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Novel *IRF6* Mutations Detected in Orofacial Cleft Patients by Targeted Massively Parallel Sequencing

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Abstract

Common variants in interferon regulatory factor 6 (*IRF6*) have been associated with nonsyndromic cleft lip with or without cleft palate (NSCL/P) as well as with tooth agenesis (TA). These variants contribute a small risk towards the 2 congenital conditions and explain only a small percentage of heritability. On the other hand, many *IRF6* mutations are known to be a monogenic cause of disease for syndromic orofacial clefting (OFC). We hypothesize that *IRF6* mutations in some rare instances could also cause nonsyndromic OFC. To find novel rare variants in *IRF6* responsible for nonsyndromic OFC and TA, we performed targeted multiplex sequencing using molecular inversion probes (MIPs) in 1,072 OFC patients, 67 TA patients, and 706 controls. We identified 3 potentially pathogenic de novo mutations in OFC patients. In addition, 3 rare missense variants were identified, for which pathogenicity could not unequivocally be shown, as all variants were either inherited from an unaffected parent or the parental DNA was not available. Retrospective investigation of the patients with these variants revealed the presence of lip pits in one of the patients with a de novo mutation suggesting a Van der Woude syndrome (VWS) phenotype, whereas, in other patients, no lip pits were identified.

Keywords: cleft lip, cleft palate, genetics, hypodontia, risk factor(s), high-throughput nucleotide sequencing

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A supplemental appendix to this article is available online.

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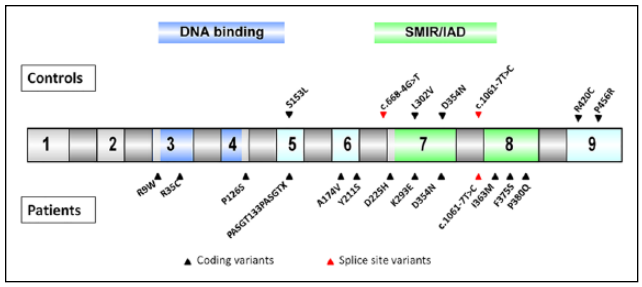

Figure 1. The structure of the *IRF6* gene. Boxes with numbers 1 to 9 represent the 9 exons of *IRF6*. Exons 1 and 2 are not translated. The predicted IRF6 protein has a helix-turn-helix DNA-binding domain (blue) and a Smad-interferon regulatory factor binding domain (SMIR/IAD) protein-binding domain (green). The arrowheads represent the relative positions of the variants in the controls (above exons) and the patients (below exons). This figure is available in color online.

Introduction

Interferon regulatory factor 6 (IRF6) belongs to a family of transcription factors that share a highly conserved N-terminal DNA-binding domain (DBD) and a less-conserved C-terminal protein-binding Smad-interferon regulatory factor binding domain (SMIR/IAD) (Fig. 1). The DBD of IRF6 facilitates its binding to DNA and its function as a transcription factor. The SMIR/IAD mediates the formation of homo- or heterodimers of IRF6. *IRF6* variants are known to cause syndromic orofacial clefting (OFC) and to contribute towards the risk of isolated or nonsyndromic cleft lip with or without cleft palate (NSCL/P) and tooth agenesis (TA). Mutations in *IRF6* have been described as a cause of Van der Woude syndrome (VWS; MIM #119300). VWS is an autosomal dominant form of OFC associated with lip pits and hypodontia, and is the most common (1 in 35,000 live births) syndromic form of CL/P (Burdick 1986; Dixon et al. 2011). Around 70% of the causal VWS mutations occur in *IRF6* (Kondo et al. 2002; de Lima et al. 2009). Another syndromic form of CL/P caused by *IRF6* mutations, popliteal pterygium syndrome (PPS; MIM #119500), additionally presents with skin and genital anomalies (Gorlin et al. 1968). PPS is estimated to affect 1 in 300,000 live births (Kousa and Schutte 2016). Most mutations leading to VWS and PPS affect the DBD or the SMIR/IAD domain of IRF6 (de Lima et al. 2009). Besides the syndromic clefting (Kondo et al. 2002), *IRF6* also contributes to NSCL/P, one of the most common congenital disorders (1 in 700 live births) (Dixon et al. 2011) and to TA. TA, which is the congenital absence of permanent teeth in the absence of any other malformation, is prevalent in 3% to 11% of the European and Asian populations, respectively (Polder et al. 2004; Vieira et al. 2007). Both NSCL/P and TA are complex disorders (Lidral and Murray 2004; Dixon et al. 2011) that are likely to be associated with multiple genetic loci in combination with environmental factors. Common variants in the *IRF6* locus have been associated with NSCL/P (Rahimov et al. 2008) and TA (Vieira et al. 2007). These variants confer a small risk toward the disorder and are not sufficient by themselves to give rise to facial clefts and TA. Mutations in *Irf6* hamper the development of tooth in mice by causing evagination of incisor epithelium (Blackburn et al. 2012). A recent study supports the role of *Irf6* in a spectrum of dental phenotypes (Chu et al. 2016). The reported *Irf6*-knock out mouse model displays variable hypodontia, occasional supernumerary, as well as crown and root patterning anomalies. A previous study on *IRF6* concluded that rare coding *IRF6* mutations may not have a major role in nonsyndromic OFC because of the misdiagnosed VWS families that are initially considered as nonsyndromic (Leslie et al. 2015). Here, we hypothesize that *IRF6*—a gene in which mutations are causative for syndromic OFC, and common variants confer risk towards NSCL/P and TA—could in some rare instances also cause nonsyndromic OFC and TA. We sequenced all exons of the *IRF6* gene in 1,072 OFC and 67 TA patients as well as in 706 controls using molecular inversion probes (MIPs) (O'Roak et al. 2012) to identify novel rare variants causal for nonsyndromic OFC and TA.

Subjects and Methods

Patients and Controls

A total of 1,139 patients, 1,072 with OFC and 67 with TA (Dreesen et al. 2014), and a population of 706 matched controls, all of European ancestry, were included in this study. The 1,072 OFC patients included 194 Cleft Palate Only (CPO), 127 Cleft Lip Alveolus (CLA), 104 Cleft Lip Only (CLO) and 647 Cleft Lip and Palate (CLP) patients. This study was part of a larger sequencing project involving subjects from Bonn, Leuven, and Nijmegen (Ockeloen et al. 2016). Nineteen of the 1,139 patients were already screened for *IRF6* variants in a previous study (Birnbaum et al. 2008).

Approval from the Institutional review boards of the respective university hospitals and informed consent from all individuals was obtained.

Sequencing and In Silico Analysis

All 9 exons of *IRF6* were sequenced using a total of 20 MIPs (Appendix Table 1) at conditions described previously (O'Roak et al. 2012). MIP sequencing of *IRF6* was performed as part of a larger study comprising 431 probes (Ockeloen et al. 2016). The final pooled MIP libraries, each consisting of 384 samples, were sequenced using 2 × 79-bp reads on a NextSeq500 sequencer (Illumina). All samples with an average coverage over all *IRF6* targeting MIPs <100-fold were excluded. MIP data were analyzed with an in-house analysis pipeline (Ockeloen et al. 2016). Briefly, reads were aligned by Burrows-Wheeler Aligner to the reference genome (GRCh37/hg19). Subsequently, MIP extension and ligation arms were removed from all the alignment files and reads were trimmed to remove overlap between the paired-ends. The alignment was split by barcode identifier resulting in a single binary alignment map (BAM) file per sample, after which small variants were called by the Genome Analysis ToolKit (GATK) (McKenna et al. 2010). Multiple sample calling using the Unified Genotyper resulted in a single Variant Call Format file. A custom annotation pipeline was used to annotate the variants (for instance, the effect on protein and variant frequency in populations).

Table 1. Filter Criteria and the Numbers of *IRF6* Variants in Orofacial Clefting and Tooth Agenesis Patients Versus Controls at Every Filter.

	In Patients (OFC+TA)	OFC	TA	In Controls
Rare variants (dbSNP freq ≤0.1 and <5 samples in MIPs screen)	22	19	3	9
Splice site or coding variants	17	15	2	7
Nonsynonymous variants	13	13	0	7
Deleterious variants (CADD Phred-like score >20)	6	6	0	1
De novo	3	3	0	NA

dbSNP, single nucleotide polymorphism database; MIP, molecular inversion probe; NA, not applicable; OFC, orofacial clefting; TA, tooth agenesis.
The above filtering steps were applied in successive order. These steps were preceded by a step where reads were filtered by depth greater than 500.

Table 2. De Novo and Inherited *IRF6* Variants Validated in Patients.

Variant cDNA	Amino acid change	Inheritance	Protein domain	SIFT (Class; Score)	PhyloP Score	Grantham Score	CADD Phred-like Score	Phenotype(s)
c.1139C>A	Pro380Gln	De novo	SMIR/IAD	Deleterious; 0.0	4.16	76	24.5	CPO
c.673G>C	Asp225His	De novo	—	Deleterious; 0.0	5.77	81	27.8	CLA, VWS
c.25C>T	Arg9Trp[a]	De novo	—	Deleterious; 0.0	6.02	101	32	CPO
c.1124T>C	Phe375Ser[b]	NA	SMIR/IAD	Deleterious; 0.0	3.35	155	24.8	CLP
c.385_398del	Thr129Leufs*3	Paternally inherited	—	—	—	—	22.1	CPO
c.103C>T	Arg35Cys	Maternally inherited	DBD	Deleterious; 0.0	3.19	180	21.4	CLA

CLA, cleft lip alveolus; CLP, cleft lip and palate; CPO, cleft palate only; DBD, DNA-binding domain; SMIR/IAD, Smad-interferon regulatory factor binding domain; VWS, Van der Woude syndrome; NA, not applicable.
[a]This variant has been previously identified (Matsuzawa et al. 2004).
[b]This variant has been previously identified (Leslie et al. 2013).

From the identified variants, only those with a quality-by-depth higher than 500 were considered. These variants were further filtered based on the criteria mentioned in Table 1. The variants obtained were selected for possible deletions, frameshifts, splice sites variants, known HGMD (Stenson et al. 2003) variants (Appendix Table 2; Fig. 1) and a high CADD Phred Score (>20; Kircher et al. 2014) (Table 2). The effect of the variants was also assessed by Alamut Visual, which integrates genetic and genomic information from different sources (phyloP, Grantham, SIFT). Those variants passing all filtering steps were validated using Sanger sequencing and segregation analysis was performed in all available family members.

Molecular Modeling

To obtain more insight into the effects of the mutations on the molecular structure of IRF6, we created a homology model. We used the YASARA (Krieger et al. 2002) & WHAT IF (Vriend 1990) twinset homology modeling script with standard parameters. The program created 2 models representing the 2 different domains of the protein. PDB file 3DSH was used to create a model for residues 202 to 455 (identity 62%), which make up the SMIR/IAD domain, and PDB file 2IRF was used to create a model for residues 1 to 123 (identity 43%), which make up the DBD.

Results

After excluding samples with an average coverage <100-fold (Appendix Table 3), the average coverage over all *IRF6* targeting MIPs was 665-fold for all patients (1,045 OFC and 67 TA) and 674-fold for all controls (n = 706). A total of 19 of 1,045 OFC patients (1.81%) and 8 of 706 controls (1.13%) were found to have at least one rare, nonsynonymous coding or splice site variant (Appendix Table 2). Six variants among the OFC patient group compared with one in the controls had CADD Phred-like scores >20 (Table 2). These variants were validated by Sanger sequencing, and segregation analysis was also performed in the parents and the other available family members of the probands.

In OFC patients, 3 de novo *IRF6* variants were identified (Table 2). Based on the gene-specific mutation rate (Samocha et al. 2014), the identification of 3 de novo mutations in a cohort of 1,112 patients is highly unlikely a chance finding ($P = 7.79 \times 10^{-5}$), after multiple testing correction for testing 13 genes in the entire MIP pool. Each of these 3 variants, c.1139C>A (p.Pro380Gln), c.673G>C (p.Asp225His) and c.25C>T (p.Arg9Trp), were present only in single patients and were not found in controls, neither in the present study nor in ExAC or GoNL.

The variant p.Pro380Gln was present in a patient who was diagnosed with nonsyndromic CPO and no lip pits (Fig. 2a), and the father and mother were not reported to have any facial clefting. The variant occurs at a highly conserved nucleotide (g.209963052G>T, phyloP: 4.16), and affects a highly conserved amino acid, up to Tetraodon, of the SMIR/IAD domain of the IRF6 protein. There is a significant physicochemical difference between the reference amino acid proline and the variant amino acid glutamine. The second variant, p.Asp225His, was identified in a patient initially diagnosed with CLA but, after in-depth phenotyping subsequent to identification of this variant, found to have lip pits in the lower lip, which is suggestive of VWS (Fig. 2b). The father and the mother have no OFC. This variant occurs at a highly conserved nucleotide (phyloP:

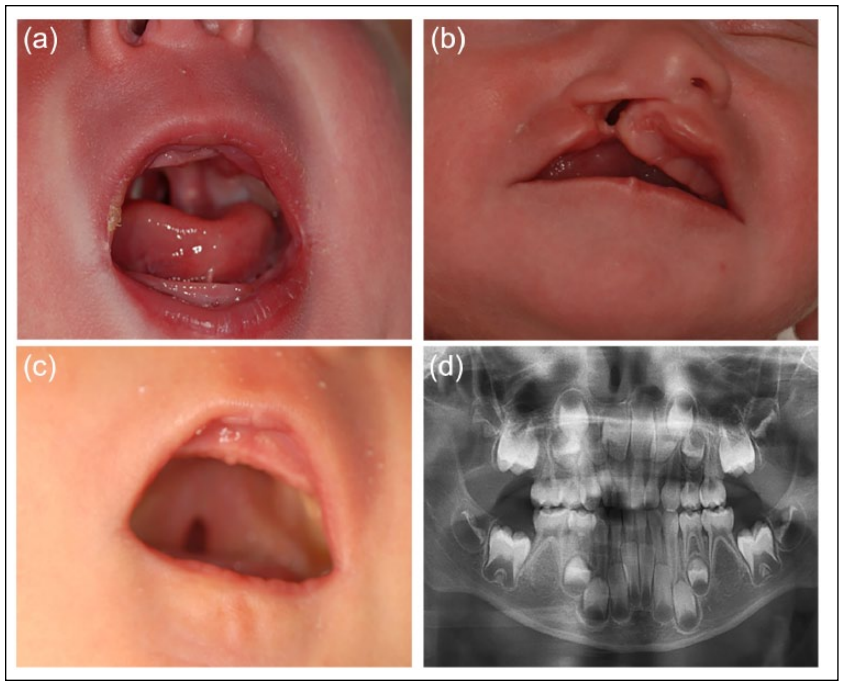

Figure 2. Clinical photographs (**a–c**) and orthopantomogram (OPT) (**d**) of patients. (a) Frontal oral photograph of the patient carrying the p.Pro380Gln de novo variant, showing cleft palate only (CPO). The patient also exhibits a submucous cleft of the hard palate. (b) Patient carrying the p.Asp225His de novo variant, showing unilateral cleft lip and cleft alveolus (CLA), and with a lip pit (VWS). (c) Patient carrying p.Thr129Leufs*3 variant, showing a cleft of the soft palate and uvula. (d) OPT of the patient carrying p.Thr129Leufs*3 variant showing tooth agenesis of 15, 25, 35 and 45.

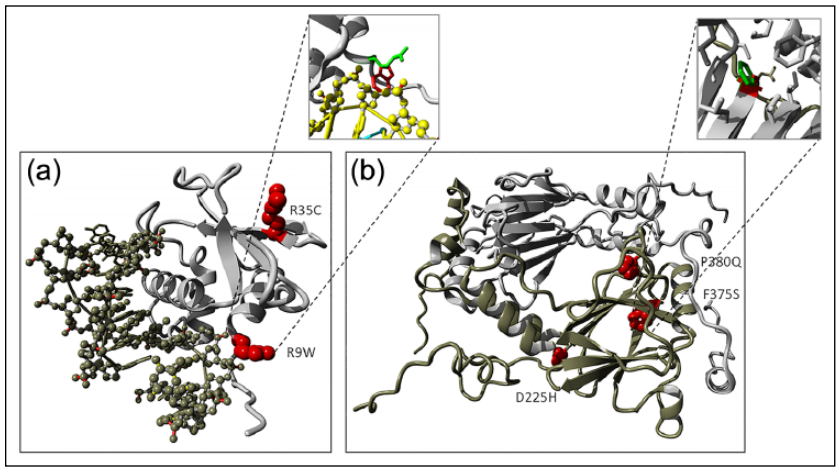

Figure 3. Homology model for the (**a**) DNA binding domain (DBD) and (**b**) the Smad-interferon regulatory factor binding domain (SMIR/IAD). Dark gray colored molecules are interacting in the (a) DNA molecule and (b) protein monomer. Mutated bases are depicted in red. The p.Arg9Trp variant changes a positively charged amino acid to a very big neutral amino acid. Attractions with negative phosphates will be lost. In the inset, the wildtype residue sidechain is indicated in green, the mutant sidechain is shown in red. The large sidechain of tryptophan will interfere with the DNA molecule. The p.Arg35Cys variant will reduce the overall stability of the protein caused by a loss of interactions with a nearby aspartate residue. The p.Asp225His variant will cause loss of some interactions because a negatively charged amino acid is changed to a neutral one. The variant p.Phe375Ser is highly deleterious. This will cause a large empty space and change the core of the protein. In the inset, the surrounding hydrophobic sidechain interactions made by this phenylalanine residue are indicated. The p.Pro390Gln variant will cause loss of hydrophobic interactions and this might have an effect on the backbone. This figure is available in color online.

5.77) and affects a highly conserved amino acid up to Tetraodon. The third variant, Arg9Trp, was found in a patient with CPO and no lip pits (picture not available). This variant also occurs at a highly conserved nucleotide (phlyoP: 6.02) and affects a highly conserved amino acid, up to Tetraodon and has been previously identified in 2 VWS patients (Matsuzawa et al. 2004), and in a CL/P patient with atypical lower lip pits (Jehee et al. 2009).

Besides the 3 de novo variants, 3 other variants with CADD Phred-like score >20 and strong functional prediction were identified in patients, 2 of which were inherited from one of the unaffected parents (Table 2). One of the inherited variants is p.Thr129Leufs*3, caused by a deletion of 14 nucleotides in exon 5. This variant was identified in a CPO patient. Review of the collected phenotypic data a posteriori revealed no lip pits (Fig. 2c, d). This deletion creates a frame shift starting at codon Thr129. The premature stop leads to the loss of the SMIR/IAD domain in the protein. The mRNA produced might be targeted for nonsense-mediated RNA decay. The other inherited variant p.Arg35Cys was also found in a nonsyndromic CLA patient, with no lip pits. This variant is a known polymorphism, rs771539601, with ExAC allele frequency 0.00003 in the European (Non-Finnish) population. It affects a highly conserved amino acid in the DBD of the IRF6 protein, and the physicochemical difference between the reference and the variant amino acid is large. A variant at the same position, Arg35Pro, was previously identified in a VWS pedigree (de Lima et al. 2009). The third variant, p.Phe375Ser, was identified in a CLP patient. Review of collected phenotypic data a posteriori revealed no lip pits. This variant has been reported previously in 3 VWS families (Leslie et al. 2013). It affects a highly conserved amino acid in the SMIR/IAD domain. The variant was not present in the mother's DNA; however, because the father's DNA was unavailable, we could not confirm whether the variant is inherited or occurs de novo in the patient.

An experimentally derived protein structure for IRF6 is not yet known. Thus, we built separate homology models for the DBD and the SMIR/IAD domain (Fig. 3).

A detailed analysis of these models revealed that none of the mutations occurred on the dimerization surface of the SMIR/IAD domain. Only one mutation, p.Phe375Ser, is positioned in the core of the domain and will severely affect the folding and function of the domain. The change from a larger hydrophilic residue into a smaller hydrophilic one will abolish many stabilizing hydrophobic interactions made by the phenylalanine sidechain. Also, mutant p.Arg9Trp is located in the DBD and is necessary for electrostatic interactions with the DNA phosphate backbone. This mutation is predicted to affect DNA binding because the ionic interaction between the negatively charged DNA backbone and the positively charged Arginine sidechain will be lost. Tryptophan has an even larger sidechain, and, more importantly, does not carry a charge, which does not allow it to make the same interactions with the DNA backbone. All other mutations occur on the surface of the protein where they may change the local structure and thereby affect interactions with other proteins in the biological complex.

Discussion

Both NSCL/P and TA are genetically complex birth defects. The phenotype can result from multiple genetic, environmental, and lifestyle factors. Several common genetic risk factors have been discovered by genome-wide association studies, but these genetic factors represent only part of the risk associated with NSCL/P (Khandelwal et al. 2013). While dominant mutations in *GRHL3* are known to cause VWS (Peyrard-Janvid et al. 2014), a recent study showed that 1.7% of the cases with nonsyndromic CPO have rare truncating mutations in *GRHL3* (Mangold et al. 2016). We aimed at identifying rare, monogenic *IRF6* variants responsible for nonsyndromic OFC or TA.

We employed MIPs for targeted resequencing of *IRF6* in 1,072 OFC and 67 TA patients and 706 controls. This massively multiplex targeted sequencing resulted in rapid and cost-effective screening of multiple genes in large cohorts, as shown in the initial reports (O'Roak et al. 2012) and numerous subsequent applications (Hildebrand et al. 2016; Schueler et al. 2016). We identified 6 variants that are likely to be deleterious variants in OFC patients, out of which 3 were confirmed to be de novo. The identification of 3 de novo mutations in a cohort of 1,112 cases is highly unlikely a chance finding ($P = 7.79 \times 10^{-5}$). These 6 variants were not present in controls in the MIPs assay. One of the OFC patients with cleft of soft palate and uvula containing the *IRF6* variant p.Thr129Leufs*3 showed TA of 4 premolars (15, 25, 35 and 45) (Fig. 2d). Hence, we show that, from the wide spectrum of dental phenotypes recently found in a novel *IRF6*-knockout mouse model (Chu et al. 2016), TA of 4 premolars is also present in a patient with OFC carrying a rare *IRF6* variant. We, however, did not identify any major contribution of rare *IRF6* variants in patients with solitary TA. A larger solitary TA cohort could probably indicate the role of rare of *IRF6* variants.

In a previous study, 1,521 nonsyndromic OFC families were screened for *IRF6* mutations (Leslie et al. 2015). The authors identified 3 de novo and 4 inherited variants. In all 3 families with de novo variants, lip pits were not explicitly reported, but participants were not checked for other signs of VWS. One of the 3 de novo variants was also previously reported in VWS families. The 4 inherited variants were present in index patients and a parent where either the index patient or a parent had VWS, or where the carrier parent was not inspected for lip pits at all. In the present study, we also identified 3 deleterious de novo variants in our OFC cohort. Out of the 3 de novo variants—Pro380Gln, Asp225His and Arg9Trp—the Asp225His variant was present in a CLA patient, in whom lip pits were also observed, thus fulfilling the diagnostic criteria of VWS. This emphasizes the need for careful and accurate phenotyping. The other patients with other de novo or inherited variants were not reported to have any lip pits. Review of the collected phenotypic data a posteriori did not reveal any lip pits. Approximately 15% of VWS patients only present with OFC as the phenotype, thus making it difficult to distinguish from nonsyndromic OFC. In such patients, phenotypic data of the family can be useful to establish the syndromic nature of the disease (Leslie et al. 2015). In large patient cohorts, however, the "genotype first" approach, as presented here, could be used, as this allows a post-test check or a posteriori refinement of the clinical diagnosis (Stessman et al. 2014).

We also identified 2 predicted deleterious variants, which were inherited from one of the unaffected parents. Besides, we also found some variants that were present in both patients and controls. These variants might have low penetrance and lead to the corresponding phenotype only in the presence of genetic modifiers; this would account for the unaffected parent and controls. Aside from the coding variants in *IRF6*, NSCL/P has also been associated with a variant 9.7-kb upstream of *IRF6* in a highly conserved noncoding regulatory element, rs642961 (Rahimov et al. 2008). This variant (rs642961) disrupts 1 of the 4 binding sites for AP-2α, which is in the same developmental pathway as IRF6. Perhaps the genetic modifiers needed to be causative for the inherited variants or the variants found in both patients and controls are present in this regulatory element. Overall, we see that, in affected patients for whom we validated the *IRF6* variants, one was found to have VWS and the rest, either nonsyndromic CLP, nonsyndromic CLA or nonsyndromic CPO.

In conclusion, our data further add to the increasing evidence that rare *IRF6* variants play a role in the etiology of nonsyndromic OFCs. Moreover, our study highlights the need for deep clinical phenotyping and medical history of families that present at genetic counseling, and the advantage of a "genotype first" approach when studying large patient cohorts.

Author Contributions

K.D. Khandelwal and N. Ishorst, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; H. Zhou, contributed to conception, design, data analysis, and interpretation, drafted and critically revised the manuscript; K.U. Ludwig, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; H. Venselaar, contributed to

design, data analysis, and interpretation, drafted and critically revised the manuscript; C. Gilissen, contributed to conception, data analysis, and interpretation, drafted and critically revised the manuscript; M. Thonissen, I.A.L.M. van Rooij, and K. Dreesen, contributed to design and data acquisition, drafted and critically revised the manuscript; M. Steehouwer, M. van de Vorst, M. Bloemen, E. van Beusekom, contributed to design and data analysis, drafted and critically revised the manuscript; J. Roosenboom, W. Borstlap, R. Admiraal, T. Dormaar, J. Schoenaers, V. Vander Poorten, G. Hens, A. Verdonck, and S. Bergé, contributed to design and data acquisition, drafted and critically revised the manuscript; N. Roeleveldt, contributed to conception and design, drafted and critically revised the manuscript; G. Vriend, contributed to conception, design, data analysis, and interpretation, drafted and critically revised the manuscript; K. Devriendt, contributed to conception, data acquisition, and interpretation, drafted and critically revised the manuscript; H.G. Brunner, contributed to conception, design, data acquisition, and interpretation, drafted and critically revised the manuscript; E. Mangold and A. Hoischen, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; H. van Bokhoven, contributed to conception, data analysis, and interpretation, drafted and critically revised the manuscript; C.E.L. Carels, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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