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ORIGINAL ARTICLE

Novel *IRF6* variant in orofacial cleft patients from Durban, South Africa

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Abstract

Background: To date, there are over 320 variants identified in the *IRF6* gene that cause Van der Woude syndrome or popliteal pterygium syndrome. We sequenced this gene in a South African orofacial cleft cohort to identify the causal *IRF6* variants in our population.

Method: Saliva samples from 100 patients with syndromic and non-syndromic CL ± P were collected. Patients were recruited from the cleft clinics at two public, tertiary hospitals in Durban, South Africa (SA), namely Inkosi Albert Luthuli Central Hospital (IALCH) and KwaZulu-Natal Children's Hospital (KZNCH). We prospectively sequenced the exons of *IRF6* in 100 orofacial cleft cases, and where possible, we also sequenced the parents of the individuals to determine the segregation pattern.

Results: Two variants were identified; one novel (p.Cys114Tyr) and one known (p.Arg84His) missense variant in *IRF6* gene were identified. The patient with the p.Cys114Tyr variant was non-syndromic with no clinical VWS phenotype expected of individuals with *IRF6* coding variants, and the patient with the p.Arg84His had phenotypic features of popliteal pterygium syndrome. The p.Arg84His variant segregated in the family, with the father also being affected.

Conclusions: This study provides evidence that *IRF6* variants are found in the South African population. Genetic counselling is essential for affected families,

particularly in the absence of a known clinical phenotype since it helps with the plans for future pregnancies.

KEYWORDS

IRF6, orofacial clefts, popliteal pterygium syndrome, South Africa, Van der Woude syndrome

1 | INTRODUCTION

Orofacial clefts (OFC) are the most common congenital anomaly of the craniofacial region with a global prevalence of 1:700 live births (Mossey & Catilla, 2003). Seventy per cent of cleft lips with or without cleft palate (CL ± P) are classified as non-syndromic or isolated. In contrast, the remaining 30% are syndromic, which means they occur together with other structural anomalies or dysmorphic features such as heart, renal, facial defects and others. (Adeyemo & Butali, 2017).

Van der Woude syndrome (VWS) (OMIM: 119300) is the most common syndromic form of CL ± P, accounting for 2% of all OFC (Murray et al., 1997; Rintala & Ranta, 1981). VWS is an autosomal dominant condition that presents with lower lip pits and clefts of the lip and/or palate (Van der Woude, 1954). Hypodontia and uvula clefts can also occur. Expression is variable; hence, the clinical presentation can be different within members of the same family. VWS is also highly penetrant, with studies estimating the penetrance to be 96.7% (de Lima et al., 2009; Janku et al., 1980).

Popliteal pterygium syndrome (PPS) (OMIM: 119500) is allelic to VWS. Clinical manifestations include lower lip pits, cleft lip and/or palate, syngnathia, skin manifestations like webbing of the lower limbs and syndactyly and genital abnormalities including hypoplasia of the labia majora and a bifid or absent scrotum (Matsuzawa et al., 2010). Worldwide, over 320 variants in the interferon regulatory factor 6 (*IRF6*) (OMIM: 607199) gene on chromosome 1q32.2 have been shown to cause both VWS and PPS (Leslie et al., 2013).

In 2002, Kondo et al (Kondo et al., 2002) showed that mutations in *IRF6* caused VWS by investigating a set of monozygotic twins who were discordant for the phenotype. The first African candidate gene study on VWS showed three novel and three known mutations in a cohort from Nigeria and Ethiopia (Butali et al., 2014). A Nigerian family presented with clinical features of PPS and had the known c.251G>A; p.Arg84His variant of the *IRF6* gene. A subsequent Moroccan PPS case report showed a different, known, missense variant, namely c.250C>T; p.Arg84Cys on exon 4 (Ratbi et al., 2014).

Studies (Birnbaum et al., 2008; Salahshourifar et al., 2012; Zucchero et al., 2004) have shown that *IRF6* variants can be detected in non-syndromic families, and in 2016, Leslie et al (Leslie et al., 2016) concluded that *IRF6* mutations are found in approximately 0.3% of apparently non-syndromic OFC families. Recently, Wang et al (Wang et al., 2021) detected a novel *IRF6* variant, namely c.961C>T; p.Val321Met, in a non-syndromic family by whole exome sequencing.

In 2013, Leslie et al. (2013) showed that mutations occurring in conserved domains are likely to result in VWS or PPS. There are nine exons of the *IRF6* gene, and exons 4 and 7 were demonstrated to be mutational hotspots for patients from Nigeria, Ethiopia and Ghana. Variants found included missense and splice site variants (Gowans et al., 2017). Non-random mutations in *IRF6* exons 3 and 4 show high combined annotation-dependent depletion (CADD) scores of between 20 and 30. These scores ranked the *IRF6* variants in the top 1% of deleterious mutations in the genome (Alade et al., 2020). Therefore, the aim of this paper was to identify the variants in the *IRF6* gene in a South African cohort.

2 | PARTICIPANTS & METHODS

2.1 | Study population

One hundred African patients born with various OFC types (Table 1) and parents of these patients were recruited and assessed at the cleft clinics from two Durban hospitals in South Africa, namely Inkosi Albert Luthuli Central Hospital (IALCH) and KwaZulu-Natal Children's

TABLE 1 Summary of Cohort phenotype

Ethnicity: African	Cleft lip only	Cleft palate only	CL ± P	Non-syndromic	Syndromic	M:F ratio	Positive family history of OFCs
100	10	39	51	73	27	48:52	6

TABLE 2 Sequence Variations for IRF6 Observed in Individuals from an Isolated South African Population

HGVS	HGVP	IRF6 exon	Nucleotide position	Nucleotide change	Variant type	Novel / known	Affected individuals	Cleft type	PolyPhen	SIFT	Provean	ACMG	CADD
c.341G>A	p.Cys114Tyr	4/9	Chr1: 209969731	G/A	Missense	Novel	1	CP-soft	PD	T	D	LP	25.7
c.251G>A	p.Arg84His	4/9	Chr1: 209969821	G/A	Missense	Known	2	Bilat CLP: child Right CLP: father	PD	D	D	P	32

Note: "c" refers to the coding sequence position within the IRF6 transcript, NM_006147.3; and "p" refers to amino acid substitutions. Amino acid substitution is for the GRHL3 isoform 2 transcript, NP_937816. Human assembly builds hg38.

Abbreviations: ACMG, American College of Medical Genetics; CADD, Combined Annotation-Dependent Depletion Score; D, deleterious; HGVS, Human Genome Variation Society; LP, Likely Pathogenic; P, Pathogenic; PD, probably damaging; S, Sorting Intolerant from Tolerant (SIFT); T, tolerated.

Hospital (KZNCH). The principal investigator, a paediatrician, conducted the assessment; plastic surgeons and an orthodontist verified all findings in the patients. Our investigation was an observational clinical study to ascertain the genetic causes of cleft lip and palate in the African population in KwaZulu-Natal. Patients were consecutively selected from the cleft clinics. Eligible children were of African ancestry and had a syndromic or non-syndromic CL ± P.

Informed consent was obtained from all participating families. A case report form was completed, and saliva samples were collected with Oragene saliva kits and sponges (DNA Genotek). These samples were sent to the Butali Laboratory at the University of Iowa for processing and analysis. DNA from all samples was extracted, followed by XY genotyping analysis for quality control purposes to ensure that the sex of the sample matched the sex of the actual donor. All 100 proband samples were analysed via Sanger sequencing. When mutations were found, parental samples were sequenced. Biomedical Research Ethics Committee clearance (BE309/18) was obtained from the University of KwaZulu-Natal.

2.2 | Sanger sequencing

Primer sequences that were used to amplify exons 1 to 9 of *IRF6* (RefSeq NM_006147.3) have been previously published (Butali et al., 2014; de Lima et al., 2009) and are available on request. All DNA processing protocols, PCR conditions and electrophoretic procedures are available at the Murray laboratory website (http://genetics.uiowa.edu/protocols.php). The amplified DNA products were sent to Functional Biosciences in Madison, Wisconsin, for sequencing using an ABI 3730XL DNA Sequencer.

To identify novel and rare mutations, variants found in our isolated population were compared to those in the 1000 Genomes database, Exome Variant Server database (http://snp.gs.washington.edu/EVS/) and exomes in the Genome Aggregation Database (gnomAD; http://gnomad.broadinstitute.org). To predict the functional effects of these mutations on the protein, we used bioinformatics tools, such as Combined Annotation Dependent Depletion (CADD; https://cadd.gs.washington.edu/; Rentzsch et al., 2019), Polymorphism Phenotyping (PolyPhen; http://genetics.bwh.harvard.edu/pph2/; Adzhubei et al., 2010), Sorting Intolerant from Tolerant (SIFT; http://sift.jcvi.org/; Kumar et al., 2009), Have Your Protein Explained (HOPE; https://www3.cmbi.umcn.nl/hope/; Venselaar et al., 2010) and Human Splicing Finder (HSF; http://www.umd.be/HSF/; Desmet et al., 2009). To assess the inheritance patterns of these mutations, we sequenced parent samples when available.

3 | RESULTS

We identified two variants; one novel (p.Cys114Tyr) and one known (p.Arg84His). The novel, missense p.Cys114Tyr variant in exon 4 was classified as "probably damaging" by PolyPhen and "deleterious" by Provean with a CADD score of 25.7, placing this variant in the top 1% of deleterious mutations in the human genome (Table 2). According to the American College of Medical Genetics (ACMG) guidelines (Richards et al., 2015), this variant is classified as likely pathogenic.

This novel variant was absent from all public exome and genome databases, including Genome Aggregation (gnomAD), Exome Variant Server (EVS) and 1000 Genomes databases that included over 6000 Africans. The p.Cys114Tyr variant occurred in a patient with no phenotypic features of Van der Woude Syndrome. The index patient's mother was tested, and the variant did not segregate in her. The father was not present at the visit. A chromatogram (Figure 1a) of the mutation is included to show the G > A change. Figure 1b shows that both mutations are found in exon 4 of the DNA-binding domain.

Concerning conservation, only this residue type was found at this position, and a 100% conserved residue mutation is usually damaging to the protein. The mutant and wild-type residues are not similar. Hence, based on this conservation information, the mutation is probably damaging to the protein (Figure 2).

Missense variant p.Arg84His segregated in this South African family with phenotypic features of popliteal pterygium syndrome (PPS), with the father carrying the same variant as his son. The father presented with a right-sided cleft lip and palate, whereas his son had a bilateral cleft lip and palate, lower lip pits and popliteal webbing (Pictures 1 and 2). The chromatogram shows the G>A change segregating in the father (Figure 1c). This variant was predicted as "damaging" by all three bioinformatics tools (namely PolyPhen, SIFT and Provean). In addition, this variant demonstrated a CADD score of 32 and is proven to be pathogenic according to the ACMG guidelines (Richards et al., 2015). Figure 1d shows a 3D close-up of this mutation and how it can cause loss of interactions and damage the protein.

4 | DISCUSSION

We sequenced the coding regions of the *IRF6* gene and found two variants: one novel (p.Cys114Tyr) and one known variant (p.Arg84His). The p.Cys114Tyr was identified only in the proband with no obvious lower lip pits classical of individuals with VWS. The lack of phenotypic information on the father limits our clinical diagnosis of

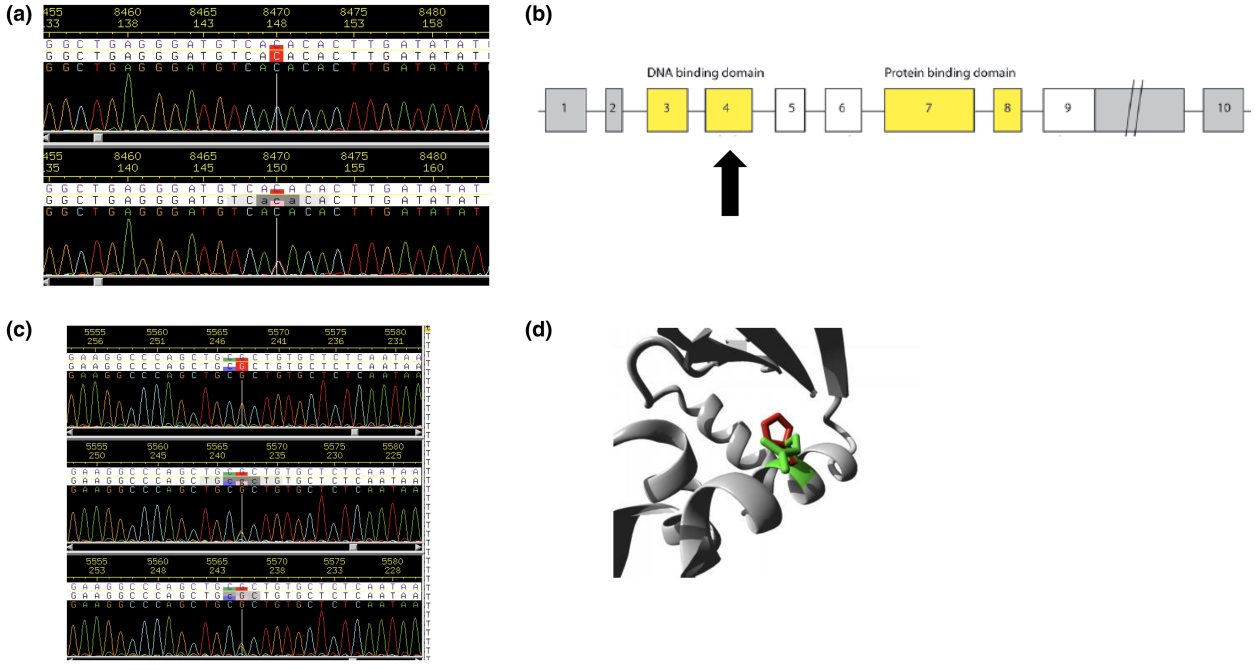

FIGURE 1 (a) IRF6 p.Cys114Tyr mutation in mum and child with nucleotide change from G>A. (b) Diagram of IRF6 gene showing exons, with DNA binding and protein binding domains colored in yellow. The black arrow shows both mutations located in exon 4. (c) IRF6 p.Arg84His mutation in mum, child and dad with nucleotide change from G>A. (d) IRF6 p.Arg84His: Protein is colored grey, mutant residue is red and wild-type residue is green. The mutant residue is smaller and differently charged which might lead to loss of interactions and hence disrupt the protein

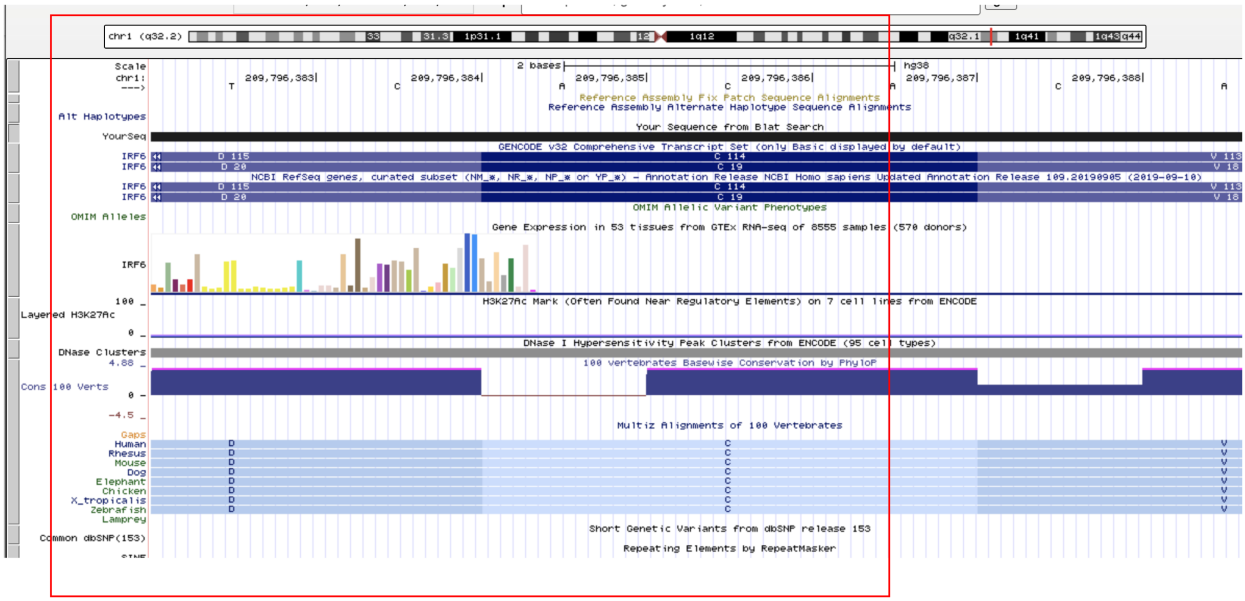

FIGURE 2 Diagram from the UCSC genome browser showing variant p.Cys114Tyr is located in a highly conserved position

PICTURE 1 Father and son with popliteal pterygium syndrome

PICTURE 2 More recent photos of dad and child and the operations to remove the popliteal pterygia.

VWS. Nonetheless, the molecular data provide evidence that the family has VWS, and perhaps the lower lip pit may be absent in the child and present in other family members not involved in the study. This will not be surprising since we know that the lower lip pit phenotype is not completely penetrant in individuals with VWS (Desmyter et al., 2010).

On review of the p.Cys114Tyr variant, the nucleotide change from G>A is classified as likely pathogenic on Varsome (Kopanos et al., 2019; Sorrentino et al., 2021). According to the ACMG evidence framework (Richards et al., 2015), for population data variant, p.Cys114Tyr is absent in population databases scoring a PM2. For the

computational and predictive data, there are multiple lines of computational evidence that support a deleterious effect on the gene/gene product (namely Provean, PolyPhen and CADD scores): PP3. With regard to functional data: Exon 4 is a mutational hotspot: PM1. There is non-segregation with the disease in this family: BS4. Under other data, the patient's phenotype is highly specific for gene: PP4. According to the rules for combining criteria: there are two moderate (PM1 and PM2) and two supporting (PP3 and PP4) pieces of evidence; which makes this variant likely pathogenic.

The p.Arg84His variant is a known pathogenic variant according to ACMG criteria and Varsome. The rationale for this conclusion is for computational and predictive data, and there is a predicted null variant in a gene where LOF is a known mechanism of disease: PVS1. For functional data, this variant occurs in exon 4 which is a mutational hotspot: PM1. There is cosegregation with disease in multiple affected family members: PP1. It is found in other databases from a reputable source as pathogenic: PP5. And lastly, the patient's phenotype and family history are highly specific for the gene: PP4. Hence, this variant is classified as pathogenic based on the following ACMG criteria: very strong (PSV1) and 1 moderate (PM1) and 1 supporting (PP1).

We noticed variable expressivity in the family with p.Arg84His where the father has a milder phenotype compared with his son who presented with additional popliteal webbing that required surgical correction. This varied presentation also highlights that the variant does not predict severity of disease (Butali et al., 2014). This family will require genetic counselling to understand the recurrence risk in future generations for this autosomal dominant trait.

Both variants presented in this study were located in exon 4. This was one of the two mutational *IRF6* hotspots documented for African patients (Gowans et al., 2017). Furthermore, these mutations in exon 4 demonstrated high CADD scores, as reported in a recent study (Alade et al., 2020). Mutations in families with VWS are usually on exons 3, 4, 7 and 9, while mutations in families with PPS are on exons 3, 4 and 9 (de Lima et al., 2009).

The *IRF6* variants identified in this South African cohort also account for a prevalence of approximately 2% (n = 2/100) consistent with the reported prevalence of VWS globally (Murray et al., 1997). However, our study suggests there is a need to correlate both the molecular and clinical evidence to classify syndromic and non-syndromic OFCs since our first patient did not have any clinical features of VWS.

5 | CONCLUSION

Our data confirm the presence of *IRF6*-related PPS with variable expressivity in South Africa as well as novel *IRF6* variant in a family with no obvious clinical features of VWS. These observations are significant because they are from African populations, which are under-represented in the literature, and because the identification of DNA variants in *IRF6* in non-syndromic cases is very rare. More genetics and genomics studies need to be conducted in South Africa to determine the different genetic aetiologies of orofacial clefts.

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AUTHOR CONTRIBUTIONS

Thirona Naicker, Colleen Aldous and Azeez Butali were responsible for the conceptualization of the study. Thirona Naicker coordinated patient recruitment, clinical data collection and sample collection in South Africa. Chinyere Adeleke, Joy Olotu, Azeez Alade, Mary Li, Waheed A. Awotoye and Tamara Busch performed the sequencing and analysis of results. Thirona Naicker wrote the initial draft. All authors contributed to reviewing and editing of the manuscript. Azeez Butali supervised the analyses. Azeez Butali and Colleen Aldous supervised the draft of the manuscript.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest concerning the research, authorship and/or publication of this article.

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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