RESEARCH LETTER

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Novel Mutations in *IRF6* in Nonsyndromic Cleft Lip With or Without Cleft Palate: When Should *IRF6* Mutational Screening be Done?

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TO THE EDITOR:

Cleft lip with or without cleft palate (CL/P) is one of the most common congenital malformations observed in humans, with an incidence of approximately 1/1,000 live births [Gorlin et al., 2001]. CL/P has been associated with more than 300 recognizable syndromes, but is more often observed as an isolated birth defect referred as nonsyndromic cleft lip with or without cleft palate (NSCL/P). The etiology of NSCL/P is multifactorial and recurrence risk varies from 4% to 10% according to the number of affected individuals in the family and the relationship to the propositus [Harper, 2004]. In contrast, Van der Woude syndrome (VWS) is the most common form of syndromic CL/P and accounts for 2% of all orofacial cleft cases [Schutte et al., 1996]. VWS is caused by mutations in the Interferon Regulatory Factor 6 gene (*IRF6*) [Kondo et al., 2002] and is inherited as an autosomal dominant disorder with an estimated penetrance of 89–99% [Burdick et al., 1985; Gorlin et al., 2001]. Clinical expressivity is variable and is characterized by the presence of CL/P or cleft palate (CP), lower-lip pits and hypodontia [Wang et al., 2003]. VWS has been considered an exception to the rule that CL/P and CP should be viewed as distinct conditions, as it is not uncommon to observe these two types of malformations segregating in the same genealogy. Orofacial clefting is not accompanied by lower lip pits in approximately 14% of VWS patients [Burdick et al., 1985]. This situation creates an overlapping phenotype between VWS and NSCL/P and can result in an incorrect recurrence risk calculation for the patient and his family. Several reports failed to detect pathological mutation in *IRF6* in NSCL/P patients [Zucchero et al., 2004; Birnbaum et al., 2008; Pegelow et al., 2008].

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We have estimated the expected number of affected individuals with VWS in our sample of familial NSCL/P, assuming the prevalence of multifactorial NSCL/P as 1:1,000, of VWS as 1:35,000, and nonpenetrance rate of lip pits in VWS as 10%. The prior probabilities favoring the hypotheses of multifactorial NSCL/P and VWS without pits are therefore in the ratio 1/1,000:1/350,000 or 350:1. This means that it is 350 times more probable that a NSCL/P

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individual without lip pits represents a case of multifactorial mechanism. The recurrence risk for a second first degree affected relative (offspring or sib to an affected individual) for cases of multifactorial NSCL/P is around 4/100 to 5/100 (average figure of 0.045). The recurrence risk for the offspring of a VWS patient is K/2, while the corresponding risk to a sib of a VWS patient with normal parents is K(1-K)/2 [Otto and Maestrelli, 2000], where K is the penetrance rate. Assuming an average value of about 0.9 for the penetrance rate K, the risks above take values 0.45 and 0.045 respectively for the offspring or for a sib of an affected isolated case of VWS. Thus, in families with two first-degree affected members, the ratio of the probabilities of multifactorial NSCL/P and VWS are: (a) 350:1 in the case of affected sib-pairs, that is exactly as the prior probabilities were, because the recurrence risks on both hypotheses are of the same order of magnitude. This means the probability that a patient without lip pits and having an affected sibling being a carrier of a VWS mutation is 1/351 (0.3%); (b) In the case of affected parent-offspring pairs, however, the ratio becomes 35:1 because the recurrence risk of VWS is ten times that of multifactorial NSCL/P in this case. Therefore, the probability that a patient without lip pits and having an affected parent or offspring relative carrying a VWS mutation is 1/36 or about 3%. Probabilities favoring both hypotheses (multifactorial NSCL/P or VWS) can be calculated for situations in which the other affected is a more distant relative. For these cases, the chances favoring the multifactorial hypothesis become greater and the corresponding recurrence risks will not differ significantly from population incidence rates.

To verify the proportion of VWS with nonpenetrance of lip pits among all NSCL/P patients, we screened 108 multiplex families with NSCL/P for mutations in *IRF6*. Inclusion criteria were based on the presence of at least two affected individuals with NSCL/P, NSCP or congenital healed cleft lip in the same family. None of the patients or their parents reported the presence of lip pits in any member of their family. This study was approved by the Research Ethics Committee of the Institute of Biosciences, University of São Paulo, Brazil. Seventy-six propositi (70.4%) had cleft lip and cleft palate, 24 had cleft lip only (22.2%), six presented with isolated cleft palate (5.6%) and two had a congenital healed cleft lip (1.9%). The number of affected individuals per family varied from two to 11, 65 families had only two affected members (60.2%). In four cases CP and CL/P were segregating in the same family. In our sample, 32 propositi (29.6%) had at least one parent or offspring affected, 21 propositi (19.4%) had at least one affected sibling, 28 (25.9%) had at least one second-degree relative affected (grandparents, uncles, aunts, nieces, and nephews), and in 27 families (25%) the relationship between affected individuals was more distant.

One affected member of each family was screened for mutations in exons 3, 4, and 7 of *IRF6*, as approximately 78% of recognized pathogenic mutations have been located in these exons [Kondo et al., 2002]. The frequency of genotypes at three known SNPs in the screened regions (rs7552506, in intron 3; rs2235371, in exon 7, that leads to the p.Val274Iso change; and rs2235373, in intron 7) in our sample did not differ from frequencies reported in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/SNP/) or in a control Brazilian sample (for SNPs rs2235371 and rs2235373—results not shown). Four distinct probable causative mutations were found in four out of the 108 screened patients, as discussed below.

Patient III:4 from family I (Figs. 1 and 2G,H) has CL/P and his brother CP while the parents are unaffected. During clinical evaluation performed by clinical geneticists, plastic surgeons and speech therapists, no lip pits were noted. The propositus is heterozygous for the missense mutation p.Arg9Trp (c.25 C>T; exon 3). We were unable to test the affected brother and other relatives. Upon close reevaluation of the patient after molecular testing we noted a little reentrance in his lower lip which could be classified as an atypical pit (Fig. 2H). The p.Arg9Trp mutation was first described in a familial case where both mother and son had CL/P and a single lip pit, but the maternal grandmother (who was also a carrier of the mutation) was unaffected [Matsuzawa et al., 2004]. Our findings of two affected siblings born from unaffected parents may corroborate the reduced penetrance of the p.Arg9Trp mutation, including atypical lip pits. We cannot exclude, however, the possibility of germline mosaicism in this family.

Patient I:1 from family II (Fig. 1) and his two sons are affected by NSCL/P. Evaluation of the family members did not reveal any alterations in their lips. All affected individuals carry the missense mutation p.Gln17Pro (c.50 A>C, exon 3) that leads to an amino acid change in the DNA-binding domain of IRF6. We considered this mutation as probably causative because it segregates with the CL/P phenotype in the family and because the glutamine residue is conserved in 24 out of 28 vertebrate species, including fishes, reptiles, amphibians, birds, marsupials, monotreme and placental mammals (UCSC Browser multiple alignment—http://genome.ucsc.edu/). Furthermore, this mutation was not observed in a control Brazilian population.

Family III (Fig. 1) has three affected members: the proposita (III:3—Fig. 2A,B), with CP, her half-sister, with CL/P and a maternal deceased half uncle affected by CP. The proposita and her unaffected mother (Fig. 2C,D), the only available members for genetic testing, are heterozygous for the novel mutation, c.1060 G>A. It was not observed in 200 control chromosomes. This change is predicted to result in the substitution p.Asp354Asn in the protein-binding domain, a possible critical functional region of the IRF6 protein. Although aspartic acid at this position is conserved in primates and other placental mammals, it was found to be replaced by glutamic acid in mouse, rat, chicken, lizard and *Xenopus* and by glycine in zebrafish and fugu. On the other hand, the guanine residue at c.1060, located one single nucleotide upstream of the exon 7 splice donor site is highly conserved among all the vertebrates (UCSC Browser multiple alignment). The p.Asp354Asn substitution decreases the predicted splicing score of mRNA from 4.40 to 1.75 (http://www.tigr.org/tdb/GeneSplicer/gene_spl.html). Alterations at this position in donor sites are observed in several diseases and result in different degrees of splicing defects [Baralle and Baralle, 2005; Krawczak et al., 2007]. The p.Asp354Asn substitution by itself is probably not causative for oral clefting in the proposita. It could, however, alter gene expression levels or IRF6 function, and be important as a predisposition factor that, combined with the genetic paternal background, is responsible for the phenotype in the child. The affected uncle from the maternal family is a result of a consanguineous relationship, which also increases the number of risk alleles inherited. The inheritance pattern in family III seems to be in agreement with a model of multifactorial mechanism.

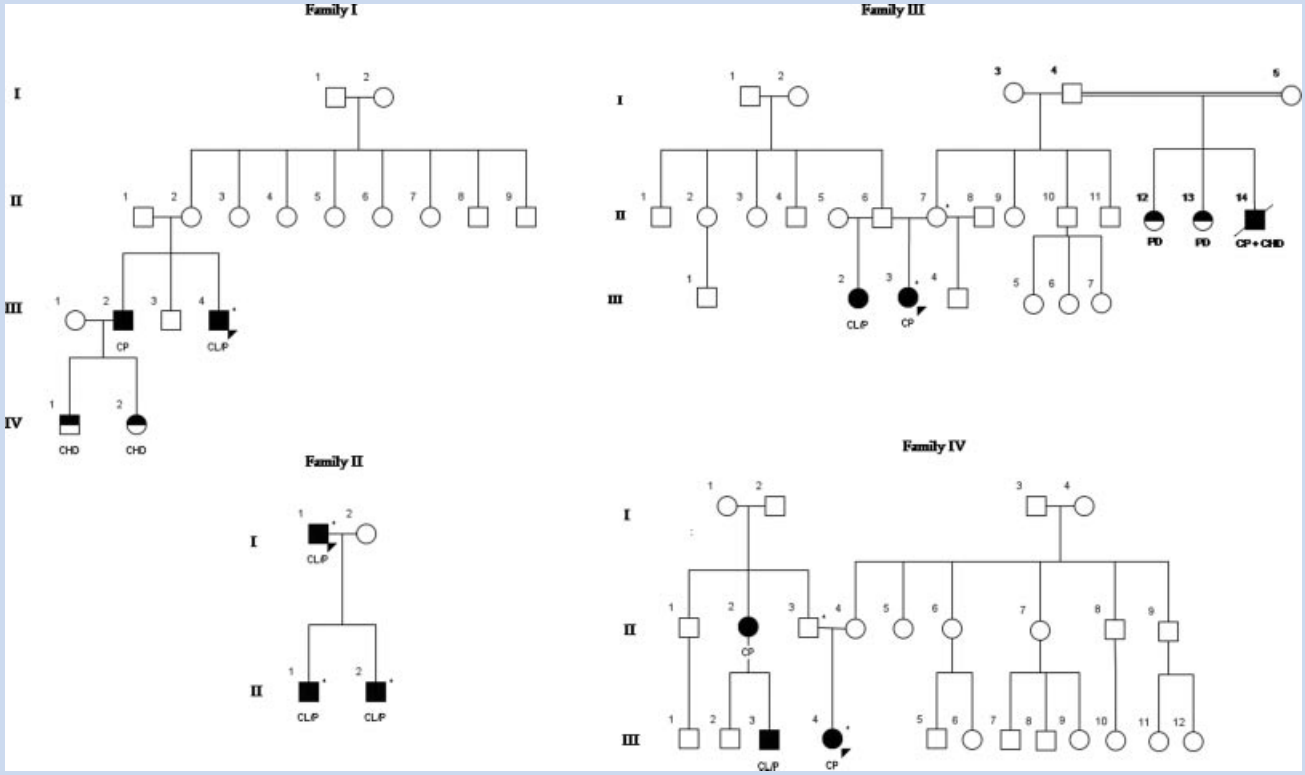

FIG. 1. Pedigrees of the families described. Arrow head indicates proband; CL/P, cleft lip with cleft palate; CP, cleft palate only, CHD, congenital heart defect, PS, psychiatric disorder; asterisks show individuals with mutations.

Patient III:4 from family IV (Figs. 1 and 2E,F) was first diagnosed as Robin sequence, presenting micrognathia, CP and glossoptosis. The paternal aunt of the propositus also presented with CP and had a son with CL/P, but they were not available for testing. The propositus and the unaffected father are heterozygous for a novel missense mutation: p.Val113Leu (c.337 G>C, exon 4). Closer reevaluation of the patient's lower lips revealed irregularities that were not considered lip pits (Fig. 2F). We suggest this change can be related to the phenotype in this family because the valine residue is located at the boundary of the winged-helix DNA-binding domain

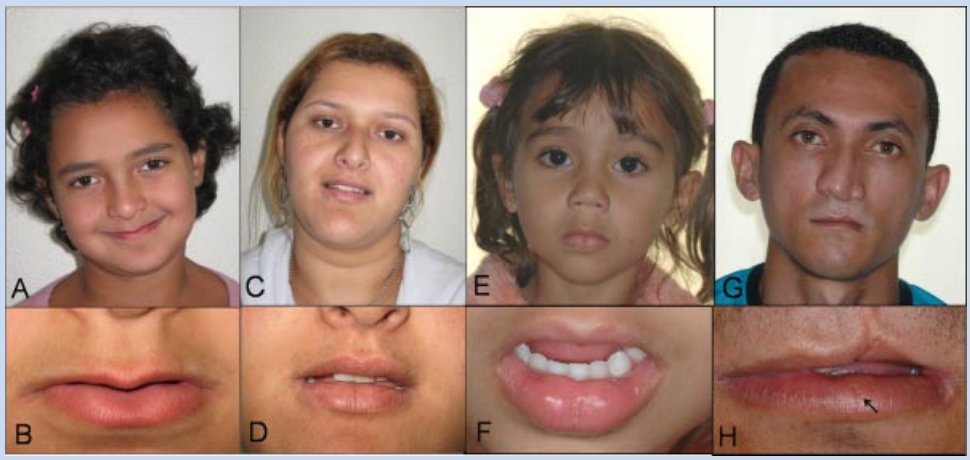

FIG. 2. Facial appearance and close view of lower lips from (A,B), patient III:3 (family III); (C,D) unaffected carrier II:7 (family III); (E,F) patient III:4 (family IV), and (G,H) patient III:4 (family I). Arrow shows single atypical lower lip pit. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and is evolutionarily conserved. Besides, this change was not found in 200 control chromosomes.

Based on our above estimates on expected frequency of VWS without presence of lip pits among NSCL/P (3% for parent-offspring pairs and 0.3% for affected sib pairs) and considering 32 of our families had a parent-offspring affected pair and 21 had affected sib pairs, we would expect one to two individuals with mutations in *IRF6* among our 108 NSCL/P propositi had we screened whole gene. Since we sequenced only three exons, where about 78% of the mutations are located, we would expect to find at least one individual with mutation in our sample. Our screening detected four pathogenic or predisposing mutations, a proportion higher than expected. Three of these mutations were found in families (I, III, and IV) segregating both CL/P and CP, which are considered to have distinct embryological origins and rarely appear in the same family, except in VWS and MSX1 cases [van den Boogaard et al., 2000]. It is noteworthy the detection rate of *IRF6* mutations was 75% (3/4) among families segregating both CL/P and CP and 3% (1/32) among those who had a parent or offspring affected with the same type of orofacial clefting.

A few other reports failed to detect *IRF6* mutations in NSCL/P patients. Zucchero et al. [2004] screened the whole coding sequence of *IRF6* in 160 NSCL/P individuals without finding any causative mutations. It is not clear, however if their sample included families with CL/P recurrence. Negative results were found by Pegelow et al. [2008] in their sample of only 17 families with NSCL/P recurrence, one of them with a mixed clefting type. These authors suggest mutations in *IRF6* are not common in NSCL/P, but their sample is extremely small considering the incidence of VWS. On the other hand, Birnbaum et al. [2008] found two families segregating causative *IRF6* mutations when 63 families with at least two consecutive affected members were screened for the whole gene. These patients were shown to have had their lip pits surgically removed prior to the genetic screening, demonstrating, similarly to the case in family I, the difficulties faced by clinicians to properly characterize VWS patients. It is possible the higher proportion of mutations in our sample results from: (a) enrichment for families segregating both CL/P and CP; (b) the penetrance of VWS mutations is actually lower than reported and (c) screening the largest sample of familial NSCL/P reported to date.

In summary, we present, for the first time, mutations in *IRF6* in NSCP/L without or with atypical lower lip pits. Only one of these mutations was previously reported in VWS patients. It is possible the mutations herein described are associated with a lower penetrance of lip pits or VWS. Based on our findings, we suggest NSCL/P families eligible for the *IRF6* screening are those with at least one affected parent-offspring pair and particularly those segregating CP and CL/P.

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