European Journal of Orthodontics 30 (2008) 169–175
doi:10.1093/ejo/cjm097
Advance Access publication 21 January 2008

Familial non-syndromic cleft lip and palate—analysis of the *IRF6* gene and clinical phenotypes

M. Pegelow*,*****, M. Peyrard-Janvid**, M. Zucchelli**, I. Fransson**, O. Larson****,*****, J. Kere**, C. Larsson*** and A. Karsten*,*****

Departments of *Orthodontics and **Biosciences and Nutrition, Karolinska Institutet, Huddinge, Departments of ***Molecular Medicine and Surgery, ****Reconstructive Plastic Surgery, and *****Stockholm Craniofacial Team, Karolinska University Hospital Solna, Sweden

SUMMARY The aim of this study was to characterize Swedish families with non-syndromic cleft lip and/or palate (NSCL/P) for mutations or other sequence variants in the interferon regulatory factor 6 (*IRF6)* gene, as well as to describe their cleft phenotypes and hypodontia. Seventeen Swedish families with at least two family members with NSCL/P were identified and clinically evaluated. Extracted DNA from blood samples was used for *IRF6* mutation screening. Exonic fragments of the *IRF6* gene were sequenced and chromatograms were inspected. Statistical analysis was undertaken with marker- and haplotype association tests.

No disease-associated *IRF6* mutation could be determined in the families analyzed. One new and seven known single nucleotide polymorphisms (SNPs) were detected. The A allele of SNP rs861019 in exon 2 and the G allele of SNP rs7552506 in intron 3 showed association with cleft lip and palate (CLP; odds ratios of 3.1 and 5.45, respectively). Hypodontia was observed more commonly in individuals affected with CL/P as compared with family members without a cleft ($P < 0.01$). The hypodontia most often affected the cleft area, possibly representing a secondary effect. The distribution of cleft phenotypes in 15 of the 17 families with NSCL/P differed from the mixed cleft types seen in Van der Woude syndrome (VWS), in that CLP did not occur together with an isolated cleft palate within the same family. It was concluded that mutations of the *IRF6* gene are not a common cause for cleft predisposition in Swedish NSCL/P families.

Introduction

A cleft lip, with or without a cleft palate (CL/P), is the most common orofacial birth defect (Marazita and Mooney, 2004). A CL/P has a major clinical impact requiring surgical, dental, and orthodontic treatment, as well as therapies related to speech, hearing, and psychological aspects. The worldwide incidence is one to two cases per 1000 live births but geographic variation is considerable (Gorlin *et al.*, 2001; Lidral and Murray, 2004). The incidence in Sweden is 1.2–2.0/1000 depending on the cleft type (Hagberg *et al.*, 1998). A CL/P occurs in approximately 70 per cent of the subjects as non-syndromic, that is without associated malformations in any other organs (Schutte and Murray, 1999). Non-syndromic cleft lip and/or palate (NSCL/P) develops during early embryogenesis. During weeks 4–7, the medial and lateral nasal processes join the maxillary process to form the primary palate and the central part of the upper lip and nose. During weeks 8–12, the secondary palatal shelves fuse to form the hard and soft palate. Failure at any of these joining points will result in a cleft, sometimes together with hypodontia (Meikle, 2002). Molecular studies of animal models have contributed to the understanding of normal lip development and several signalling pathways have been linked to these processes (Jiang *et al.*, 2006). Families of genes encoding for proteins such as bone morphogenetic proteins, fibroblast growth factors, wingless-type integration families, and sonic hedgehogs are such examples.

Molecular studies of syndromic CL/P have identified approximately 30 human genes in which mutations could lead to CL/P as a major or an associated phenotype (Schutte and Murray, 1999; Cobourne, 2004; Jugessur and Murray, 2005). Given the concept that mutations in a single gene can contribute to a spectrum of phenotypes, the known disease genes for syndromic CL/P are also good candidates for involvement in familial forms of NSCL/P (Wong and Hägg, 2004). This is exemplified by the X-linked *TBX22* gene involved in familial and isolated cleft palate (CP; Marçano *et al.*, 2004). The human interferon regulatory factor 6 gene (*IRF6*) from chromosomal region 1q32.2 has previously been shown to be responsible for the majority of patients with Van der Woude syndrome (VWS, Online Mendelian Inheritance in Man: #119300; Kondo *et al.*, 2002). VWS patients present a CL/P phenotype (approximately 50 per cent) and additional malformations such as lip pits (approximately 80 per cent) and hypodontia (approximately 25 per cent; Van der Woude, 1954; Janku *et al.*, 1980; Shprintzen *et al.*, 1980; Ranta and Rintala, 1983; Rizos and Spyropoulos, 2004). A higher recurrence risk for NSCL/P

has been reported for individuals carrying the C allele at the single nucleotide polymorphism (SNP) rs2235371 coding for amino-acid Val274 of the *IRF6* gene product (Ghassibe *et al.*, 2004; Zucchero *et al.*, 2004; Blanton *et al.*, 2005; Srichomthong *et al.*, 2005).

The aim of this study was to characterize Swedish families with NSCL/P for mutations or other sequence variants in the *IRF6* gene, as well as to describe their cleft-phenotypes and hypodontia.

Subjects and methods

NSCL/P patients

The research was approved by the local ethical committee in Stockholm (dnr.163/93) and informed consent was obtained from all participants or their parents.

The study included families with familial NSCL/P (two or more family members with NSCL/P) identified and treated by the Stockholm Craniofacial Team, Sweden. Initially, 19 families were selected. Affected individuals from two of the families were later found to have features associated with Sticklers syndrome or VWS and were therefore excluded. The pedigrees and individual phenotypes of the 17 families are shown in Figure 1 and Table 1. The diagnosis of CL/P and hypodontia were based on clinical examination, medical records, telephone interviews, and information from other family members. The detected NSCL/P phenotypes included bilateral cleft lip and palate (BCLP), unilateral cleft lip and palate (UCLP—CLP) when combined in one group, CL, and isolated CP (Gorlin *et al.*, 2001). A subset of patients with NSCL/P also had hypodontia. One family member without CL/P had hypodontia only (family 16). The pedigrees of families 1, 2, and 4 have previously been reported (Wong *et al.*, 2000). Pedigrees were drawn using the Cyrillic 2.1 software (www.cyrillicsoftware.com). For sequencing of the *IRF6* gene, peripheral blood was obtained from 43 individuals (23 affected and 20 healthy). Of the 20 healthy individuals, three were obligate carriers (families 9, 11, and 16; Figure 1).

Sequencing of IRF6

Genomic DNA was extracted from peripheral blood using a standard method (Lahiri and Nurnberger, 1991). *IRF6* mutation screening was performed using previously published primers and laboratory conditions (Peyrard-Janvid *et al.*, 2005). In summary, exonic fragments of the *IRF6* gene were amplified by polymerase chain reaction and directly sequenced on both DNA strands. The following parts of the *IRF6* gene were targeted for sequencing: the entire open reading frame (exons 3–9), the entire 5′-untranslated region (5′-UTR; exons 1–2), parts of the 3′-UTR (exon 9), and corresponding exon–intron splice sites with up to 150 bp of flanking intronic sequences. The sequences were analysed with the Sequence Analyser 3.0 software (Amersham Biosciences, GE Heathcorp, Amersham, Buckinghamshire, UK) and sequences were aligned using the Pregap and Gap4 software from the Staden package (www.staden.sourceforge.net). Chromatograms were visually inspected by two independent researchers in order to detect sequence variations.

Statistical analyses

Statistical analysis was performed on the 17 families screened for *IRF6* mutations in order to detect possible linkage disequilibrium between SNPs and to build the subsequent block structure (Haploview software, www.broad.mit.edu/mpg/haploview; Barrett *et al.*, 2005). Marker- and haplotype–disease association were tested using the family-based association test software FBAT (Lange *et al.*, 2003). Association analysis with independent affected and healthy family members was performed. Chi-square test was undertaken to compare cleft phenotypes and frequencies of hypodontia.

Results

Phenotypes of the NSCL/P families

In 14 families, individuals affected by CL/P were observed in two, three, or four generations. In the remaining three families (families 6, 14, and 15), the affected individuals were from the same generation, as siblings or cousins. In three of the families with a BCLP phenotype (families 4, 12, and 17), BCLP was observed in both parents and children, suggesting vertical transmission of the predisposition. Two families (6 and 11) presented only CP. In the 12 remaining families, both CL and CLP phenotypes were observed within each family (Figure 1).

Hypodontia was registered in 13 patients with UCL, UCLP, BCL, or BCLP from 10 of the 17 families (Tables 1 and 2). It was significantly more common in the affected family members (13/50 individuals or 26 per cent) than in relatives without a cleft (1/105 individuals or 0.95 per cent) (Table 2). In addition, hypodontia was significantly ($P < 0.001$) more frequent in individuals with a UCLP phenotype, as eight of 18 UCLP individuals had hypodontia, all in the cleft region. In five of the eight affected individuals, hypodontia was seen at the lateral incisor (12 or 22) on the cleft side. One patient (affected with BCLP) had hypodontia also in the lateral segments (multiple aplasias). Hypodontia was not found in children with isolated CP. In family 16, one relative without a cleft, but who was an obligate carrier, had hypodontia.

IRF6 sequence variants and linkage disequilibrium

No *IRF6* mutation was detected in the 17 NSCL/P families analysed. However, normally occurring sequence variants

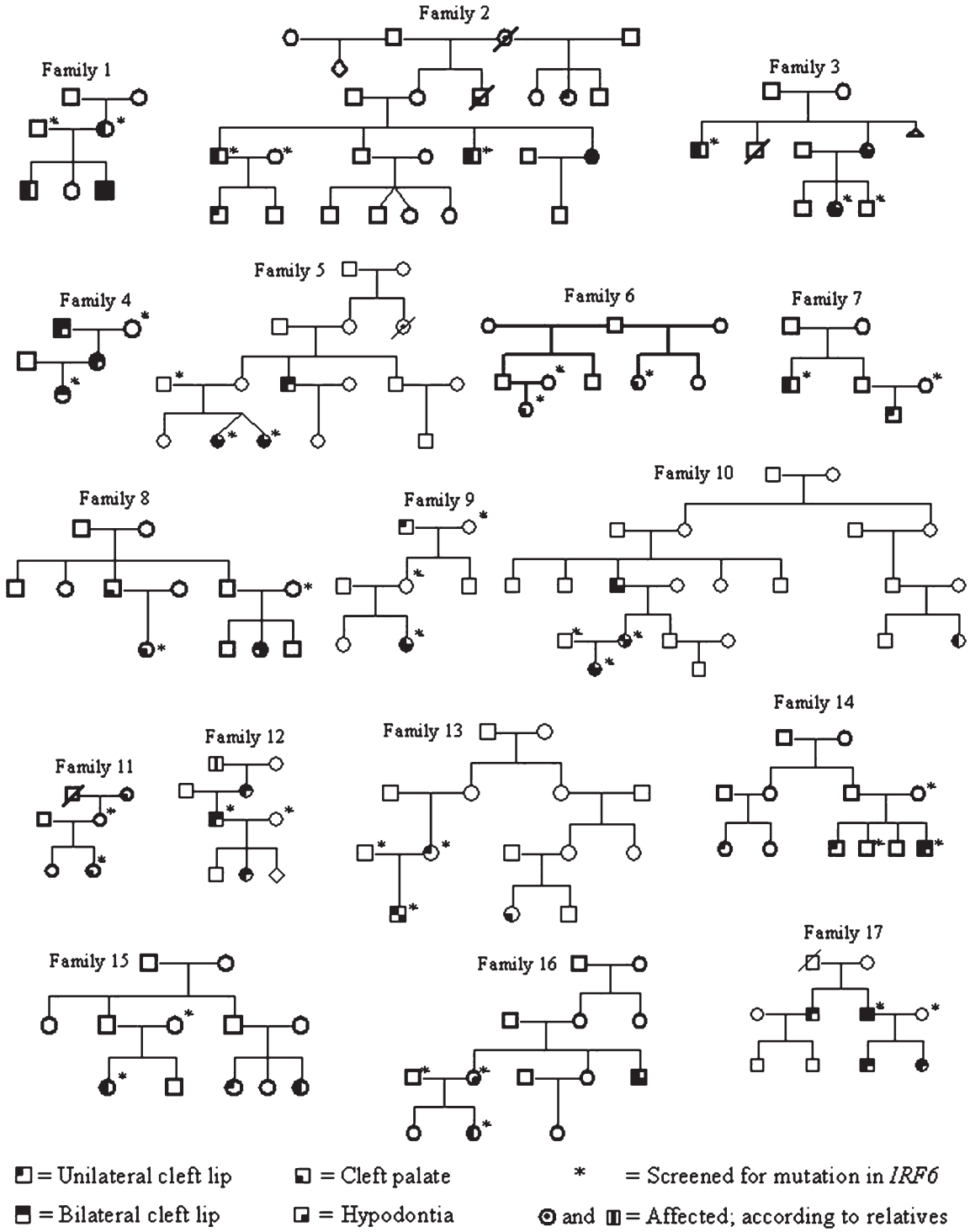

Figure 1 Pedigrees and phenotypes of families 1–17 with familial non-syndromic cleft lip and palate. Filled symbols represent individuals affected by cleft lip and/or palate or hypodontia.

in the form of SNPs were frequently observed, as detailed in Table 3 and Figure 2. Seven SNPs were observed in the gene promoters, exons 2 and 5, and introns 3, 6, and 7 of the *IRF6* gene. SNP1 is a newly detected polymorphism not previously reported in the database (rs34743335 in the SNP database, www.ncbi.nlm.nih.gov/projects/SNP/). SNP5 (rs2013162), results in a silent polymorphism in exon 5 which does not change the protein product (Ser153). A common *IRF6* polymorphism in exon 7 (SNP7, rs2235371, C>T, pVal274Ile) was not found to be polymorphic in the

Table 1 Details of hypodontia (HY) in relation to the cleft phenotype in the non-syndromic cleft lip and palate (NSCL/P) families.

Family		No. of individuals included	Individuals with HY	HY in relation to*					
No.	Lab. ID			UCL	BCL	UCLP	BCLP	CP	Individuals with no cleft
1	F2	7	LKG12				12		
2	F3	8	LKG13				14, 12, 11, 21, 24, 37, 36, 45		
3	F5	6	LKG521			12			
			LKG28			22			
4	F8	5	—						
5	F10	6	LKG62			12			
			LKG51			22			
6	F14	7	—						
7	F16	3	—						
8†	F26	5	LKG134		n.a				
9	F30	5	LKG503			23			
10‡	F204	4	LKG518	12.22					
			LKG517			62			
			LKGM519			n.a.			
11	F42	5	—						
12	F49	4	—						
13‡	F206	8	LKG527	52					
14	F207	8	—						
15	F209	12	—						
16	F210	5	LKG548			22			
			LKG549						41
17†	F211	7	—						
Total		105	14	2	1	8	2	0	1

UCL, unilateral cleft lip; BCL, bilateral cleft lip; UCLP, unilateral cleft lip and palate; BCLP, bilateral cleft lip and palate; CP, isolated cleft palate; n.a, Not available. Hypodontia information obtained only from relatives.
*Numbers correspond to tooth positions.
†Only limited information could be obtained from families 8 and 17.
‡Families 10 and 13; two children under the age of five with missing primary lateral incisor (52, 62) are included.

Table 2 Summary of hypodontia in the families with non-syndromic cleft lip and palate (NSCL/P).

No. of individuals	NSCL/P patients affected with					Total	Members without cleft
	UCL	BCL	UCLP	BCLP	CP		
With hypodontia	2	1	8	2	0	13	1
Without hypodontia	6	1	10	12	8	37	104
Total	8	2	18	14	8	50	105
Hypodontia (%)	25	50	44	14	0		
P value	0.1	0.01	<0.001	<0.01			

UCL, unilateral cleft lip; BCL, bilateral cleft lip; UCLP, unilateral cleft lip and palate; BCLP, bilateral cleft lip and palate; CP, isolated cleft palate.

present material as all individuals were homozygous for the most common C allele, Val 274 (Table 3). Analysis of linkage disequilibrium between all SNPs revealed a two-block structure (Figure 2). Block 1 includes SNP1 and SNP2, while block 2 covers SNP4–6. SNP5 and SNP6, in exon 5 and intron 6, respectively, were found to be in high linkage disequilibrium ($D' > 0.9$). As SNP7 was always seen as homozygous, it was not included in the block representation (Figure 2C).

The alleles of SNPs1–8 were tested for association with the cleft and hypodontia phenotypes. The family-based association test software showed no significant *P* values, while the CLP phenotype showed significant association with SNP3 (rs861019) and SNP4 (rs7552506) with odds ratios of 3.1 (95 per cent confidence level; 1.08–8.88) and 5.45 (95 per cent confidence level; 1.07–27.69), respectively. Taken together, the putative risk alleles (A at SNP3 and G at SNP4) co-segregated in 12 of the 43 (28 per cent) individuals.

Discussion

The aim of this study was to characterize Swedish families with NSCL/P for mutations or other sequence variants in the *IRF6* gene as well as to describe cleft phenotypes and hypodontia.

When all CL/P phenotypes were included, hypodontia was significantly more common (26 per cent) in affected individuals ($P < 0.01$) as compared with family members without a cleft (less than 1 per cent). The prevalence of hypodontia in the general population (excluding third molars) has been reported to range between 3 and 10 per cent. (Graber, 1978; Ranta and Tulensalo, 1988). In the investigated families, hypodontia was commonly seen in

Table 3 Details of the *IRF6* SNPs studied in the families.

Variant	Name	Location	Database*	Protein effect	Alleles	Allele frequencies (in %)		
						Reported†	This study total‡	This study affected
SNP1	rs34743335	Promoter	dbSNP208046152:g.T>A	—	T/A	—	35/65	36/64
SNP2	rs12403006	Promoter	dbSNP208046141:g.A>T	—	A/T	25/75	35/65	36/64
SNP3	rs861019	Exon 2	dbSNP208042009:c.-73A>G	—	A/G	64/36	68/32	62/38
SNP4	rs7552506	Intron 3	dbSNP208036525:g.C>G	—	C/G	35/65	26/74	23/77
SNP5	rs2013162	Exon 5	dbSNP208035307:c.C>A	Ser153Ser	C/A	63/37	70/30	68/32
SNP6	rs2235375	Intron 6	dbSNP208032210:g.G>C	—	G/C	63/37	76/24	77/23
SNP7	rs2235371	Exon 7	dbSNP208030703:c.C>T	Val274Ile	C/T	96/4	100/0	100/0
SNP8	rs2235373	Intron 7	dbSNP208030426:g.G>A	—	G/A	88/12	93/7	98/2

IRF6, interferon regulatory factor 6.
*www.ncbi.nlm.nih.gov.
†Reported in the Caucasian reference population named 'CEPH' (www.hapmap.org).
‡From 43 individuals, e.g.86 chromosomes.

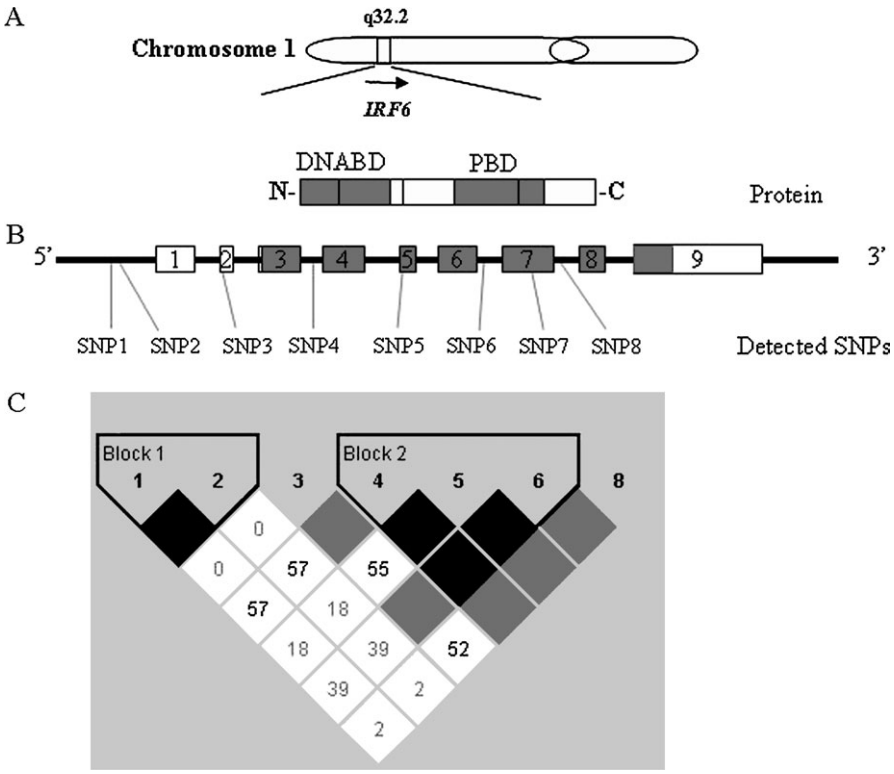

Figure 2 Structure of the *IRF6* gene from the chromosomal region 1q32.2, with the location of all single nucleotide polymorphisms detected in this study. The genomic organization of the gene is shown in (A) with its encoded protein containing a DNA-binding domain and a protein-binding domain (in grey). The amino-and carboxy-terminus of the protein are labelled N- and C-, respectively. Coding exons are marked in grey while untranslated regions are shown in white. The location of SNPs 1–8 is shown. (B) Linkage disequilibrium plot of the genomic region of the *IRF6* gene. (C) Black squares show 100 per cent linkage disequilibrium (LD) between markers while grey squares are non-informative. In the white squares, the numbers indicates percentage LD. SNP7 (rs2235371) is not shown in the block structure since all individuals were homozygous for the most common C allele.

UCLP subjects, where the lateral incisor on the cleft side (12 or 22) was most often missing (28 per cent). Similar observations have previously been reported (Tsai *et al.*, 1998; Eerens *et al.*, 2001). Several possible explanations can be proposed: hypodontia can be a secondary effect of the cleft itself due to lack of tissue for the tooth morphogenesis. Furthermore, it is possible that the genetic defect predisposing to the development of a cleft could also

predispose to hypodontia. Finally, the possibility of a random association just by chance must also be considered. The aetiology of hypodontia is not fully understood but teeth that erupt in the terminal areas of the dental lamina and those located in the embryonic fusion areas are the most frequently affected, such as the upper lateral incisors 12, 22, second premolars, 15, 25, 35, 45, and third molars (Kjær, 1997; Svinhufvud *et al.*, 1988; Kjær and Daugaard-Jensen, 2000; Thesleff, 2003). In the present investigation, hypodontia was not seen in individuals with isolated CP (four families). This finding is in contrast to previous studies, where 30–45 per cent of isolated CP subjects had hypodontia of the second premolars (Jiroutova and Mullerova, 1994; Larson *et al.*, 1998; Karsten and Larson, 2004). The present results are most probably due to the small sample size.

In VWS, the most common syndromic form of CL/P, a mixture of cleft types (CP and CL/P) is often observed within pedigrees. (Janku *et al.*, 1980; Shprintzen *et al.*, 1980; Kondo *et al.*, 2002). In the present study, a mixed pattern of CP and CLP phenotypes was observed in two families only (families 8 and 10), while the remaining 15 pedigrees had either CP or CLP (Figure 1).

Mice carrying a heterozygous missense mutation in the *IRF6* gene resemble the human disorders VWS, and popliteal pterygium syndrome (Richardson *et al.*, 2006). Popliteal pterygium syndrome patients show a cleft phenotype together with webbed skin, genital anomalies, and syndactylies. The *IRF6* mutant mice also exhibit a hyper-proliferative epidermis that fails to undergo terminal differentiation, resulting in soft tissue fusion, suggesting that *IRF6* is a key determinant of the keratinocyte proliferation–differentiation switch.

No mutation that changed the protein product was detected in the *IRF6* gene, suggesting that *IRF6* is not a high-risk disease gene for NSCL/P (Houdayer *et al.*, 2001). None of the mutations identified earlier in VWS families could be found in this NSCL/P material (Kondo *et al.*, 2002; Kayano *et al.*, 2003; Gatta *et al.*, 2004; Ghassibe *et al.*, 2004; Peyrard-Janvid *et al.*, 2005). In a study of the *IRF6* gene (Zucchero *et al.*, 2004), 8000 individuals from 10 populations in Asia, Europe, and South America were screened for a specific polymorphism changing amino-acid 274 from valine to isoleucine (rs2235371, C>T, Val274Ile). This corresponds to SNP7 in the present study. Zucchero *et al.* (2004) found a higher risk of recurrence of NSCL/P for carriers of the C base (Val274) of rs2235371. They demonstrated strong evidence for overtransmission of the valine allele from the parent to the affected child in CL/P families, and that individuals heterozygous for this specific polymorphism (CT genotype) have a lower recurrence risk of CL/P than individuals homozygous for the C allele (CC genotype). In Asian populations, rs2235371 is very polymorphic in contrast to populations in Europe and Africa (Zucchero *et al.*, 2004). In the present study, rs2235371 was seen as monomorphic (C allele only) and can therefore contribute to the NSCL/P phenotype.

A high linkage disequilibrium between SNPs 5 and 6 (rs2013162 and rs2235375) confirms previously published data (Blanton *et al.*, 2005; Scapoli *et al.*, 2005). Scapoli *et al.* (2005) showed linkage disequilibrium between markers rs2013162 and rs223537 and, in addition, association with the NSCL/P phenotype. No association between the two SNPs and the NSCL/P phenotype was found in the present study, probably due to the small sample size. When combining unilateral and BCLP, an association between SNPs 3 and 4 (rs861019 and rs7552506) and the CLP phenotype was found. However, since only a limited number of patients were studied, a possible association needs to be verified in a larger series of cases.

Conclusions

Mutations of the *IRF6* gene are not a common cause for cleft predisposition in Swedish NSCL/P families. A genome-wide approach is recommended to follow up the findings of this study and detect disease genes for NSCL/P (Marazita and Mooney, 2004; Vieira *et al.*, 2005).

Address for correspondence

Marie Pegelow
Department of Orthodontics
Section for Jaw Orthopaedics
Box 4064
Karolinska Institutet
141 04 Huddinge
Sweden
E-mail: marie.pegelow@ki.se

Funding

The Swedish Research Council, the Karolinska Institutet, and the Stockholm County Council (K2002).

Acknowledgements

We are grateful to the family members for their participation in this study, to the staff of the Department of Orthodontics, Karolinska Institutet, and the Department of Plastic Reconstructive Surgery, Karolinska University Hospital, Solna for help with the material collection.

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