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Genetic Variants in *IRF6* and the Risk of Facial Clefts: Single-Marker and Haplotype-Based Analyses in a Population-Based Case-Control Study of Facial Clefts in Norway

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Abstract

Mutations in the gene encoding interferon regulatory factor 6 (*IRF6*) underlie a common form of syndromic clefting known as Van der Woude syndrome. Lip pits and missing teeth are the only additional features distinguishing the syndrome from isolated clefts. Van der Woude syndrome, therefore, provides an excellent model for studying the isolated forms of clefting. From a population-based case-control study of facial clefts in Norway (1996–2001), we selected 377 cleft lip with or without cleft palate (CL/P), 196 cleft palate only (CPO), and 763 control infant-parent triads for analysis. We genotyped six single nucleotide polymorphisms within the *IRF6* locus and estimated the relative risks (RR) conferred on the child by alleles and haplotypes of the child and of the mother. On the whole, there were strong statistical associations with CL/P but not CPO in our data. In single-marker analyses, mothers with a double-dose of the 'a'-allele at rs4844880 had an increased risk of having a child with CL/P (RR = 1.85, 95% confidence interval: 1.04–3.25; $P = 0.036$). An RR of 0.38 (95% confidence interval: 0.16–0.92; $P = 0.031$) was obtained when the child carried a single-dose of the 'a'-allele at rs2235371 (the p.V274I polymorphism). The P -value for the overall test was <0.001 . In haplotype analyses, several of the fetal and maternal haplotype relative risks were statistically significant individually but were not strong enough to show up on the overall test ($P = 0.113$). Taken together, these findings further support a role for *IRF6* variants in clefting of the lip and provide specific risk estimates in a Norwegian population.

Keywords

birth defects; facial clefts; genetic epidemiology; *IRF6*; case-control; case-parent triad; log-linear model; association analysis; haplotype analysis; haplotype relative risk; HAPLIN; HapMap

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Introduction

Facial clefts comprise a heterogeneous group of birth defects with a birth prevalence of approximately 1–2 per 1,000 [Gorlin et al., 2001]. The majority of clefts manifest as isolated defects, where affected individuals have no other structural, behavioral, or cognitive abnormalities [Jugessur and Murray, 2005]. It is now well established that genetic aberrations play a decisive role in the causation of clefts [Lidral and Moreno, 2005], and findings from multiple candidate gene analyses indicate that variants in a handful of genes may collectively account for as much as 15–20% of the genetic contribution [Riley et al., 2007; Vieira et al., 2005; Zuccherro et al., 2004].

We previously reported that mutations in interferon regulatory factor (*IRF6*) cause a common form of syndromic cleft known as Van der Woude syndrome (VWS) [Kondo et al., 2002]. Patients with VWS have clefts of the lip and palate, missing teeth in approximately 25% of cases, and pits in the lower lip in approximately 85% of cases. VWS thus represents a Mendelian form of clefting, in which around 15% of affected individuals will present as phenocopies of isolated clefts. This led us to hypothesize that genetic variants in *IRF6* underlying VWS might also be involved in the etiology of isolated clefts, which we subsequently demonstrated in a large data set of nearly 2,000 families made up of 10 populations of diverse ancestry [Zuccherro et al., 2004]. Since then, five independent studies have confirmed the statistically strong associations between genetic variants in *IRF6* and the risk of cleft lip with or without cleft palates (CL/P) [Blanton et al., 2005; Ghassibe et al., 2005; Park et al., 2007; Scapoli et al., 2005; Srichomthong et al., 2005].

Great strides have also been made in deciphering the functions of *IRF6*. This gene belongs to a family of nine transcription factors that share a highly conserved winged-helix DNA-binding domain and a less conserved protein-binding domain [Kondo et al., 2002]. *IRF6* is highly expressed in the leading edge ectoderm of the palatal shelves prior to and during formation of the primary palate [Ben et al., 2005; Knight et al., 2006; Washbourne and Cox, 2006]. IRF family members are known to regulate host defense mechanisms. For example, mice deficient in *Irf1*, 2, 3, 4, 5, 7, 8, or 9 have an impaired immune response [Ingraham et al., 2006]. These mice, however, do not manifest any embryologic abnormalities. *Irf6*-null mice, on the other hand, have abnormal skin, limb and craniofacial development [Kondo et al., 2002]. A significant role for *Irf6* in epidermal development was recently reported in two independent studies [Ingraham et al., 2006; Richardson et al., 2006]. *Irf6*-null mice were shown to lack a normally stratified epidermis, and gene-expression analyses indicated that the primary defect was in the keratinocyte proliferation-differentiation switch.

A recurrent theme in genetic epidemiological studies of complex traits, such as facial clefts, is that identifying etiologically relevant risk factors with adequate statistical power necessitates large and well-characterized data sets. Norway provides an attractive setting in which to conduct such a study, because the treatment of all cleft-related defects is centralized at two surgical departments, enabling a nation-wide study of these defects with virtually complete case ascertainment. Moreover, Norway has an ethnically homogeneous population and one of the highest prevalences of cleft lip in Europe (1.9/1,000) [Harville et al., 2007].

Using this population-based resource, we explored the role of six single nucleotide polymorphisms (SNPs) within the *IRF6* locus, previously shown to be strongly associated with CL/P. We also discuss the added value of having control infant-parent triads in conjunction with case-parent triads in exploring these genetic effects.

Materials and Methods

Study design and participants

The treatment of babies born with clefts in Norway is centralized at two surgical departments responsible for all cleft repairs (Oslo and Bergen). All expenses related to hospitalization and surgery, traveling, loss of income, and later follow-ups are covered by the Norwegian national health-care system. From 1996 to 2001, we contacted the families of all newborn babies referred for corrective surgery of clefts. Of the nearly 300,000 women who gave birth in this period, 676 mothers had a baby born with a cleft. Of these mothers, we excluded 24 who either did not speak Norwegian or whose baby had died after birth. Of the remaining 652 eligible case mothers, 573 (377 CL/P and 196 cleft palate only (CPO)) agreed to participate (88%). During the same years, we randomly selected 1,022 controls (an average of 4 per 1,000 live births) from the Medical Birth Registry of Norway to serve as controls for both CL/P and CPO. After excluding 16 mothers who did not speak Norwegian or whose baby had died, 1,006 control mothers were eligible, of whom 763 (76%) agreed to participate. Control fathers were asked to provide a swab sample only after November 1998. Hence, a substantial proportion of the control families initially recruited consisted of only mother-child dyads ($N = 347$). See Table I for details.

We used three sources of information to verify the cleft status of cases. Medical records from the hospitals that performed the corrective surgery provided detailed information on the type of cleft and diagnoses of accompanying syndromes or defects other than clefts. We also examined records from the Medical Birth Registry of Norway and the mothers' questionnaires (see below). Cases without an accompanying birth defect or a syndrome diagnosis were labeled "isolated"; all other cases were classified as 'non-isolated'. We further separated cases into CPO and CL/P. The term 'other birth defects' in Table I refers to cases that had accompanying congenital malformations, chronic medical conditions, or vaguely described dysfunctions registered in their medical records.

Questionnaire

All mothers consenting to the study were mailed a self-administered questionnaire shortly after the birth of their babies. We collected information on demographics, reproductive history, maternal health, and family history of clefts. The questionnaire also queried a range of environmental exposures such as smoking, alcohol, vitamin use, food frequency, occupational exposures, use of medication, and stress during the periconceptional period. On average, mothers completed the questionnaire within three months after the baby's birth in both the case and control groups. For more details, see [Wilcox et al., 2007]. An English translation of the questionnaire can be downloaded from our website at <http://niehs.nih.gov/research/atniehs/labs/epi/studies/ncl/index.cfm>.

Biological specimens for DNA analysis

After mothers and fathers provided informed consent, whole-blood samples were drawn from case babies during their first surgical operation. The parents too donated blood samples at the time of the baby's surgery. Control families were mailed cheek swab kits, with sterile cotton-tipped sticks and alcohol-containing plastic vials. The swabs were preserved in alcohol and the vials returned by mail. We also asked permission from case and control mothers to claim the remainder of the capillary blood sample that is routinely used to screen all newborn babies for metabolic and endocrine disorders such as phenylk-etonuria.

IRF6 SNPS and Genotyping

We examined the association of six SNPs in and around the *IRF6* gene with facial clefts (Table II). These SNPs were selected based on strong statistical associations with isolated CL/P reported in [Zuccherro et al., 2004] and in an Italian population [Scapoli et al., 2005]. Figure 1 (A,B) shows the approximate physical and genetic locations of these SNPs in relation to *IRF6*, and the pattern of linkage disequilibrium (LD) they produce based on the genotypes of the population-based sample of control infant-parent triads. The LD plot was generated using the HAPLOVIEW software (see below for details). Genotyping was performed using the TaqMan™ assay (Foster City, CA) according to published protocols [Zuccherro et al., 2004]. The raw genotype data for the 6 *IRF6* SNPs are retrievable from our website at <http://www.cleftsresearch.com> (under “supplementary materials”).

Pairwise LD Plot

Pairwise LD plots were created using HAPLOVIEW (version 3.32). This software calculates allele frequencies for each marker, generates a test for deviations from Hardy-Weinberg equilibrium (HWE), and screens for Mendelian inconsistencies within the control infant-parent triads. In total, 416 control infant-parent triads were available for this part of the analysis. The exact positions of the SNPs were determined using the UCSC genome browser, March 2006 assembly (<http://genome.ucsc.edu/>). Genotype data in linkage pedigree file format for the control infant-parent triads were fed into HAPLOVIEW and the marker positions were entered as specified (for details, see <http://www.broad.mit.edu/mpg/haploview/>).

Multi-Species Nucleotide Sequence Alignment

Using UCSC's Human Genome Browser of March 2006 assembly (<http://genome.ucsc.edu/>), we searched for the genotyped SNPs and obtained multi-species sequence alignments of the corresponding orthologous regions from the following organisms: chimp, rhesus, mouse, rat, rabbit, dog, cow, armadillo, elephant, tenrec, opossum, chicken, and frog. Specific coordinates for the 17 bp-long tracks of each SNP in the human sequence are: rs4844880, chr1:207937531–207937547; rs669694, chr1:208017375–208017391; rs2235371, chr1:208030695–208030711; rs2235375, chr1:208032202–208032218; rs2013163, chr1:208035299–208035315; rs126280, chr1:208086439–208086455, respectively.

Statistical Analyses

We used the statistical software HAPLIN [Gjessing and Lie, 2006] (<http://www.uib.no/smis/gjessing/genetics/software/haplin>) to reconstruct haplotypes from infant-parent triad data and to estimate relative risks associated with these haplotypes. This method is based on a log-linear model that provides a flexible tool for measuring the penetrance of alleles or haplotypes as relative risks [Gjessing and Lie, 2006]. HAPLIN assumes HWE in all analyses and provides separate estimates for the effects of a single and double-dose of an allele or haplotype, when carried either by the child or the mother. Triads with missing genotype information were handled by the Expectation Maximization algorithm, and incomplete case-parent triads were thus included in the estimation.

In single-marker analyses, HAPLIN uses the major allele as reference by default (Table II). The reference category for a designated “risk” or target haplotype was obtained by pooling all the remaining haplotypes into a joint reference [denoted as “reciprocal reference”; see Gjessing and Lie, 2006]. Here, we estimate both single-marker and haplotype relative risks for the child and the mother. The haplotype frequency cut-off was set to 2% in order to exclude rare haplotypes that may complicate double-dose estimations. All analyses on the case-parent triads were done exclusively on isolated clefts and HAPLIN was run using the *R* statistical software

[R Development Core Team, 2006] (<http://www.r-project.org/>). A *P*-value of 0.05 or less was considered statistically significant.

Study Approval

Approval for this study was obtained from the Norwegian Data Inspectorate, the Regional Committee on Research Ethics for Western Norway, the National Institute of Environmental Health Sciences, Institutional Review Board, and the University of Iowa Institutional Review Board. Mothers and fathers provided informed consent.

Results

Participants

Table I provides descriptive characteristics of the case and control infant-parent triads. The number of babies born with CL/P was roughly twice the number with CPO (377 compared with 196), consistent with findings in other populations of European ancestry. Scandinavians have traditionally been positive toward health-related research, which is reflected in the particularly high participation rates in the case (88%; *N* = 573) and control (76%; *N* = 763) groups. Around 3–4% of mothers and fathers reported having had a cleft themselves. Among children born with CPO, 40% had other birth defects in addition to the cleft, compared with 17% among children with CL/P.

SNPS, HWE, LD, and Sequence Conservation

Characteristics of the six *IRF6* SNPs are outlined in Table II and illustrated in Figure 1(A)–(C). Note that *IRF6* is transcribed from the reverse strand, but for the sake of convention, alleles are shown relative to the forward strand of the chromosome (Fig. 1A). The population-based control triads (*N* = 416) were used to compute estimates of minor allele frequencies for each SNP and the corresponding *P*-values for deviation from HWE. The latter was done to verify the transmission patterns of alleles among the controls, in order to justify the use of the HWE assumption in HAPLIN (to enhance power). As seen in Table II, none of the SNPs showed any deviation from expected proportions under Mendelian transmission.

Three of the SNPs were intragenic: rs2235371 in exon 7 alters the conserved amino acid valine to isoleucine at codon 274 (p.V274I) in the SMIR-binding domain of *IRF6*; rs2013162 in exon 5 is a silent variant (Ser153Ser); and rs2235375 is located in intron 6. These are highlighted in green in Figure 1(B). In order to facilitate comparison with previous studies, we have indicated the archaic name rs17015215 for the p.V274I polymorphism. This SNP ID has recently been merged into rs2235371.

The LD plot in Figure 1(B) indicates that alleles at rs669694, rs2235371, rs2235375, and rs2013162 are strongly correlated. *D'* values for pairwise LD comparisons between alleles at these SNPs approach 1.0 (for strong LD). Because of their being in strong LD, HAPLOVIEW groups this SNP quartet into a common haplotype block (Fig. 1(B)). By being physically farthest away from the immediate regions of *IRF6* and the rest of the SNPs, rs4844880 displays a lack of LD, which is as expected, given distance is inversely correlated with the strength of LD.

Variants that confer susceptibility to common diseases are generally thought to reside in transcriptional regulatory regions that tend to be highly conserved across species. To assess evolutionary conservation of the genotyped SNPs and their flanking nucleotide sequences, we obtained alignments of 17 bp-long orthologous sequences centered at each SNP from multiple species. Figure 1(C) shows the multiple alignment. Compared to the rest of the SNPs, a lower

degree of cross-species sequence homology was evident for rs2235371, rs2235375, and rs126280.

Isolated CPO

In single-marker analyses of the CPO triads (Table III), none of the estimated relative risks were statistically significant on their own. However, the overall likelihood ratio *P*-values for SNPs rs2235375 and rs2013162 were 0.022 and 0.025, respectively. Interestingly, these were entirely accounted for by a maternal effect (*p*-maternal = 0.022 and 0.023). In haplotype analyses (Table IV), no fetal or maternal effects were observed with any of the haplotypes tested.

Isolated CL/P

Mothers carrying two copies of the 'a'-allele at rs4844880 had an increased risk of having a baby with CL/P (relative risks (RR) = 1.85, 95% confidence interval (CI): 1.04–3.25; *P* = 0.036) (Table III). For rs2235371 (the p.V274I polymorphism), HAPLIN estimates a fetal RR of 0.38 (95% CI: 0.16–0.92; *P* = 0.031) with a single-dose of the 'a'-allele. The fetal RR rose to 7.25 (95% CI: 1.26–37.3; *P* = 0.026) with a double-dose of the 'a'-allele (Table III). The *P*-value for the overall test was 0.00087. We also see evidence of a maternal effect with this SNP, but the RR is large and the CI is wide.

In haplotype analyses, the following four haplotypes were particularly noteworthy: T-c-G-G-C-a, a-G-G-c-a-G, T-c-G-G-C-G, and a-G-G-G-C-G (Table IV). Focusing on fetal effects first, we observed a statistically significant increase in the risk of CL/P with a single-dose of T-c-G-G-C-a (RR = 1.81; 95% CI: 1.20–2.70; *P* = 0.005). In contrast, carrying the T-c-G-G-C-G haplotype in single-dose appeared to be protective (RR = 0.42; 95% CI: 0.20–0.90; *P* = 0.028). The global *P*-value for the overall test was (*P* = 0.113). Several of the double-dose estimates for fetal and maternal haplotype relative risks were implausibly large and had wide confidence intervals, which may be a consequence of the low frequencies of these haplotypes (only a few homozygotes are available for analysis).

Discussion

With about 120 children referred annually for surgery, cleft lip and palate ranks among the most common birth defects in Norway (1.9/1000 live births). The national health-care system covers all expenses related to the treatment of clefts at the two surgical departments appointed to treat all cleft patients in the country. As a result, we had virtually a 100% case ascertainment in this study. In other countries without such centralized treatment, an ascertainment bias may be introduced if specialized treatment centers tend to attract the more severe and complicated cases. In less developed countries, for instance, these more severe cases may have a higher initial mortality, and through biased exclusion from birth registries, may lead to apparently lower predictive rates.

Our main objective here was to test the association between six SNPs in and around the *IRF6* gene and the risk of isolated clefts. To this end, we used a large population-based case-control study of facial clefts in Norway (1996–2001). In order to facilitate comparison across studies, a synopsis of the other six studies that have examined genetic variants in *IRF6* and the risk of clefts is provided in Table V.

Single-Marker Analyses

Consistent with studies in other Caucasian populations [Blanton et al., 2005; Ghassibe et al., 2005; Park et al., 2007; Scapoli et al., 2005], the heterozygosity of the p.V274I polymorphism (rs2235371) was low in the Norwegian population-based controls. Despite this, we saw a

decreased risk of CL/P among children with one copy of the less common 'a'-allele at this SNP and a 7.25-fold increased risk with two copies of the same allele (Table III). This apparently paradoxical result can be explained by the particularly low frequency of the 'a'-allele at this SNP (2.7% among 313 CL/P triads and 2.1 among the 416 population-based control triads; see Tables II and III). Estimates of double-dose effects may become imprecise due to a lack of homozygotes [Gjessing and Lie, 2006]. Estimates of single-dose effects are generally more reliable because relatively larger numbers of heterozygotes are available for analysis despite the low frequency of alleles.

The decreased risk we observed here with a single-dose of the minor 'a'-allele at rs2235371 is consistent with the increased risk seen with the major "G"-allele at this SNP in [Zuccherro et al., 2004]. Also, the effect of the p. V274I allele was found in CL/P only and not in CPO. This apparent specificity may be rooted in the distinct genetic predispositions of the two cleft subtypes [Marazita and Mooney, 2004]. CL/P may be more susceptible to genetic aberrations in *IRF6* than CPO, which may also partly explain why previous studies have tended to report exclusively on this category of clefts (Table V).

Among the six SNPs genotyped in this study, only three were in common with those of the other reports listed in Table V: namely, rs2235371, rs2235375, and rs2013162. Scapoli and co-workers reported statistically significant overtransmissions of the common alleles at rs2235375 and rs2013162 ($P = 0.002$ with the 'G'-allele and $P = 0.004$ with the 'T'-allele, respectively) among 219 Italian CL/P triads [Scapoli et al., 2005]. Overtransmission of the 'C'-allele at rs2013162 was further confirmed in a sample of 216 European-American families with CL/P ($P = 0.05$) [Blanton et al., 2005], and in 195 Belgian families ($P = 0.014$) [Ghassibe et al., 2005]. We did not observe this among our CL/P triads, which is particularly intriguing considering the close proximity and strong LD between rs669694, rs2235371, rs2235375, and rs2013162 (Fig. 1(A,B)).

Haplotype Analyses

Analyzing haplotypes is more informative than analyzing one marker at a time, because haplotypes increase the information content at a locus, and thus the power of the study, by creating a larger number of heterozygous families available for analysis [Zuccherro et al., 2004]. Haplotype-based analyses can also exploit the LD structure between markers in order to reduce haplotype complexity [Shi et al., 2007]. Zuccherro and colleagues showed that haplotypes carrying the common V-allele of p. V274I and common alleles at all the other SNPs had significant transmission distortions in a Filipino ($P < 0.001$) and a combined Danish and Iowa CL/P triad sample ($P < 0.001$) [Zuccherro et al., 2004]. Compared to our results, only markers rs2235371 (p. V274I) and rs2013162 intersect with those in Zuccherro et al. (numbered in their paper as SNP 17 and SNP 15, respectively). In haplotype analyses of CL/P triads, we observed the following: (a) an increased risk among infants carrying one copy of the T-c-G-G-C-a haplotype, and (b) a decreased risk with T-c-G-G-C-G (SNP 17 and SNP 15 reported in [Zuccherro et al., 2004] are underlined and boldfaced here). Thus, carrying the most common allele at rs2235371 and rs2013162 does not produce a consistent pattern of risk in our CL/P triads compared to those in Zuccherro et al. Upon closer inspection of haplotypes T-c-G-G-C-a and T-c-G-G-C-G above, the only difference is the allele at the last SNP (rs126280, a/G). The observed association is therefore explained entirely by rs126280, as the same alleles of all the other SNPs form part of both risk and protective haplotypes (fetal HRRs of 1.81 and 0.42; Table IV).

The other two SNPs in our study that overlap with those in Scapoli et al. and Blanton et al. are rs2013162 and rs2235375 [Blanton et al., 2005; Scapoli et al., 2005]. Note that, in our study, all alleles are reported on the forward strand of the chromosome and their frequencies are according to a European population (Norwegian). We thus designated the alleles for rs2013162

as (a,C), with the uppercase 'C' representing the major allele in our population. In haplotype analyses, Scapoli and colleagues found that the G-T-G-A haplotype (rs2013162 and rs2235375 being the 2nd and 3rd SNP, respectively) showed significant undertransmission ($P = 0.0003$) among 219 Italian CL/P triads. This was consistent with findings in Blanton et al. where all haplotypes including the minor allele 'A' at rs2013162 were significantly undertransmitted to cases. The C-A-X-T haplotype in particular produced a P -value of 0.002 among the 216 Caucasian CL/P triads. We did not find a consistent pattern of risk for alleles at these two SNPs in our CL/P triads. Common alleles at these two SNPs gave a fetal RR of 1.81 in T-c-G-G-C-a and 0.42 in T-c-G-G-C-G in single-doses of the haplotypes (Table IV). Again, these two haplotypes differ only in the allele at the last SNP (rs126280).

HWE Issues and Control Infant-parent Triads

Although the Norwegian population is ethnically highly homogeneous, we specifically tested for HWE in our population-based controls instead of simply relying on the assumption that alleles at the *IRF6* locus would most likely be in HWE in this unselected population. Indeed, no deviations from HWE were seen among the control triads, compatible with a population in which there is random mating and essentially no population structure. We therefore used the HWE assumption in all analyses in order to enhance the power for detecting a genetic effect. This situation is different for case-parent triads, because, unless there are multiplicative dose-response effects, children with clefts cannot be assumed to be in HWE for alleles at *IRF6* if there is evidence to suggest a deleterious effect of this gene [Gjessing and Lie, 2006; Lee, 2003]. As predicted, we did observe deviations from HWE in the CL/P subgroup for SNPs rs4844880, rs669694, and rs2235371 (results not shown). The effects observed with rs2235371 can be ascribed to the low frequency of the minor allele, which makes effect estimates less precise, but this cannot be said of rs4844880 whose minor allele frequency was above 20%. Moreover, the case population is not random, as it has been highly selected for having the birth defect. Therefore, alleles or haplotypes that increase the risk of clefts would be expected to be overrepresented among cleft patients and produce deviations from HWE.

In conclusion, our study confirms, at least in part, the previously reported associations between genetic variants at the *IRF6* locus and isolated cleft lip and palate. However, no clear patterns emerged in terms of the direction of risk from comparisons with previously published reports, despite comparisons being restricted to studies done on European-derived populations. In agreement with the study by Zuccherro et al. [2004], the V-allele of p. V274I does not appear to be causal by itself, but most likely resides in a genetic background responsible for the observed elevated risks. The next logical step is to undertake targeted functional studies to refine this genetic background in order to uncover the hitherto undisclosed causal variant(s) responsible for the strong associations seen with *IRF6*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Fig. 1.

(A) *Structure of the IRF6 gene* (not drawn to scale). The gene is located on chromosome 1q32.3–q41 and its exons are numbered 1–10. Coding regions are indicated by black rectangles; untranslated regions by white rectangles. The predicted IRF6 protein contains a highly conserved winged-helix DNA-binding domain encoded by exons 3 and 4 and a less conserved Smad-interferon regulatory factor (SMIR/IAD) binding domain encoded by exons 7 and 8 (striped lines). Arrows indicate the approximate positions of the six genotyped SNPs. The p.V274I polymorphism, formerly known as rs17015215, is now called rs2235371. Note that the gene is transcribed from the reverse strand, but alleles for each SNP are displayed according to the forward strand of the chromosome. (B) *Linkage disequilibrium (LD) between the genotyped SNPs*. The pairwise LD plot was created by HAPLOVIEW (see text for details), using the population-based control infant-parent triads for generating D' values (an LD statistic). Intragenic SNPs rs2235371, rs2235375, and rs2013162 are shown in green. As indicated by the color scheme (bottom left), each diamond is color coded and indicates the

level of LD between a given pair of SNPs. A D' value less than 1.0 is indicated by the number inside a given diamond. (C) *Multi-species sequence alignments of the regions containing the genotyped SNPs*. To evaluate sequence conservation at each of the six *IRF6* SNPs, alignments were obtained from the conservation track of the UCSC Genome Browser using the March 2006 assembly (<http://genome.ucsc.edu/cgi-bin/hgGateway>). The vertical yellow line corresponds to the allele that is most prevalent at the given SNP across the different species. SNPs, single nucleotide polymorphisms; IRF, interferon regulatory factor. [Color figure can be viewed in the online edition, which is available at www.interscience.wiley.com]

TABLE I
Characteristics of mothers, fathers, and children in the case and control groups (Norway, 1996–2001)

	<i>Cases^a</i>		<i>Controls (N = 763)</i>
	<i>CL/P (N = 377)</i>	<i>CPO (N = 196)</i>	
MOTHERS	<i>N (%)</i>	<i>N (%)</i>	<i>N (%)</i>
<i>Current marital status</i>			
Married	182 (48)	91 (46)	405 (53)
Living as if married	177 (47)	96 (49)	329 (43)
Single ^b	18 (5)	8 (4)	28 (3)
Missing	0 (0)	1 (1)	1 (1)
<i>Age (years)</i>			
<20	8 (2)	6 (3)	12 (1)
20–29	199 (53)	105 (54)	408 (54)
30–39	165 (44)	81 (41)	328 (43)
≥40	5 (1)	4 (2)	15 (2)
<i>Parity</i>			
1	151 (40)	88 (45)	292 (38)
2	138 (37)	63 (32)	290 (38)
3	63 (17)	36 (18)	132 (17)
≥4	25 (6)	9 (5)	49 (7)
<i>Education</i>			
< High school	70 (19)	23 (12)	87 (11)
High school	94 (25)	48 (25)	211 (28)
Technical college	69 (18)	41 (21)	153 (20)
2–4 year college	124 (33)	72 (36)	265 (34)
University	19 (5)	11 (6)	46 (6)
Other	1 (<1)	1 (<1)	1 (<1)
<i>Country of birth</i>			
Norway	354 (94)	177 (90)	720 (94)
Other	23 (6)	19 (10)	43 (6)
<i>Mother with clefts</i>			
Yes	10 (3)	6 (3)	2 (0.2)
No	367 (97)	190 (97)	761 (≥99)
FATHERS			
<i>Age (years)</i>			
<20	3 (1)	0 (0)	3 (1)
20–39	341 (91)	179 (92)	696 (91)
≥40	30 (8)	16 (8)	63 (8)
<i>Yearly income (Nkr)</i>			
No income	7 (2)	1 (1)	15 (2)
<150,000	20 (5)	11 (5)	44 (6)
151,000–200,000	63 (17)	31 (16)	96 (12)

	Cases ^a		Controls (N = 763)
	CL/P (N = 377)	CPO (N = 196)	
201,000–250,000	94 (25)	59 (30)	186 (24)
≥251000	166 (44)	85 (43)	380 (50)
Missing	27 (7)	9 (5)	42 (6)
<i>Country of birth</i>			
Norway	348 (92)	182 (93)	709 (93)
Other	29 (8)	14 (7)	54 (7)
<i>Father with clefts</i>			
Yes	14 (4)	6 (3)	2 (0.2)
No	363 (96)	190 (97)	761 (≥99)
INFANTS			
<i>Other birth defects^c</i>			
Yes	63 (17)	78 (40)	38 (5)
No	314 (83)	118 (60)	725 (95)

^aCL/P, cleft lip with or without cleft palate; CPO, cleft palate only.

^bIncludes divorced, separated, and never-married.

^c“Other birth defects” denotes infants with other accompanying congenital malformations, chronic medical conditions, or vaguely described dysfunctions registered in their medical records.

Characteristics of the *IRF6* markers and tests for Hardy-Weinberg equilibrium (HWE) in the Norwegian population-based control triads (*N* = 416^a)

SNP name	SNP order ^b	Genetic location	Physical location ^c	Alleles ^d	Ma ^e (%)	HWE <i>P</i> -value	<i>P</i> -value for overall test of Mendelian transmission ^f
rs4844880	28	90kb 3' of <i>IRF6</i>	207937539	a/T	22.1	0.86	0.24
rs669694	20	10kb 3' of <i>IRF6</i>	208017383	c/G	22.6	0.42	0.57
rs2235371 ^g	17	In <i>IRF6</i>	208030703	a/G	2.1	0.43	0.43
rs2235375	—	In <i>IRF6</i>	208032210	c/G	36.7	0.66	0.24
rs2013162	15	In <i>IRF6</i>	208035307	a/C	35.4	0.62	0.18
rs126280	7	40kb 5' of <i>IRF6</i>	208086447	a/G	23.1	0.35	0.55

^aWe did not use the 347 control families in whom paternal DNA was not collected (see Study design and participants in Materials and Methods). Of the total 763 control families, 416 were available for these analyses.

^bAccording to the single nucleotide polymorphism (SNP) order in [Zuccherro et al., 2004].

^cBased on UCSC Human Genome Browser, March 2006 assembly (NCBI Build 36.1 draft of the human DNA sequence).

^dUppercase denotes the more frequent allele in the Norwegian sample population.

^e“Ma^e” stands for minor allele frequency.

^fThis represents an overall test for deviation from Mendelian transmission among the 416 control infant-parent triads.

^gThis single nucleotide polymorphism (SNP), which is the same as the p.V274I polymorphism described in [Zuccherro et al., 2004], was formerly known as rs17015215. This has now been changed to rs2235371 in the dbSNP database (see <http://www.ncbi.nlm.nih.gov/projects/SNP/>).

TABLE III

Single-marker analyses of six *IRF6* SNPs in relation to the risk of isolated CPO and CL/P

Cleft type/triads left ^a	SNP name	Alleles ^b	Maf (%)	Fetal RR (95% CI); <i>P</i> -value ^c		<i>P</i> -fetal ^d	Maternal RR (95% CI); <i>P</i> -value ^c		<i>P</i> -maternal ^d	<i>P</i> -overall ^d
				Single-dose	Double-dose		Single-dose	Double-dose		
<i>Isolated CPO (N = 118)</i>										
115	rs4844880	a,T	20.8	1.03 (0.61–1.74); 0.904	1.05 (0.32–3.16); 0.939	0.995	1.06 (0.64–1.77); 0.828	1.03 (0.34–3.04); 0.962	0.970	0.999
116	rs669694	c,G	23.2	1.05 (0.64–1.73); 0.832	0.92 (0.31–2.53); 0.870	0.927	1.24 (0.77–2.02); 0.381	0.89 (0.29–2.56); 0.838	0.532	0.842
117	rs2235371 ^e	a,G	3.0	0.77 (0.28–2.08); 0.610	Not estimable	0.746	1.78 (0.63–4.88); 0.276	Not estimable	0.468	0.717
114	rs2235375	c,G	36.3	1.72 (0.99–2.98); 0.053	1.81 (0.76–4.18); 0.188	0.147	1.60 (0.96–2.65); 0.069	0.76 (0.30–1.84); 0.548	0.022	0.022
113	rs2013162	a,C	35.2	1.61 (0.97–2.68); 0.063	1.87 (0.84–4.05); 0.130	0.163	1.60 (0.99–2.60); 0.053	0.81 (0.33–1.90); 0.645	0.023	0.025
115	rs126280	a,G	21.5	0.72 (0.43–1.20); 0.205	0.69 (0.22–2.01); 0.491	0.440	1.59 (0.97–2.69); 0.067	0.57 (0.12–2.70); 0.475	0.050	0.107
<i>Isolated CLP (N = 314)</i>										
309	rs4844880	a,T	20.9	0.95 (0.69–1.30); 0.760	1.19 (0.62–2.21); 0.606	0.710	0.92 (0.67–1.26); 0.597	1.85 (1.04–3.25); 0.036	0.022	0.081
311	rs669694	c,G	25.3	1.23 (0.91–1.66); 0.175	1.44 (0.81–2.51); 0.207	0.335	0.90 (0.67–1.21); 0.459	1.13 (0.65–1.92); 0.655	0.474	0.451
313	rs2235371 ^e	a,G	2.7	0.38 (0.16–0.92); 0.031	7.25 (1.26–37.3); 0.026	2.8e-03	0.96 (0.42–2.27); 0.919	17.1 (2.45–119); 0.003	0.030	8.7e-04
311	rs2235375	c,G	33.8	0.84 (0.62–1.14); 0.258	0.92 (0.55–1.53); 0.764	0.480	1.06 (0.79–1.44); 0.692	1.09 (0.65–1.84); 0.737	0.909	0.799
313	rs2013162	a,C	34.1	0.81 (0.60–1.08); 0.150	0.83 (0.50–1.36); 0.457	0.349	1.08 (0.81–1.47); 0.598	1.18 (0.72–1.96); 0.527	0.794	0.632
312	rs126280	a,G	27.0	1.27 (0.94–1.71); 0.119	1.49 (0.85–2.56); 0.155	0.242	0.90 (0.68–1.19); 0.442	0.74 (0.42–1.29); 0.307	0.575	0.414

Salient results are boldfaced.

^aThe remaining number of triads after Mendelian inconsistencies and rare haplotypes have been removed.

^bBy default, HAPLIN uses the minor allele (lowercase) as target ‘risk’ allele and the major allele (uppercase) as reference in relative risk estimation.

^c“Not estimable” means that the confidence intervals (CI) of the point estimates are extremely wide.

^d*P*-values for likelihood ratio tests were computed on the basis of comparisons between the following three models: (I) the *null* model (for no effects); (II) the *fetal effects* model; and (III) the combined *fetal and maternal* effects model. “*P*-fetal” is the *P*-value (2 degrees of freedom (df)) for the comparison between models (I) and (II), showing an effect of fetal alleles (without “correcting” for maternal effects). “*P*-maternal” is the *P*-value (2 df) obtained when comparing models (II) and (III), showing that maternal alleles contribute beyond the effects of fetal alleles (i.e., maternal effects adjusted for fetal effects). “*P*-Overall” is the *P*-value (4 df) for the comparison between models (I) and (III), which tests whether there is any effect at all of any allele, fetal, or maternal.

^eThe p.V274I polymorphism was formerly known as rs17015215. This has now been changed to rs2235371 in the dbSNP database.

CPO, cleft palate only; CL/P, cleft lip with or without cleft palate; SNP, single nucleotide polymorphism; RR, relative risk.

TABLE IV
Haplotype analyses of six *IRF6* SNPs in relation to the risk of isolated CPO and CL/P

Cleft type/triads left ^b	Target haplotype ^c	Freq. (%)	Fetal HRR (95% CI); <i>P</i> -value ^a		Maternal HRR (95% CI); <i>P</i> -value ^a		<i>P</i> -maternal ^d	<i>P</i> -overall ^d
			Single-dose	Double-dose	Single-dose	Double-dose		
<i>Isolated CPO (N = 118)</i>								
75	a-c-G-G-C-a	3.1	1.11 (0.38–3.23); 0.861	Not estimable	2.32 (0.73–7.40); 0.147	Not estimable	0.600	0.853
	T-c-G-G-C-a	10.8	0.79 (0.36–1.76); 0.570	Not estimable	1.47 (0.67–3.22); 0.327	Not estimable		
	a-G-G-c-a-G	2.7	1.71 (0.47–6.41); 0.413	Not estimable	1.65 (0.53–5.26); 0.396	Not estimable		
	T-G-G-c-a-G	30.4	1.68 (0.83–3.33); 0.154	1.22 (0.39–3.83); 0.734	1.13 (0.60–2.09); 0.697	0.78 (0.26–2.36); 0.675		
	a-c-G-G-C-G	3.3	1.86 (0.50–6.80); 0.371	Not estimable	0.54 (0.15–1.92); 0.349	Not estimable		
	T-c-G-G-C-G	5.1	0.51 (0.12–2.28); 0.379	Not estimable	0.32 (0.06–1.57); 0.169	Not estimable		
	a-G-G-G-C-G	9.1	0.68 (0.23–2.00); 0.484	Not estimable	0.48 (0.17–1.38); 0.183	Not estimable		
	T-G-G-G-C-G	32.2	0.67 (0.34–1.30); 0.232	0.64 (0.21–1.96); 0.442	0.93 (0.48–1.80); 0.844	0.86 (0.27–2.72); 0.796		
<i>Isolated CL/P (N = 314)</i>								
258	a-c-G-G-C-a	11.6	1.13 (0.72–1.75); 0.582	1.40 (0.44–4.48); 0.578	0.76 (0.49–1.20); 0.238	1.27 (0.44–3.65); 0.666	0.169	0.113
	T-c-G-G-C-a	10.2	1.81 (1.20–2.70); 0.005	2.47 (0.88–7.05); 0.086	0.88 (0.58–1.34); 0.557	1.86 (0.75–4.49); 0.180		
	T-G-G-G-C-a	4.8	0.74 (0.37–1.49); 0.398	2.62 (0.31–22.9); 0.380	0.65 (0.32–1.32); 0.239	Not estimable		
	a-G-G-c-a-G	2.7	1.03 (0.49–2.20); 0.940	Not estimable	1.08 (0.48–2.47); 0.850	11.5 (1.95–67.6); 0.006		
	T-G-G-c-a-G	28.5	0.93 (0.66–1.32); 0.676	1.04 (0.57–1.88); 0.904	1.22 (0.85–1.75); 0.278	1.16 (0.62–2.18); 0.639		
	T-c-G-G-C-G	4.1	0.42 (0.20–0.90); 0.028	Not estimable	1.24 (0.59–2.57); 0.564	6.13 (0.62–58.6); 0.116		
	a-G-G-G-C-G	5.1	0.87 (0.51–1.49); 0.613	3.51 (0.93–12.8); 0.065	1.54 (0.85–2.81); 0.152	4.87 (1.15–20.5); 0.033		
	T-G-G-G-C-G	32.1	0.89 (0.63–1.25); 0.503	0.88 (0.48–1.61); 0.676	0.91 (0.64–1.29); 0.604	0.94 (0.51–1.70); 0.840		

Salient results are boldfaced.

^aWhen alleles or haplotypes are exceedingly rare, double-dose estimates become increasingly imprecise due to a lack of homozygotes. “Not estimable” means that the confidence intervals of the point estimates are extremely wide. HRR, haplotype relative risk.

^bThe remaining number of triads after Mendelian inconsistencies and rare haplotypes have been removed.

^cThe specific order of the single nucleotide polymorphisms is: rs4844880(a,T)–rs669694(c,G)–rs2235371(a,G)–rs2235375(c,G)–rs2013162(a,C)–rs126280(a,G). Uppercase denotes the major allele. The reference category for a designated target ‘risk’ haplotype consists of all the remaining haplotypes pooled into a joint reference (“reciprocal reference”).

^d *P*-values for likelihood ratio tests were computed on the basis of comparisons between the following three models: (I) the *null* model (of no effects); (II) the *fetal effects* model; and (III) the combined *fetal and maternal effects* model. “*P*-fetal” is the *P*-value (15 df) for the comparison between models (I) and (II), showing an effect of fetal haplotypes (without “correcting” for maternal effects). “*P*-maternal” is the *P*-value (15 df) obtained when comparing models (II) and (III), showing that maternal haplotypes contribute beyond the effects of fetal haplotypes alone (i.e., maternal effects are adjusted for fetal effects). “*P*-overall” is the *P*-value (30 df) for the comparison between models (I) and (III), which tests whether there is any effect at all of any haplotype, fetal, or maternal.

CPO, cleft palate only; CL/P, cleft lip with or without cleft palate; CI, confidence interval.

TABLE V
Summary studies that have investigated the role of *IRF6* variants in the risk of facial clefts

Study and population ^a	Cleft type ^b	Study design	Genotyped SNPs ^c	Haplotypes analyzed?	Summary of results ^d
[Zuccherro et al., 2004]	NS CL/P	Case-control and case-parent triad:	36 SNPs (see original article for details).	Yes	There was strong statistical evidence of overtransmission of the 'G'-allele at rs2235371 in predominantly Asian and South American populations, but far less so in European populations. Haplotypes harboring the common 'G'-allele produced a 3-fold increased risk of cleft among Filipino case-parent triads. The proportion of NS CL/P in the population which is attributable to the 'G'-allele was estimated to be approximately 12%.
East Asia		Affected families ($N = 1968$):	SNPs overlapping with our study:		
South America		790 East Asia	1. <i>rs4844880</i>		
Europe		643 South America	2. <i>rs669694</i>		
India		485 Europe	3. <i>rs2235371</i>		
		50 India	4. <i>rs2013162</i>		For SNPs rs2013162 and rs2235375, it was the common allele that was significantly overtransmitted to affected members, both individually ($P = 0.004$ and $P = 0.002$, respectively), and when carried on haplotypes. These two SNPs also showed strong LD.
		Controls families ($N = 949$):	5. <i>rs126280</i>		
		647 CEPH diversity panel			
		262 East Asia			
		40 India			
[Scapoli et al., 2005]	NS CL/P	Case-parent triad ($N = 219$)	1. <i>rs1319435</i>	Yes	OR for patients with GG vs. those with (GA + AA) was 1.7 (95% CI: 1.1-2.5). TDT analysis gave a P-value of 0.874. SNP rs2235371 was responsible for 16.7% of the genetic contribution to CL/P. The common 'G'-allele was associated with elevated risk and there was a recessive effect with this allele. The GA and AA genotypes were not associated with clefting.
Italian (White)			2. <i>rs2013162</i>		
			3. <i>rs2235375</i>		
			4. <i>rs2235543</i>		
[Srichomthong et al., 2005]	NS CL/P	Case-control and case-parent triad:	1. <i>rs2235371</i>	No	Significant association was seen with rs2013162 but not with rs2235543. Note that rs2235543 is 100 Kb 3' of <i>IRF6</i> . Only the haplotype made up of the common alleles at both SNPs showed the most significant transmission asymmetry ($P = 0.004$ with the G-G haplotype).
Thai or Thai-Chinese		192 case and 278 controls	'G' = common allele		
		53 complete triads			
[Ghassibe et al., 2005]	NS CL/P	Case-parent triad ($N = 195$)	1. <i>rs2013162</i>	Yes	Significant overtransmission of the 'C'-allele at rs2013162 ($P = 0.05$), as well as for all haplotypes carrying this allele. A matching decrease in transmission was seen with haplotypes carrying the 'A'-allele at that same SNP. The most significant results were
Belgian (White)			2. <i>rs2235543</i>		
[Blanton et al., 2005]	NS CL/P	Case-parent triad:	1. <i>rs1319435</i>	Yes	Significant overtransmission of the 'C'-allele at rs2013162 ($P = 0.05$), as well as for all haplotypes carrying this allele. A matching decrease in transmission was seen with haplotypes carrying the 'A'-allele at that same SNP. The most significant results were
US (Texas)		51 multiplex families	2. <i>rs2013162</i>		
White		184 simplex child-parent triads	3. <i>rs2235375</i>		

Study and population ^a	Cleft type ^b	Study design	Genotyped SNPs ^c	Haplotypes analyzed?	Summary of results ^d
Hispanic		21 child-parent triads with family history	4. rs2235543		obtained for the C-A-X-T haplotype ($P = 0.009$).
African-American [Park et al., 2007]	NS CL/P	Case-parent triad:	1. rs599021	Yes	rs2235371 was not genotyped in the European-American sample due to low heterozygosity.
European-American		77 European-American	2. rs2073485		Linkage and association were observed in all four populations. SNPs rs2073485 and rs2235373 (not in LD with rs2235371) yielded statistical significance both individually and as haplotypes in the Taiwanese triads. Two specific haplotypes, G-C of rs2235373-rs2235371 and A-A-G of rs599021-rs2235373-rs2013162, showed significant transmission asymmetry in the Taiwanese sample. The SNPs showing statistical significance differed across ethnic groups.
Taiwanese		146 Taiwanese	3. rs2235373		
Singaporean		34 Singaporean	4. rs2235371		
Korean		40 Korean	5. rs674433		
			6. rs595918		
			7. rs2013162		
			8. rs2236907		
			9. rs2294408		
			10. rs861019		
			11. rs2073487		
			12. rs3753517		
			13. rs1005287		
<i>This study</i>	NS CL/P and NS CP	Case-control, infant-parent triad:	1. rs4844880	Yes	Mothers with two copies of the 'a'-allele at rs4844880 had a 1.8-fold risk of having a baby with CL/P (95% CI: 1.0–3.2; $P = 0.036$). For rs2235371, a fetal RR of 0.4 (95% CI: 0.2–0.9; $P = 0.031$) was obtained with a single-dose of the 'a'-allele. The corresponding P -value for the overall test was <0.001 .
Norwegian (White)		118 CPO	2. rs669694		
		314 CL/P	3. rs2235371		
		724 Control	4. rs2235375		
			5. rs2013162		
			6. rs126280		

In haplotype analyses, infants with a single-dose of T-c-G-G-C-a had a RR of 1.8 (95% CI: 1.2–2.7; $P = 0.005$). In contrast, carrying T-c-G-G-C-G in single-dose seemed protective (RR = 0.4; 95% CI: 0.2–0.9; $P = 0.028$). The P -value for the overall test was, however, not statistically significant ($P = 0.113$).

^aThe p.V274I polymorphism (SNP rs2235371) was not genotyped in [Blanton et al., 2005; Scapoli et al., 2005], because the T-allele was not detected in these populations due to low heterozygosity of this marker.

^b „NS”: Non-syndromic or isolated.

^c The p.V274I polymorphism was formerly known as rs17015215. This has now been changed to rs2235371 in the dbSNP database (see <http://www.ncbi.nlm.nih.gov/projects/SNP/>). The SNPs in common with those in the present study are shown boldfaced and italicized.

^d OR: odds ratio; RR: relative risk; HRR: haplotype relative risk; TDT: transmission disequilibrium test.

CPO, cleft palate only; CL/P, cleft lip with or without cleft palate; SNP, single nucleotide polymorphism; CI, confidence interval.