular diagnostic settings. NGS analysis may reveal more than one distinct mendelian disease leading to a dual diagnosis. An initial molecular diagnosis may be incomplete when an apparently new clinical phenotype or a phenotypic expansion of a single disease is assumed. Our data challenge the notion that a diagnostic investigation is not necessarily complete after a single genetic mutation has been identified.

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Statement of Ethics

This study was performed under the tenets of the Declaration of Helsinki and was approved by the Ethics Commission of the University of Würzburg. Informed written consent was obtained from participating subjects.

Disclosure Statement

All authors declare no conflict of interests.

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