

Differential expression of the *IRF6* gene in the signs of Van der Woude Syndrome: Are distinct genetic modifiers operating?

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Abstract

Objective: The purpose of this study was to report a new variant in the Interferon Regulatory Factor 6 gene (*IRF6*) and to determine phenotype-genotype correlations in a family segregating Van der Woude syndrome.

Methods: A five-generation family of 80 individuals segregating VWS was investigated using a tabulated pedigree but considering that three individuals registered in the pedigree died shortly after birth, the final sample size was 77 individuals. Five individuals had a complete dental clinical examination and molecular analysis performed using direct sequencing of the exon 4 and an adjacent region with 23 base pairs of the *IRF6* gene.

Results: Features of VWS reported in family history were present in 36.4% (28/77) of all family members; of these 57% (16/28) had pits in the lower lip, 36% (10/28) had both pits and orofacial clefts and 7% (2/28) had only orofacial clefts. Developmental dental anomalies were observed in three individuals. The sequence analysis of exon 4 of the *IRF6* gene carried out for 4 family members revealed the presence of the SNP rs7552506 (c.175-5C>G) located in five base pairs before the start of exon. The analysis of exon 4 of the *IRF6* gene also revealed a new variant c.269G>C (p.Ser90Thr) which causes exchange of the Serine amino acid residue for the Threonine residue.

Conclusion: The c.269G>C(p.Ser90Thr) can interfere with multimeric interactions and with protein conformation that will be slightly destabilized, because the mutant residue is bigger than the wild-type

residue. The phenotypic variations in the cases studied, despite carrying the same genetic mutation, suggest that distinct genetic modifiers operate on the formation of clefts and dental development.

Keywords: Van der Woude syndrome; Orofacial clefts; *IRF6* gene

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Introduction

Orofacial clefts may occur in association with other birth defects or be isolated. The Van der Woude Syndrome (VWS) (Online Mendelian Inheritance in Man [OMIM]119300) is the most common and well-known syndrome associated with orofacial clefts, being present in approximately 2% of orofacial cleft cases [1, 2]. VWS consists of a rare congenital malformation and presents clinical features in addition to orofacial clefts, such as lower lip pits and occasionally, hypodontia. The frequency of VWS in the population varies from 1: 100,000 to 1: 40,000, and there are no reports of differences between males and females [3,4].

The expression of VWS is highly variable, both interfamilial and within the same family. In other words, there is a range of associated phenotypes, from the complete absence of visible abnormalities, only the presence of pits in the lower lip, to the combination of these pits with different types of clefts. These types may include aspects such as laterality (unilateral or bilateral), extension (complete and incomplete) and may involve lip, palate or both [2, 5-7].

The etiology of VWS has been attributed to mutations in the *IRF6* gene (Interferon Regulatory Factor 6). However, from a molecular point of view, mutations in the *IRF6* gene can explain 70% of the cases of VWS. Several studies also point to the possibility of other genes causing VWS, despite the limitations of molecular biology techniques in identifying mutations in many of these genes [8-10].

The *IRF6* gene is located on the long arm of chromosome 1 (1q32.3 - q41) and contains 10 exons; exons 1, 2 and 10 are not translated. The product encoded by the *IRF6* gene is a 467 amino acid polypeptide [8, 11, 12].

In contrast to the other members of the IRF family (interferon regulating factors), *IRF6* does not participate in the innate immune response, however it is essential for embryonic development, can act as a tumor suppressor and participate in the differentiation of ectodermal tissues [13-15]. Recently, a new role assigned to the *IRF6* ortholog gene is participation in the development of the exocrine gland. This information can be used to investigate the suspicion of disorders in the salivary glands of patients with the two forms of syndrome caused by mutations in

the *IRF6* gene, the VWS and Popliteal Pterygium Syndrome (SPP), as well as to understand the presence of dental problems, for example, caries, in patients with orofacial clefts [16].

The aim of this study was to report a new variant in the *IRF6* gene and to determine phenotype-genotype correlations in a family segregating Van der Woude syndrome.

Material and Methods

Genealogical data

The family participating in the study was located in the Service of Assistance to the Patients with Orofacial Clefts at Ophir Loyola Hospital (Belém - Pará, Brazil). At the time, five members of this family were present, a mother and four children (two girls and two boys). All five had congenital pits in the lower lip and only one of them did not present orofacial cleft but instead presented excess of lower lip. The research was carried out in accordance with the basic ethical principles of the guidelines and standards that regulate research in human beings: autonomy, beneficence, non-maleficence and justice. The project was previously approved by the Ethics Committee of the Health Science Center of the

Federal University of Pará (CAAE 4879.0.000.073-10, no140 /10).

To obtain genealogical data, the tabulated pedigree method according to Polleta et al. (2014) [17] was used. The graphical construction of the pedigree was performed using an online tool from the Progeny program, available at: www.progenygenetics.com.

Information about other members of this family was obtained during three interviews at different times, resulting in the registration of 80 individuals in the pedigree, belonging to five generations of the index family. It was not possible to specify the gender and the presence of any manifestations of VWS in only three cases, all of whom had died shortly after birth; of these three cases, two were Siamese twin siblings. Of the final sample size of 77 individuals analyzed, 36 (45%) were females and 41 (51%) were males (Figure 1).

Phenotypic characteristics

Family members with one or more phenotypic characteristics of VWS were considered affected individuals. These characteristics included cleft lip with or without palate, cleft palate, and the presence of pits, whether bilateral or unilateral. The types of

orofacial clefts found were grouped into: cleft lip (CL), cleft lip with cleft palate (CLP) and cleft palate (CP). Dental information was gathered through a complete dental clinical examination.

DNA analysis

Four family members underwent molecular analysis of exon 4 of the *IRF6* gene. The samples of biological material, 3 to 4 mL of blood, were collected through venipuncture and transferred to test tubes containing EDTA. For the extraction of DNA, the Invirsob spin blood mini kit (Invitek) was used. For the amplification of the sequence of interest in the polymerase chain (Polymerase Chain Reaction, PCR), the primers used were forward – GCTCTGGGCAATGATAGGAC and reverse - ACCTTCTCCCCAGCACCT. The PCR products were subjected to direct sequencing analysis of exon 4 of *IRF6* and its exon-intron boundaries based on capillary electrophoresis, using the ABI PRISM BigDye kit, Terminator Cycle Sequencing. The same primers were used for PCR reactions and the ABI PRISM® 3500 Genetic Analyzer Capillary Sequencer by Applied Biosystems.

Data analysis

The family history information obtained from the tabulated

pedigree was inserted in the spreadsheet of the Microsoft Excel 2010 program and later analyzed with the aid of the PEDINFO program available at S.A.G.E. (Statistical analysis for genetic epidemiology) version 6.3 (2012) (<http://darwin.cwru.edu>). The analysis of the occurrence of polymorphic mutations or variations in exon 4 of the *IRF6* gene was performed using the BioEdit program version 7.1.9 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). For the in silico prediction of functional effects on the protein, the bioinformatics tools HOPE (<http://www.cmbi.ru.nl/hope>) [18], Provean (available at: <http://provean.jcvi.org/index.php>) [19], SIFT (available at: <http://sift.jcvi.org/>) [20] and PolyPhen-2 (available at: <http://genetics.bwh.harvard.edu/pph2/>) [21] were utilized. The data obtained was analyzed using descriptive statistics.

Results

The family did not present a history of consanguineous marriage and from the second generation on, where a family member has a cleft lip and pits, all subsequent generations have more than one affected relative. It is observed through the pedigree that the affected individuals belong only to the paternal, ascending and descending sides in relation to the index case

(indicated with an arrow in the pedigree, Figure 1).

Lip pits were present in 26 of the 28 affected individuals and 12 out of 28 individuals had orofacial clefts, with six of them having the lip and palate affected. Ten individuals had both pits and orofacial clefts (Table 1).

Out of the five individuals that had a dental examination (Table 2), one had permanent dentition and four had primary dentition. Three out of five had developmental dental anomalies.

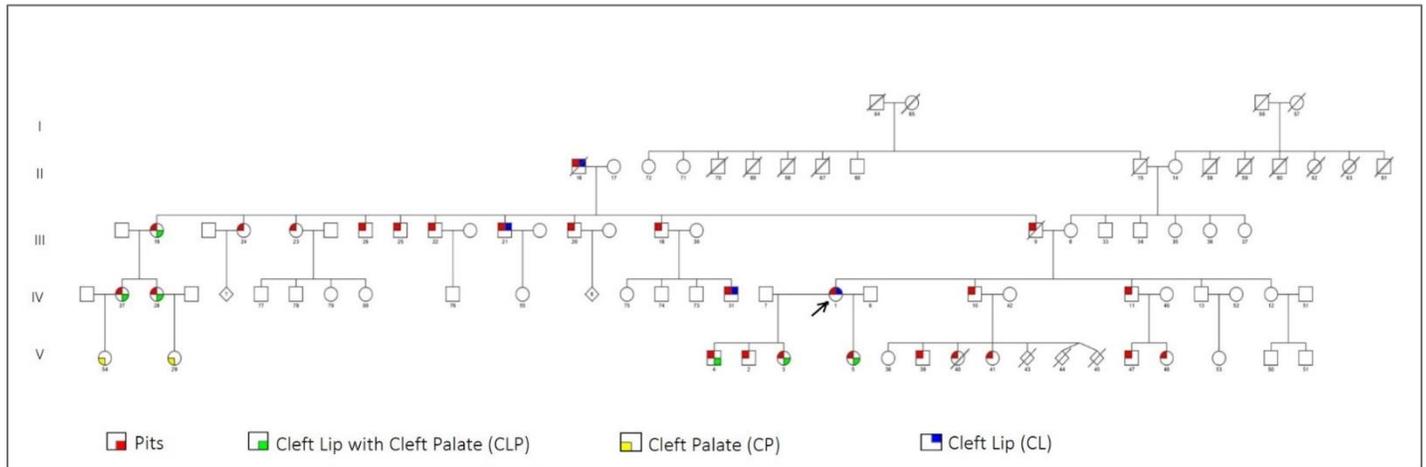


Figure 1. Pedigree showing family members affected by Van der Woude Syndrome.

Table 1. Phenotypic characteristics of Van der Woude Syndrome present in affected family members.

Phenotype	n	%
Pits	16	57
Pits and cleft lip	4	14
Pits and cleft lip with cleft palate	6	21
Cleft palate	2	7
Total	28	100

Table 2. Phenotype variation of Individuals with *IRF6* Ser90Thr variant and Van der Woude syndrome.

Affected Individuals	Pits	Orofacial clefts	Dental anomalies
 <p>27-year-old female, mother of 2, 3, 4, and 5</p>	Yes	Left cleft lip	Microdontia of right maxillary lateral incisor and agenesis of right mandibular lateral incisor
 <p>7-year-old female, half-sister of 3, 4, and 5*</p>	Yes	Bilateral cleft lip with cleft palate	Fusion of mandibular primary left lateral incisor and canine
 <p>4-year-old male, brother of 4 and 5</p>	Yes	No	Agenesis of mandibular primary left lateral incisor
 <p>2-year-old female, sister of 5</p>	Yes	Bilateral cleft lip with cleft palate	None evident
 <p>1-year-old male</p>	Yes	Bilateral cleft lip with cleft palate	None evident

*Biological sample not collected.

The sequencing analysis of exon 4 of the *IRF6* gene, performed for 4 family members (Table 2), revealed the presence of the SNP rs7552506 (NM_006147.3: c.175-5C> G), located five base pairs before exon and revealed a new variant c.269G>C (p.Ser90Thr) present in heterozygosity in the 4 affected family members (Figure 2).

The in silico analysis using the PROVEAN tool, pointed out that the c.269G> C variant (p.Ser90Thr)

should be considered as deleterious since this variant had a score of -2.704; according to the platform parameters, a variant below the score of -2.5 is considered deleterious. As for the prediction result obtained by the Poliphen-2 tool, the new variant was considered potentially deleterious, with a score of 1,000 (sensitivity: 0.00; specificity: 1.00). According to the SIFT tool this variant is predicted as tolerated with a score of 0.37.

One of the most informative prediction tools was HOPE. The analysis derived from it provided information about the structure of the mutant amino acid residue compared to the wild type of residue, showing that the mutant residue is structurally larger, a factor that can interfere in the multimeric interactions and in the conformation of the protein (Figure 3).

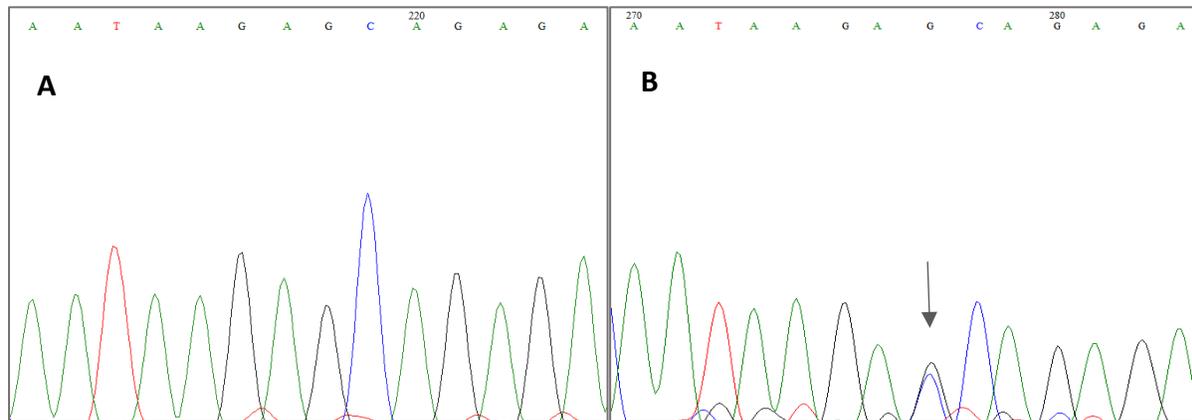


Figure 2. Electropherograms showing sequence without variant (A) and with the new mutation in exon 4 of the *IRF6* gene (B). An arrow indicates the altered nucleotide.

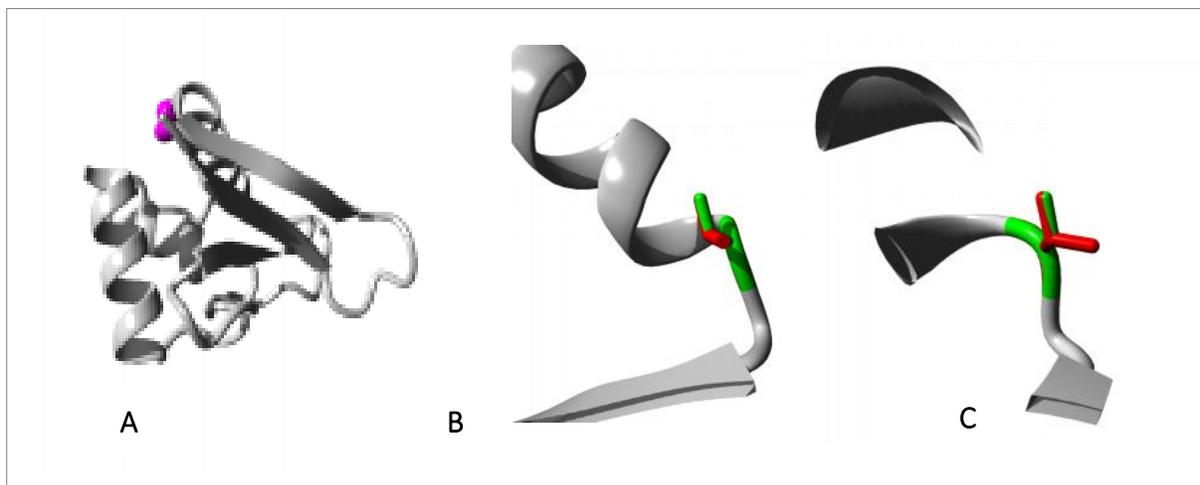


Figure 3. Images representing parts of the protein structure with the mutant residue. A. Ribbon overview of the protein, in gray, with the mutant residue represented in purple. B and C. Detailed view of the representation of the side chains of the mutant amino acid residue, in red compared to the wild residue, in green. Source: generated by the HOPE tool report.

Discussion

For the clinical diagnosis of VWS, lower lip pits must be present in one or more members of a family, together with the presence of cleft lip with or without cleft palate and cleft palate [22]. The family investigated in this study presented both the classic VWS triad and mixed types of clefts. Compared to other families, the percentage of individuals affected in the studied family (36.4%, 28/77) differs little from the percentages found by Martelli-Júnior et al. (2007) [23] who studied two Brazilian families with VWS, one with 54 and the other with 17 descendants, where the phenotypic characteristics of the syndrome were present in 22.23% and 47.06% of the family members, respectively.

Due to the fact that in each generation of the studied family there is at least one affected member and they also had at least one of their parents affected, it's clear that this family follows the expected high penetrance that can vary from 80% to 100% according to previous studies in the literature [2, 24].

Variable expression was observed in the studied family, with the main phenotypic manifestation of VWS in the affected individuals

being congenital lip pits. According to the literature, this type of pits occurs in around 85% of individuals affected by VWS and in 64% of cases it is the only manifestation of the syndrome [4, 7]. Regarding orofacial clefts, the studied family presented mixed types of clefts, with the most prevalent type (50%) being the CLP, a finding similar to other studies [2, 7, 25].

The presence of mixed types of orofacial clefts in the same family is often observed in families with VWS. In an attempt to explain this occurrence, it has been proposed that individuals with genetic components that cause VWS are subject to some type of multifactorial influence, similar to the non-syndromic form of orofacial clefts [8, 26-28]. Furthermore, another possible explanation could be the fact that another gene could be involved in this difference, for example variants in the gene *GRHL3* (Grainyhead Like Transcription Factor 3) are more likely to have CP and less likely to have CL or lip pits than individuals with an *IRF6* mutations [10]

As for the molecular analysis in exon 4, the SNP rs7552506 identified in the investigated family members is a polymorphism already described

in the literature in studies with families with non-syndromic orofacial clefts and also in studies with families with VWS [28, 29]. In the study by Pegelow et al. (2008) [30] with 17 Swedish families this SNP showed an association with CLP. In addition, in the same study it was found that when there was combined unilateral and bilateral CLP in the same family, there was an association of this polymorphism. This information corroborates the findings in the present study.

According to the analysis performed by the HOPE tool, the c.269G> C variant (p.Ser90Thr) found in the present study results in a mutant amino acid residue that is larger than the wild type residue, which can influence the conformation of the protein that will be slightly destabilized and, in addition, there may be disturbances in the multimeric interactions. The Ser90 position of the polypeptide chain is located in the DNA-binding domain (structural motif of the helix-gyro-helix type), which is important for the binding of other molecules and is in contact with the residue of a regulatory domain. Therefore, this mutation can affect this interaction and disrupt protein regulation. Variants that occur in the *IRF6* gene are mostly missense-type and occur mainly in

the DNA-binding or protein-binding domains; variants in these domains are critical to the function of the *IRF6* gene [8, 29, 31]. In addition, the silico analysis to verify the effect on the protein conformation of *IRF6*, suggests that this mutation is potentially pathogenic.

More specifically, the wild-type residue interacts with the amino acid residue Alanine at position 86 of the polypeptide chain; the size difference observed in the mutant residue prevents it from being in the correct position to perform the hydrogen bonding possible in the wild type of residue. Only two mutations for position 90 of the protein chain encoded by the *IRF6* gene are described in the literature, one of which is mutation c. A268G (p.Ser90Gly) as described by Kondo et al. (2002) [8] that causes an exchange of the amino acid residue Serine for the amino acid residue Glycine. The other mutation, c.G269T (p.Ser90I12) as described by Lima et al. (2009) [29], promotes the exchange of the amino acid residue Serine for the amino acid residue Isoleucine.

Among the dental anomalies associated with VWS, the most common among patients is hypodontia [32]. In our study, individuals who presented dental

anomalies, as tooth agenesis were in the mandibular arch, which is something already predicted in the literature. Syndromic agenesis generally appears associated with other manifestations such as microdontia and the upper lateral incisor is the most frequently affected tooth in the cleft area [33, 34]. Considering the data shown in Table 2, it's possible to see the phenotypic variation of the cases presented despite carrying the same genetic mutation, suggesting that distinct genetic modifiers operate on the formation of clefts and the dental development.

Despite being one of the most studied syndromic forms of orofacial clefts, this study reveals how much remains to be discovered in terms of how genetic factors act underlying the phenotypic expression of VWS. In addition, we emphasize that families with this syndrome still go unnoticed in health systems. Observationally, many families in turn see congenital lower lip pits as a family trait and therefore fail to seek specialized care and appropriate treatment. Thus, it is also expected that this study will contribute in the future to the genetic counseling for these families and also will improve the knowledge and qualifications of

health professionals who care for them.

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Conflicts of interest

The authors declare no competing interest.

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