SHORT REPORT

Interferon regulatory factor-6: a gene predisposing to isolated cleft lip with or without cleft palate in the Belgian population

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Cleft lip with or without cleft palate is the most frequent craniofacial malformation in humans (~1/700). Its etiology is multifactorial; some are a result of a genetic mutation, while others may be due to environmental factors, with genetic predisposition playing an important role. The prevalence varies widely between populations and the mode of inheritance remains controversial. The *interferon regulatory factor-6* (*IRF6*) gene has been shown to harbor mutations in patients with van der Woude syndrome, a dominant form of clefts associated with small pits of the lower lip. Moreover *IRF6* has been associated with nonsyndromic cleft of the palate (CL/P) in two separate studies. We investigated the role of *IRF6* in a set of 195 trios from Belgium. Cleft occurred as an isolated feature. We studied association of the *IRF6* locus using two variants: one in the *IRF6* gene and the other 100 kbp 3' of the gene. Our independent study group confirms that the *IRF6* locus is associated with nonsyndromic cleft lip with or without palate. This result, with previous studies performed in the United States and Italy, shows for the first time the implication of *IRF6* in isolated CL/P in northern Europe. It is likely that association to this locus can be identified in various populations and that the *IRF6* locus thus represents an important genetic modifier for this multifactorial malformation.

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Introduction

Normal development of lips and palate is a highly regulated and complex process.¹ On the basis of murine models, human syndromes, association and expression

studies, a wide range of transcription factors, growth factors and signaling molecules have been shown to be involved in this process.^{2–5} When the structure or expression of one or more of these genes is disrupted, a cleft of the lip with or without a cleft of the palate (CL/P) may occur. Despite recent investigations on the mode of inheritance and interaction of different factors, genetics of orofacial clefts remains controversial.

Approximately 70% of CL/P cases are nonsyndromic, occurring as an isolated condition without any other associated recognizable anomaly. The remaining 30% are

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syndromic, associated with other abnormalities.^{6,7} The nonsyndromic forms of orofacial clefts are likely due to gene–environment interactions, whereas the syndromic forms are mainly due to genetic alterations.

Inconsistent results have accumulated from genetic studies of complex traits, such as nonsyndromic CL/P, over the past decade. Numerous linkage and association studies performed to search for genes predisposing to CL/P could not be replicated.⁷ This is not unexpected, given the complicated heterogeneous nature of isolated CL/P and the number of confounding factors. In addition, the resolution of genetic scans has not been sufficient. An interesting approach to study genetics of clefts is to evaluate genes known to contribute to syndromic forms of CL/P and especially to those phenotypically closest to nonsyndromic CL/P. They may serve as a starting point to unravel genetic variants for nonsyndromic CL/P.

The best phenocopy for isolated clefts is probably the van der Woude syndrome (VWS, OMIM# 119300). It is a dominantly inherited disorder in which pits of the lower lip (85% of cases) and hypodontia (25% of cases) are the only features distinguishing it from isolated CL/P.^{8,9} Linkage studies pointed to a locus on 1q32–q41.¹⁰ Recently, the *interferon regulatory factor-6* (*IRF6*) gene on 1q32.2 was shown to harbor mutations in patients with van der Woude and/or popliteal pterygium syndrome,¹¹ and this has been since reproduced in other studies.¹² Moreover, studies investigating the role of *IRF6* in the occurrence of isolated clefts showed strong evidence of overtransmission of the V allele of an intragenic *IRF6* polymorphism (V274I) in Asian and South American populations.¹³ Moreover, linkage disequilibrium with CL/P was detected from 40 kbp 5' of *IRF6* to 135 kbp 3' of *IRF6* in the Iowan and Danish populations.¹³ This result was confirmed by a study supporting the involvement of *IRF6* in nonsyndromic CL/P in an Italian population.¹⁴ Here, we show that *IRF6* gene is likely implicated in nonsyndromic CL/P across different populations as we show study association between the intragenic SNP and CL/P in the Belgian population.

Materials and methods

Family assessment

Families were collected in collaboration with the Cleft Lip and Palate Center, Cliniques universitaires Saint Luc,

Brussels, Belgium. Families are from Belgium, which has a heterogeneous population with immigrants from various origins.

Informed consent was obtained from each participant prior to the participation in the study, as approved by the local ethics committee. All patients were ascertained by contributing physicians for the absence of other anomalies and only isolated clefts were included in this study. Patients were asked about familial cleft history. Cleft occurred as a sporadic feature in 190 children, and in five families, the affected child had one affected parent or sibling. As cleft palate is genetically different from cleft lip with or without cleft palate, no cleft palate-only patients were included in this study. Overall, 195 families (190 as trios) with altogether 498 persons were included in this study.

Venous blood samples were drawn for the majority of adult participants. Genomic DNA was extracted from the buffy coats using 'QIAamp DNA Blood Mini Kit, Qiagen Inc., Valencia, CA, USA' or from whole blood using 'DNA purification kit, Gentra systems Inc., Minneapolis, MN, USA'. When whole-blood collection was not possible, DNA was extracted from a buccal-cell sample.

Genotyping

Two single-nucleotide polymorphisms (SNPs) rs2013162 in *IRF6* gene and rs2235543 100 kbp 3' of the gene were genotyped (Table 1, Figure 1). The same SNPs were used in the two previous studies,^{13,14} which allows an easy comparison of the results. These SNPs were highly associated with CL/P in the two reports.^{13,14} Genotyping was performed using the Taqman chemistry (Applied Biosystems) as described previously.¹⁴

Statistical analysis

Hardy–Weinberg equilibrium was assessed in both patients and parents. Transmission disequilibrium test (TDT) was used to measure the distortion of the inheritance of alleles

Table 1 SNPs information

	SNP i.d.	Position	Alleles ^a
1	rs2013162	in <i>IRF6</i>	G/T
2	rs2235543	100 kbp 3' of <i>IRF6</i>	G/A

^aCommon allele listed first, in bold.

Figure 1 Structure of the *IRF6* gene. Exons (rectangles) are drawn to scale, except for exon 9, which is longer than shown. Untranslated regions are smaller rectangles. Exons 3 and 4 encode the DNA-binding domain and exons 7 and 8 code for the protein-binding domain (both in gray). Line connecting exons represents introns and 5'-, 3'-untranslated regions. Arrows indicate the position of the two SNPs in exon 5 of *IRF6* and 100 kbp 3' of the gene, respectively.

from parents to affected offspring. The null hypothesis was that the transmission of alleles from parents to child was at random.¹⁵ Association between the 2 markers and CL/P was tested using Genehunter¹⁶ and the Family Based Association Test (FBAT).^{17–19} Haplotype transmission disequilibrium was calculated using haplotype-based association test.¹⁹ Linkage disequilibrium between the two markers was assessed using the ldmax program from the GOLD software package.²⁰

Results

Hardy–Weinberg equilibrium was assessed for the two SNPs in the Belgian population to ascertain if there was a deviation that would bias our findings. χ^2 tests were performed and Hardy–Weinberg equilibrium was observed in both patients and parents.

Results of the TDT showed a significant association of the intragenic rs2013162 SNP with clefting in both Genehunter ($P=0.02$; results not shown) and FBAT analysis (0.01) (Table 2).

No significant association was obtained when we genotyped the marker 100 kbp 3' of the gene. Nevertheless, there was a trend towards borderline positive association ($P=0.3$) (Table 2). This marker lacked a high degree of heterozygosity in our sample (20.2%), and thus most parents were uninformative. For both markers, the common allele (G) was the one that was overtransmitted.

To increase informativeness, we also conducted haplotype-based transmission disequilibrium analysis using genotype data. The haplotype made of the two frequent alleles at each SNP had the most significant distortion ($P=0.004$), whereas the haplotype made of the two rare alleles showed no association with the condition (Table 3).

Linkage disequilibrium between markers genotyped in this study showed that their association was not random ($D^2=0.014$; $D'=0.427$).

Discussion

The majority of genetic variants underlying susceptibility to isolated clefts are yet to be discovered. Several genes accounting for a sizeable fraction of the syndromic forms of CL/P occurring in a Mendelian pattern have been identified (*TBX22*, *MSX1*, *P63*, *FGFR1*, *TGFBR*, etc).^{21–25} Mutations in these genes, however, account for less than 15% of the total genetic susceptibility for isolated CL/P and the remaining component is likely to be polygenic in nature – the interaction of mutations in multiple genes, each with a weak effect, in combination with environmental influences.

The identification of all these genes will likely require large case–control association studies. Until the time when complete genome sequence information is feasible to obtain all study subjects, interim strategies involving a

Table 2 TDT summary

	SNP	Allele ^a	A. frequency ^b	T/NT ^c	P^d
1	rs2013162	G	0.659	64/41	0.014
2	rs2235543	G	0.856	23/16	0.303

^aCommon allele.

^bCommon allele frequency.

^cTransmission/nontransmission of the common allele.

^d P -value for TDT.

Table 3 Haplotypes showing transmission distortion

	Haplotype	H. frequency	P^a
1	GG	0.556	0.004
2	TG	0.315	0.036
3	GA	0.1	0.16
4	TA	0.028	

^a P -value for transmission distortion.

subset of all common genetic variations (primarily SNPs) need to be used. Assuming the 'common disease–common variant' theory of complex disease susceptibility, a very dense set of genetic markers is likely to identify susceptibility genes, by including either the biologically relevant variant or one closely associated with it.

VWS closely mimics isolated CL/P, with lip pits and dental abnormalities being the only features distinguishing this syndrome from the isolated condition. Moreover, previous studies have implicated *IRF6*, the VWS causative gene, in isolated cleft lip or palate.^{13,14} We used the TDT to see if we can replicate these results in our CL/P cohort collected within the Belgian population. We found strong evidence for association supporting the role of *IRF6* in nonsyndromic CL/P in different populations.

In this study, we analyzed the two SNPs in the *IRF6* locus that have earlier shown in haplotype analysis the strongest association with CL/P in two different populations. Whether these SNPs are causative themselves or whether they are only in linkage disequilibrium with the disease-causing variants is yet to be elucidated. In our study population, linkage disequilibrium between the two markers was stronger than in the Filipinos, but less significant than in the Italian study. The polymorphism V274I located in exon 7 of *IRF6* showed a significant association between the V allele and nonsyndromic CL/P in Asian and South American populations, but not in Europeans.¹³ We could not identify the rare I allele in 110 tested controls from Europe, nor in a set of European individuals with VWS.¹² Based on these two observations, the role of this polymorphism was not investigated in this study, and it is likely not the disease-causing variant.

In this study, we were able to reproduce the results that implicate the *IRF6* gene in a complex trait. The 195 trios

was clearly a big enough sample to observe the association, which is a necessity for studies of complex traits. As *IRF6* shows such a strong association with CL/P, it is interesting to speculate that it may also be a modifier to the phenotypic variability within families with a single *IRF6* mutation causing VWS and PPS.

After implicating *IRF6* in the complex etiology of isolated CL/P, the question remains: how does *IRF6* cause CL/P? Which are the target genes? A better understanding of the function, the targets and the pathways implicating this transcription factor is essential for a better comprehension of the cleft formation as well as for unraveling other CL/P-associated factors.

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