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CASE REPORT

Concurrent de novo ZFHX4 variant and 16q24.1 deletion in a patient with orofacial clefting; a potential role of ZFHX4 and USP10

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Abstract

A girl with a unilateral cleft lip, alveolus and palate, tooth agenesis, and mild dysmorphic features, without a specific underlying syndrome diagnosis, was genotypically characterized and phenotypically described. Cleft gene panel analysis, single-nucleotide polymorphism (SNP) array, whole genome sequencing (WGS), whole exome sequencing, and quantitative PCR (Q-PCR) analysis were used as diagnostic tests. SNP array revealed a maternal deletion at 16q24.1, encompassing the cleft candidate gene *USP10*. WES revealed an additional de novo Loss-of-Function variant (p.(Asn838fs)) in the Zinc-Finger-Homeobox-4 (*ZFHX4*) gene. Q-PCR was performed to explore the effect of the ZFHX4 variant and the deletion in 16q24.1. The mRNA expression of a selection of putative target genes involved in orofacial clefting showed a lowered expression of *USP10* (52%), *CRISPLD2* (31%), and *CRISPLD1* (1%) compared to the control. *IRF6* showed no difference in gene expression. This case supports *ZFHX4* as a novel cleft gene and suggests *USP10* may contribute to the etiology of orofacial clefts in humans.

KEYWORDS
cleft palate, CRISPLD1, CRISPLD2, IRF6, USP10, ZFHX4

1 | INTRODUCTION

Cleft lip, alveolus, and/or palate (CL(A)/P) are among the most common heterogeneous craniofacial birth defects. The incidence of CL(A)/P varies throughout the world. In Europe one to two infants per 1000 births is affected (Leslie & Marazita, 2013; Mossey & Modell, 2012).

In approximately 70% of the newborn with CL(A)/P, the orofacial cleft occurs as an isolated anomaly. In the remaining cases, the cleft can be classified as syndromic, associated with additional symptoms and/or developmental delay, caused by a Mendelian pathogenic gene variant (e.g., *IRF6*; Van der Woude syndrome (OMIM # 119300)), chromosomal defect (e.g., 22q11.2 deletion) and/or embryopathy due to teratogens. The etiology of isolated nonsyndromic CL(A)/P is complex involving an interplay of genetic and environmental factors (Beaty et al., 2016; Bishop et al., 2020; Cox et al., 2018).

There is increasing evidence that rare and common variants are involved in both Mendelian and complex forms of CL(A)/P (Leslie &

Marazita, 2013). Genetic association studies and mouse models suggest an interaction network in which *IRF6* plays a central role in the etiology of nonsyndromic clefting (Kousa & Schutte, 2016; Velázquez-Aragón et al., 2016). Moreover, de novo protein-altering variants in syndromic cleft genes (e.g., *IRF6*, *GRHL3*, *TFAP2A*, *CDH1*, *CTNND1*, and *COL2A1*) were identified in 6% of isolated nonsyndromic CL(A)/P cases in a large cohort ($n = 756$) (Bishop et al., 2020; Leslie & Marazita, 2013).

However, in most individuals with isolated CL(A)/P, the exact genetic factors remain unknown, making it difficult to understand the underlying molecular mechanisms (Dixon et al., 2011; Gundlach & Maus, 2006; Leslie & Marazita, 2013; Mossey et al., 2009). Advanced DNA techniques, association studies, and mouse models are powerful tools to reveal new gene candidates responsible for CL(A)/P (Lustosa-Mendes et al., 2021). Recently, WGS revealed *ZFHX4* as a novel orofacial cleft candidate gene (Bishop et al., 2020). Identifying genetic variants at novel loci can help understand the complex interaction of genes involved in facial development and the molecular mechanisms determining normal and abnormal development of the lip, alveolus, and palate.

In this report, a girl with unilateral cleft lip, alveolus, and palate (CLAP) and some dysmorphic features is presented with a combination of genomic aberrations, consisting of a de novo frameshift variant in *ZFHX4*, a maternal deletion at 16q24.1, and a paternal duplication at 22q12.3. The possible role of these copy number variants and the involved genes in the etiology of OFC will be discussed.

1.1 | Case description

The patient, an 11-year-old girl, was referred to our clinic in 2017 and was seen by an interdisciplinary orofacial anomaly specialist team at the Wilhelmina Children's Hospital of the University Medical Centre in Utrecht, The Netherlands.

She was born after an uneventful pregnancy with a birthweight of 3140 g. At birth, an oral cleft on the left side was noted. No additional congenital anomalies were identified then. Her growth and motor development were uneventful. There were no signs of hypo-/anhidrosis, suggestive of ectodermal dysplasia. She had no associated ophthalmologic abnormalities nor severe hearing loss. After visiting specialized primary education, because of mild learning disabilities, she attends regular secondary education.

On physical examination at our clinic, a unilateral cleft lip and palate on the left side was noted. In addition, she had mild dysmorphic features, without a suspected underlying syndrome diagnosis.

She had a round face, thin eyebrows, malar flattening, micro/retrognathia and low-set, posterior rotated and protruding ears with a forward-facing earlobe. No lip pits were present. Her hands were slender with clinodactyly of digit 5, and mild syndactyly between digits 4 and 3. The hypothenar eminence was small. Her feet show short and broad halluces and a clinodactyly of digit 4. Her skin, hair, and nails show no specific abnormalities. No other cleft syndrome-related features or signs were noted.

The panoramic radiograph shows that tooth numbers 2.2, 2.5, 3.5, and 4.5 were missing congenitally. Tooth numbers 3.6 and 4.6 were extracted due to caries. There is a transposition of tooth numbers 1.3 and 1.4. The lateral cephalogram shows a vertical growth pattern and retrognathic mandible. All clinical features of the patient are presented in Figure 1a,b.

1.2 | Family history

The family history is negative for orofacial clefts or specific cleft syndrome-related symptoms. Similar to the proband, the mother shows micrognathia (Figure 1c). Additional physical examination revealed a high palate without signs of a submucous cleft palate. The father has no orofacial abnormalities.

2 | MATERIAL AND METHODS

Genomic DNA from the patient's and parents' blood were extracted using standard methods. Diagnostic single nucleotide polymorphism (SNP) array analysis and diagnostic next-generation sequencing were performed.

2.1 | Cleft gene panel analysis

Whole exome sequencing (WES)-based gene panel analysis, targeting 191 cleft-related genes, was performed (OWS02v17.2; more details in Data S1, Supporting Information). This method detects more than 95% of the variants in the CLP gene panel. Details on the methods and reliability of the analysis can be found at: https://www.umcutrecht.nl/nl/next-generation-sequencing-ngs?lang=en

2.2 | SNP array analysis and whole genome sequencing

To better understand the etiology of the CLAP phenotype of the patient an SNP array was performed on the patient and her parents. A SNP array analysis was performed with an Affymetrix Cytoscan HD array platform. The whole genome was analyzed with an average resolution of 20 Kb (Humane Genome Build hg19 UCSC genome browser February 2009).

2.3 | Trio whole-exome sequencing

After targeted gene panel analysis, a routine diagnostic trio-based Whole-exome analysis was performed for the patient and her parents. Sequencing was performed as described above.

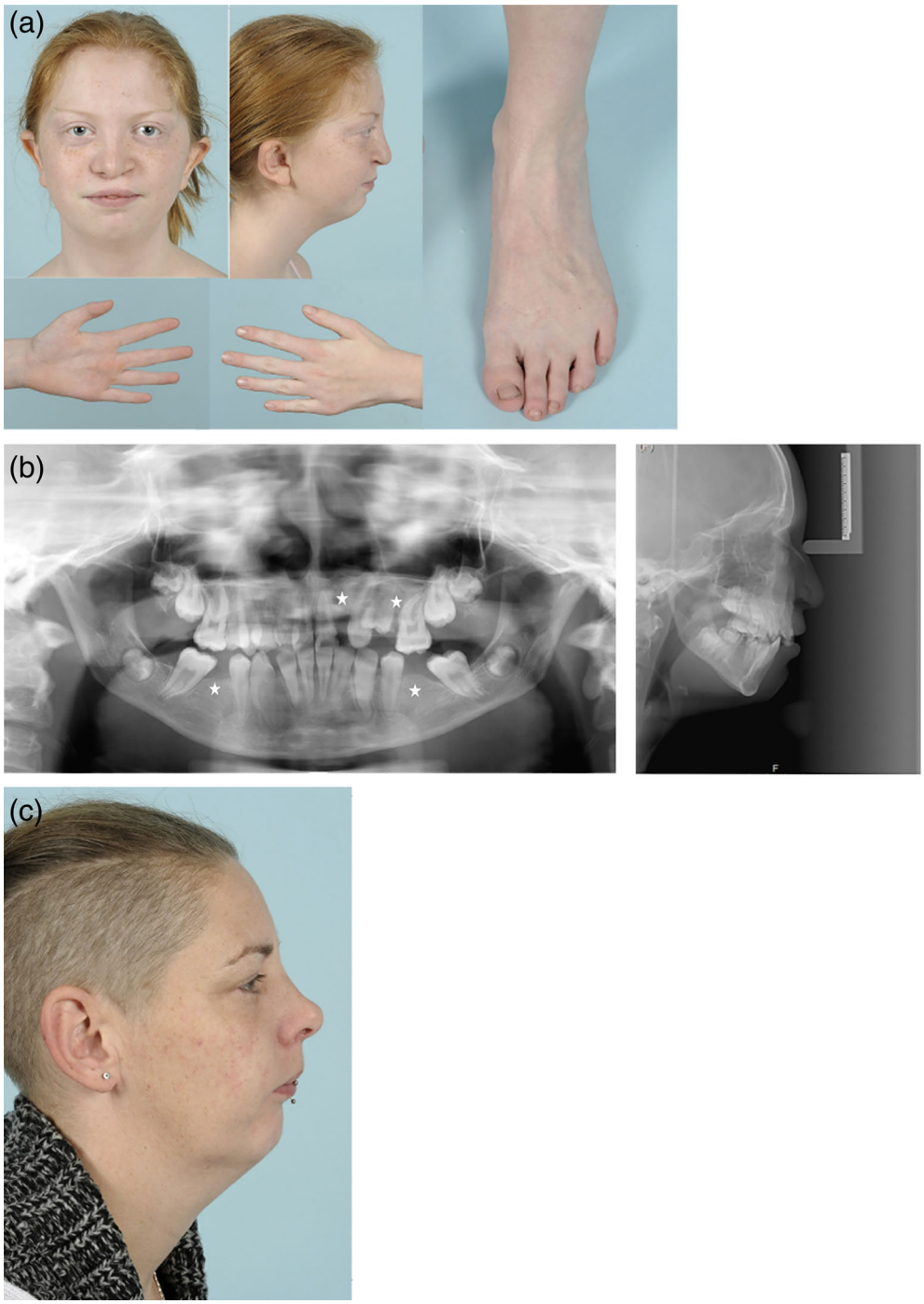

FIGURE 1 (a) Clinical features of the patient are as described above. (b) The panoramic radiograph shows the congenitally missing teeth and a transposition of tooth numbers 1.3 and 1.4. The lateral cephalogram shows a retrognathic mandible. (c) The mother of the patient shows the same micrognathic mandible

2.4 | Quantitative PCR

Fibroblasts of the patient's palate were analyzed with a polymerase chain reaction (PCR) test and compared with palatal fibroblasts of an age-matched healthy control. RNA was extracted, and a reverse transcriptase reaction was performed, followed by quantitative PCR (Q-PCR) for the genes Ubiquitin Specific Peptidase 10 (USP10), Cysteine Rich Secretory Protein LCCL Domain Containing 2 (CRISPLD2), Interferon Regulatory Factor 6 (IRF6), and Cysteine Rich Secretory Protein LCCL Domain Containing 1 (CRISPLD1). The 2^{-ddCt} values were calculated for all mentioned genes.

3 | RESULTS

3.1 | Cleft gene panel analysis

The patient showed no pathogenic variants in the WES-based gene panel analysis of 191 cleft-related genes (Data S1).

3.2 | SNP array analysis and whole genome sequencing

The SNP array revealed a maternal ~85 kb deletion in 16q24.1 (92 array probes), encompassing one gene (USP10) and a paternal

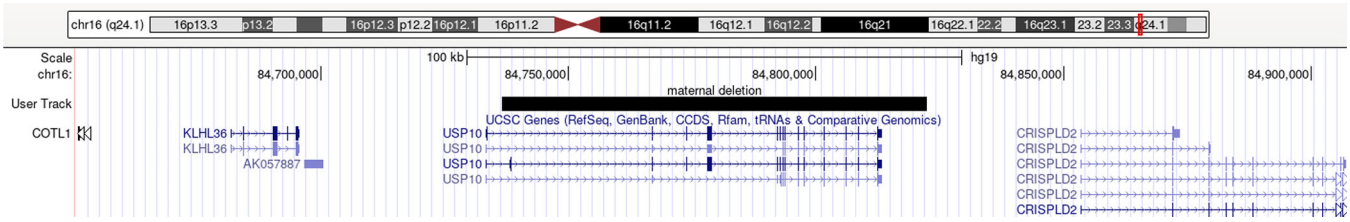

FIGURE 2 The region containing the maternal ~85 kb deletion in 16q24.1 identified by the SNP array depicted in a screen shot of the UCSC Genome Browser Build 37/hg19 representing gene structures. The black bar represents the maternal deletion in 16q24.1

duplication (~90 kb) in 22q12.3 (249 array probes, including 1 partial gene deletion (*LARGE1*)) (Figure 2). The genomic coordinates according to GRCh37/hg19 are: 16q24.1 (84,737,619-84,822,855)x1 mat, 22q12.3(33,645,415-33,737,634)x3 pat.

The paternal duplication 22q12.3 is not considered causal for the orofacial cleft. The gene (*LARGE1*) is associated with muscular dystrophy-dystroglycanopathy (congenital with brain and eye anomalies), type A, 6 (ADDGA10; OMIM 613154) and muscular dystrophy-dystroglycanopathy (congenital with mental retardation), type B, 6 (MDDGB6; OMIM 608840). The girl shows no features of these autosomal recessive disorders.

Based on whole genome sequencing, the maternal deletion and paternal duplication coordinates were identified as chr1 6:84736595-84822374 (hg19) and chr22:33644240-33737416 (h19).

3.3 | Trio whole exome sequencing

Since the patient and mother harbor the same deletion in 16q24.1 but do not both demonstrate orofacial clefting, we postulated that additional de novo genetic and/or environmental factors should be responsible for the patient's phenotype. Therefore, we performed WES on the patient and her parents. WES revealed a heterozygous de novo pathogenic variant in the Zinc Finger Homeobox 4 gene (*ZFHX4*; OMIM 606940); (*ZFHX4* (NM_024721.4):c.[2513del];[=] p.[(Asn838fs)];[(=)] (Chr8(GRCh37):g.[77618836del];[=]) (3VUS)). This variant can lead to premature termination in exon 2 of 11 in the *ZFHX4* gene. This de novo frameshift variant is expected to cause loss-of-function (LOF). This variant is not present in the healthy population (gnomAD) and was not described previously in ClinVar. Moreover, this gene is strongly conserved against LOF variants (pLI = 1; gnomAD).

No rare or possible pathogenic variants were identified in the remaining allele of *USP10* and *CRISPLD2*; (CRISPLD2 coverage >15x 100% c.[=];[=] p.[(=)];[(=)] Normal; USP10 coverage >15x 98,95588% c.[=];[=] p.[(=)];[(=)] Normal)

3.4 | Quantitative PCR

To explore how the ZFHX4 variant and the deletion in 16q24.1 could lead to disturbed changes in gene expression and resulting in the patient's phenotype, we probed the mRNA expression of a selection of putative target genes involved in orofacial clefting.

Q-PCR analysis of the *USP10* and CRISPLD2 gene expression was performed, these two genes are located in or close to the 16q24.1 deletion, respectively (Ge et al., 2018; Girardi et al., 2011; Messetti et al., 2017; Neela et al., 2020; Shen et al., 2011). *USP10* is located proximal to *CRISPLD2*, *and* contains a *CRISPLD2* regulatory element (Fishilevich et al., 2017; Stelzer et al., 2016). The analysis showed lower expression of the 2^{-ddCt} values in *USP10* (52%) and *CRISPLD2* (31%) in the patient when compared to the control.

In addition, expression levels of candidate genes were determined using Q-PCR in order to formulate novel hypotheses by detecting possible down-stream effects of the mutated *ZFHX4* and the maternal deletion on genes involved in clefting.

CRISPLD1 is the main paralog of CRISPLD2 (70% at nucleotide level and 58% at protein level) and located 2.4Mb proximally to *ZFHX4*. Previous studies suggest involvement of CRISPLD1 in the etiology non-syndromic (CL(A)/P) through the interaction with CRISPLD2 and folate pathway genes (Chiquet et al., 2011). The expression 2^{-ddCt} values outcome of *CRISPLD1* was 138 times lower than the control (0,01%).

IRF6 Q-PCR tests were performed because its importance in the etiology of CL(A)/P (Kousa & Schutte, 2016; Li et al., 2013) and the proposed interaction with the folate pathway CRISPLD2 and CRISPLD1 (Chiquet et al., 2011; Velázquez-Aragón et al., 2016). *IRF6* showed no difference in gene expression.

4 | DISCUSSION

This report describes a girl with a unilateral cleft lip, alveolus and palate, tooth agenesis, and mild dysmorphic features. Genetic testing revealed a 16q24.1 maternal deletion, encompassing the gene *USP10*, and a concurrent de novo pathogenic variant in the gene *ZFHX4*. This case supports *ZFHX4* as a novel cleft gene and suggests *USP10* may contribute to the etiology of orofacial clefts in humans.

Bishop et al. recently identified an increased number of de novo LOF variants in *TFAP2A* and *ZFHX4* in a large cleft population, supporting *ZFHX4* as a novel CL(A)/P risk gene (Bishop et al., 2020). Fontana and colleagues published a patient with a heterozygous variant in *ZFHX4* with greatly overlapping clinical features with our patient, like clinodactyly of digit 5, low-set and prominent ears, thin eyebrows, high-arched palate and microretrognathia, however without CL(A)/P (Fontana et al., 2021). Previously, eight patients with an 8q21.11 deletion were reported, with *ZFHX4* in the smallest region of overlap, of

which one case showed a cleft palate (Palomares et al., 2011). The absence of an orofacial cleft in the other cases might represent a reduced penetrance for CL(A)/P and for *ZFHX4*.

We hypothesized that in our case the maternal deletion, including deletion of the gene *USP10*, plays a contributing role. Particularly given the mother, with the same *USP10* deletion but without the *ZFHX4* LOF frameshift variant, showed some dysmorphic facial features (micrognathia and high palate) without resemblance to the cases with a *ZFHX4* deletion. The gene expression studies we performed in palatal fibroblasts of the presented case and an age-matched control showed a reduced mRNA expression of the gene *USP10* supporting haploinsufficiency of *USP10*. In addition, a reduced expression of the genes *CRISPLD2* and its paralogue *CRISPLD1* was noted (Kent et al., 2002; Wan et al., 2018). Although to our knowledge the gene *USP10* has not yet been reported as a candidate gene for oral clefting, several studies support the involvement of USP10 in orofacial development. First, recent studies demonstrated that USP10, like the major cleft gene *IRF6*, regulates epithelial-to-mesenchymal transition (EMT) factor Slug/SNAI2 (Ouchida et al., 2018), significant in the formation of the facial prominences and palate during embryonic development (Ke et al., 2015; Tak et al., 2018; Zeuner et al., 2018). Second, *USP10* contains a regulatory enhancer element for *CRISPLD2* (Fishilevich et al., 2017; Stelzer et al., 2016). Crispld2 knockdown zebrafish showed altered neural crest cell migration patterns resulting in abnormal jaw and palate-like development (Chiquet et al., 2011; Swindell et al., 2015). Moreover, recent studies reported an association between *CRISPLD2* and nonsyndromic CL(A)/P in a Chinese and Brazilian population (Ge et al., 2018; Girardi et al., 2011; Messetti et al., 2017; Neela et al., 2020; Shen et al., 2011).

The reduced mRNA expression of *CRISPLD2* in the fibroblasts of our case will not be a direct result of the identified 16q24.1 deletion, since this deletion did not comprise the gene *CRISPLD2*. One might postulate that deletion of the *CRISPLD2* regulatory element in *USP10* reduces the expression of *CRISPLD2*, located distally of USP10. In our case, the strongly reduced CRISPLD1 mRNA expression cannot yet be explained. To the best of our knowledge, no regulatory enhancer element for *CRISPLD1* was identified in *ZFHX4* or *USP10*, and no specific topologically associating domain (TAD) is disrupted.

The presented case is unique, and no additional cases with the same combination of genetic defects have yet been reported. However, we realize this study has limitations. The mRNA studies performed in age-matched palatal fibroblasts may not reflect the effect of the deletion during embryonic development. Nevertheless, the specific combination of genetic defects in this patient might initiate further studies and contribute to novel insights into the role of *ZFHX4* and *USP10*, *CRISPLD1* and *CRISPLD2* in the etiology of orofacial clefting.

In summary, this case report describes a female CLAP patient with a de novo *ZFHX4* LOF frameshift variant and a *USP10* deletion. This de novo frameshift variant in ZFHX4 supports *ZFHX4* as a cleft candidate gene with a reduced penetrance. In addition, the performed mRNA studies in the presented case may indicate that both genes *USP10* and *CRISPLD2* contribute to the etiology of clefting in humans, and a sole *USP10* deletion may influence the expression of the adjacent gene *CRISPLD2*. Identifying more patients with similar genetic and phenotypic defects is necessary to support our hypotheses and additional studies are required to reveal the possible underlying pathogenic mechanisms, involving *ZFHX4*, *USP10*, and *CRISPLD2*.

AUTHOR CONTRIBUTIONS

Marijn Créton, Frank Wagener, and Marie José van den Boogaard designed the study, coordinated the project, and contributed in preparing the manuscript. Aebele Mink van der Molen contributed in the study design and collected essential clinical material data. Maarten Massink and Gijs van Haaften performed and analyzed the data from the WES, SNP array, and WGS. Marijn Créton, Frank Wagener, Marjon Bloemen, and Marie José van den Boogaard performed the Q-PCR tests and analyzed the data. Willem Fennis, Jan Schols, and Miranda Aarts contributed in preparing and designing the manuscript. All authors have contributed in re-drafting and revising the manuscript and have seen and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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