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# Evaluating *SKI* as a candidate gene for non-syndromic cleft lip with or without cleft palate

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Non-syndromic cleft lip with or without cleft palate (NSCL/P) is one of the most common of all congenital malformations and has a multifactorial etiology. Findings in mice suggest that the v-ski sarcoma viral oncogene homolog (*SKI*) gene is a candidate gene for orofacial clefting. In humans, a significant association between rs2843159 within *SKI* and NSCL/P has been reported in patients from the Philippines and South America. In the South American patients, the association was driven by the subgroup of patients with non-syndromic cleft lip only (NSCLO). Here we investigated the association with rs2843159 in a Mayan Mesoamerican population (172 NSCL/P patients and 366 controls). In addition, we analyzed the phenotypic subgroups NSCLO and non-syndromic cleft of lip and palate (NSCLP). A trend towards association between rs2843159 and NSCL/P was observed in the Mayan cohort ($P = 0.097$), and we found a stronger association in the NSCLP subgroup ($P = 0.072$) despite a limited sample size. To investigate whether other common variants within the *SKI* gene contribute to NSCL/P susceptibility in European and Asian populations, we also analyzed genotypic data from two recent genome-wide association studies using set-based statistical approaches. These analyses detected a trend toward association in the European population. Our data provide limited support for the hypothesis that common *SKI* variants are susceptibility factors for NSCL/P.

Key words: association study; case–control design; Native American population; orofacial clefting; v-ski sarcoma viral oncogene homolog

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Orofacial clefts are a common type of congenital malformation and represent a major public health challenge in terms of patient care. The estimated worldwide prevalence of orofacial clefts among newborns is 1 in 600 (1). Cleft lip with or without cleft palate (CL/P) is the most frequent type of orofacial clefting. Although CL/P may occur as part of a complex malformation syndrome, most cases are non-syndromic and have a multifactorial etiology that includes both genetic and environmental factors (2). In terms of genetic factors, conclusive evidence for involvement in non-syndromic CL/P (NSCL/P) has been obtained for the interferon regulatory factor 6 (*IRF6*) gene and for five chromosomal loci identified through genome-wide association studies (GWAS) (2). There is some indication that the strength of the associations might vary between populations, and not all loci identified by GWAS appear to be involved in both European and Asian populations (3). However, these susceptibility loci explain a considerable proportion of all NSCL/P cases. Nevertheless, additional risk variants still await identification.

The v-ski sarcoma viral oncogene homolog (*SKI*) gene was first implicated as a candidate gene for orofacial clefting by Berk *et al.* (4). Their study showed that $Ski^{-/-}$ murine mutants display a complex phenotype that includes severe defects in the patterning of

vertebral and craniofacial skeletal structures. The occurrence of exencephaly or midline facial clefting, but never both, in these mice suggests that the genetic background influences the penetrance of the mutation (4). This hypothesis was confirmed in a subsequent analysis of $Ski^{-/-}$ mice, which showed that the incidence of facial clefting increased from generation to generation when mutated mice were backcrossed (5). Notably, the human *SKI* gene maps to the genomic region that is commonly deleted in the 1p36 deletion syndrome. The phenotypic spectrum of this syndrome includes orofacial clefting (6).

On the basis of this evidence, the *SKI* gene has been included in a comprehensive resequencing and linkage study of multiple clefting candidate genes (7). A highly significant association between a common single-nucleotide polymorphism (SNP) within *SKI* (rs2843159), and NSCL/P was reported. This association was found predominantly in patients from the Philippines ($P = 0.000004$) and was replicated in a South American NSCL/P sample ($P = 0.02$).

Epidemiologic and embryologic data support the hypothesis that NSCL/P can be subdivided into non-syndromic cleft lip only (NSCLO) and non-syndromic cleft of lip and palate (NSCLP), the latter affecting both lip and palate, and that distinct genetic factors may be involved in their respective development (2). Interestingly, in the above-mentioned South American NSCL/P sample, the association with the *SKI* gene was driven by the subgroup of patients with NSCLO) ($P = 0.004$).

A strong Native American maternal contribution has been reported in the South American clefting population (8). This finding, together with the observation of variable penetrance depending on the genetic background, suggests that a replication attempt in a Native American cleft sample is warranted.

The aim of the present study was to analyze the association with rs2843159 in a case–control cohort of Mayan descent. To investigate whether other common variants within the *SKI* gene might contribute to NSCL/P susceptibility in European and Asian populations, we also extracted genotypic data from two GWAS (9, 10) and analyzed them using set-based statistical approaches.

## Material and methods

### Mayan sample

The study included 172 NSCL/P patients (152 with NSCLP and 20 with NSCLO; 111 male patients and 61 female patients) and 366 unaffected controls (127 male subjects and 239 female subjects). Patients and controls were recruited from confined areas of San Cristóbal de las Casas and Tuxtla Gutiérrez in the State of Chiapas (Mexico). Most patients were ascertained within the context of surgical outreach programs. A detailed patient questionnaire was completed to identify possible prenatal contributory factors, such as maternal ingestion of known teratogenic medications or toxins, and no affected families were identified. Unaffected controls were recruited at two outpatient clinics, which they were attending for various medical indications. The ethnic background of patients and controls was assessed on the basis of the grandparents' descent. Only those individuals whose four grandparents were all of Mayan descent (Mestizo; Mestizo admixed with Chol, Tzotzil, Tzeltal, Mam; or Chol, Tzotzil, Tzeltal, Mam) were included. We were not aware of any specific environmental risk factors or protective factors in the controls.

Ethical approval for the study was obtained from the respective Medical Faculty Ethics Committees, and all individuals provided written informed consent. For children <18 yr of age, consent was obtained from their parents. All participating individuals were clinically assessed by one of four medical geneticists (A.R.M., H.R., O.C.C., or S.N.) to determine cleft status and, if affected, to exclude any underlying syndrome. Patients with cleft palate only (CPO) were excluded from the present study. None of the controls displayed orofacial clefting or any minor form of clefting, such as small defects of the lip or alveolar arch, or scarlike ridges above the lip or submucosal cleft palate.

### Genomic DNA preparation

A whole-blood sample of 10–30 ml was collected into ethylenediaminetetraacetic acid (EDTA)-coated vacutainer tubes according to standard venipuncture methods. Blood was drawn from all affected individuals, from their parents (if possible), and from all controls. Genomic DNA was extracted from peripheral leukocytes using the salting-out method and a standardized genomic DNA isolation protocol.

### Genotyping

Genotyping was performed using restriction fragment length polymorphism (RFLP) analysis. The SNP rs2843159 (NM_003036.3:c.1475-60T>C) was genotyped after initial amplification of the *SKI* intron 4 by PCR. The PCR was performed using the FastStart *Taq* DNA Polymerase kit (Roche Applied Science, Indianapolis, IN, USA) under standard conditions and with the primers SKI_intron4_F: 5′-TCCTACAAGAGCTTTGAGACAG-3′ and SKI_intron4_R: 5′-GAGCTGGATGAGGTAAAG-GAC-3′, yielding a product length of 498 bp. The PCR product was then subjected to restriction digestion with *Bst*NI (restriction site: CCWGG; New England Biolabs, Ipswich, MA, USA). Based on human reference sequence data, two *Bst*NI restriction sites are present in the amplicon, yielding restriction fragments of 112, 165, and 221 bp. In the presence of the T allele, one restriction site is lost, yielding fragments that are 165 and 333 bp in size. The digested PCR products were separated by electrophoresis through 3% agarose gels, and genotypes were assigned by two independent investigators. Detailed information of PCR amplification and the genotyping procedure are obtainable on request.

### *In-silico* set-based tests on GWAS data

The GWAS data were available from (i) our own case–control GWAS of 399 patients and 1,318 controls of European descent (10) and (ii) the publically available database

dbGaP. The latter data comprised case–parent data from European and Asian people (9) and were retrieved from dbGaP upon approval of data access (dbGaP accession number phs000094.v1.p1.). The SNP rs2843159 was not represented on any of the SNP arrays used in these GWAS. Set-based analysis was performed with VEGAS (11), which can be used for both case–control and family-based data. Standard settings were applied, including boundaries of ±50 kb for 5′ or 3′ untranslated regions (UTRs). For *SKI*, SNPs in the range of 2,099,993 to 2,281,416 (positions according to hg18) were entered in the gene-based analysis.

### Statistical analysis

All statistical analyses were performed using SAS software (version 9.1, Cary, NC, USA). A standard chi-square test was used to test for deviation from Hardy–Weinberg equilibrium, and a two-sided Armitage trend test was used to compare genotype distributions between cases and controls. Analysis was performed: (i) for the entire sample of NSCL/P patients; (ii) with respect to gender in the NSCL/P group; and (iii) for each of the two subgroups NSCLP and NSCLO. The power of the present study to detect an effect size of 1.2 (for $\alpha = 0.05$) was calculated on the basis of the allele frequency of rs2843159 observed in the Mayan controls.

## Results

### Sample of Mayan descent

The distribution of *SKI* genotypes observed among NSCL/P cases and controls was consistent with Hardy–Weinberg equilibrium ($P_{\text{HWE_controls}} = 0.431$, $P_{\text{HWE_NSCL/P}} = 0.965$). We observed a trend toward association between rs2843159 and NSCL/P ($P_{\text{NSCL/P}} = 0.097$), with ORs (95% CI) of 1.36 (0.86–2.13) for the heterozygous genotypes and 1.54 (0.96–2.57) for the homozygous genotypes (Table 1). No evidence was obtained for a gender-specific contribution to the association between rs2843159 and NSCL/P. Comparison of genotypic distribution between male cases and controls ($P = 0.105$), and between female cases and controls ($P = 0.635$), revealed no significant differences.

Analyses of the subgroups NSCLO and NSCLP revealed evidence for a stronger effect of rs2843159 in patients with NSCLP (Table 1). Compared with the overall analysis of NSCL/P, the *P*-value in the NSCLP subgroup decreased to 0.072, despite the smaller sample size. No significant association, or trend toward association, was observed in the NSCLO group (Table 1).

### Set-based analysis of GWAS data

The results of the gene-based analysis for *SKI* are shown in Table 2. A trend toward association was observed in the European population in both the case– = 0.0612) and the trio ($P = 0.108$) data. The lowest *P*-value was observed for rs9039160 – an intronic variant within *SKI* – in the European case–control data ($P = 0.00633$). In the family-based data set the lowest *P*-value was observed for rs4648831 ($P = 0.00592$), which is located downstream of *SKI*. However, these single SNP results did not remain significant after Bonferroni correction for the number of included SNPs ($n = 41$).

## Discussion

We followed up on previous findings reporting strong evidence for an association between rs2843159, a SNP within the *SKI* gene, and NSCL/P (7). The association was identified in two independent samples of Filipino and South American subjects. As research suggests that there is a strong maternal Native American contribution to clefting in samples from South America (8), we investigated the role of this common variant in a Native American case–control cohort of Mayan descent.

Although we observed a trend towards association for rs2843159 in the NSCL/P group, the *P*-value fell short of nominal significance. To address whether this may be attributed to power issues, we recalculated the power for the present study assuming an OR of 1.2, as observed in the original report (7). This analysis yielded a power of 28.5%, which supports the hypothesis that the failure to reach statistical significance was a result

Table 1

*Case–control association result for the single nucleotide polymorphism (SNP) rs2843159 (T/C) in the sample of Mayan descent*

| Sample | Sample size | Genotype (cases) (TT/TC/CC) | T-allele frequency (cases) | Genotype (controls) (TT/TC/CC) | T-allele frequency (controls) | *P*-value* | $OR_{het}$ (95% CI)† | $OR_{hom}$ (95% CI)† |
|---|---|---|---|---|---|---|---|---|
| NSCL/P | 172 | 48/86/38 | 0.529 | 86/175/105 | 0.474 | 0.097 | 1.36 (0.86–2.13) | 1.54 (0.92–2.57) |
| Subgroup | | | | | | | | |
| NSCLO | 20 | 5/9/6 | 0.475 | 86/175/105 | 0.474 | 0.991 | – | – |
| NSCLP | 152 | 43/77/32 | 0.536 | 86/175/105 | 0.474 | 0.072 | 1.44 (0.90–2.33) | 1.64 (0.96–2.81) |

NSCL/P, non-syndromic cleft lip with or without cleft palate; NSCLO, non-syndromic cleft lip only; NSCLP, non-syndromic cleft lip and palate; OR, odds ratio (for the T-allele); $OR_{het}$, odds ratio for the heterozygous genotype; $OR_{hom}$, odds ratio for the homozygous genotype.

*The *P*-value was calculated using the Cochran–Armitage trend test.

†The OR is shown for analyses with $P < 0.1$.

Table 2

*Gene-based test of the v-ski sarcoma viral oncogene homolog (SKI)* gene in data sets from genome-wide association studies (10)

| Sample (study) | Number of SNPs | Number of simulations* | Gene-based test statistics | Gene-based *P*-value | Best SNP | Position (in base pairs, according to hg18) | Best SNP *P*-value | Location relative to *SKI* |
|---|---|---|---|---|---|---|---|---|
| European case–control sample (9) | 41 | 100,000 | 71.48 | 0.0612 | rs903916 | 2,221,323 | 0.00633 | Intron 1 |
| European case–parent trios (8) | 42 | 1,000 | 63.06 | 0.108 | rs4648831 | 2,272,927 | 0.00592 | Downstream of *SKI*, intronic of *MORN1* |
| Asian case–parent trios (8) | 42 | 1,000 | 46.48 | 0.327 | rs263526 | 2,163,364 | 0.0234 | Upstream |

Membrane Occupation and Recognition Nexus 1 gene *(MORN1)*; SNP, single nucleotide polymorphism.
*VEGAS increases the number of simulations, depending on the result of the previous round of simulations.

of the limited sample size. Notably, the association observed in the present study is in the same allelic direction as that reported by Vieira *et al.*, with the T allele being more frequently observed in cases. This further supports the possible contribution of rs2843159 to NSCL/P in samples from South America and Middle America.

When analyzing the NSCL/P subgroups NSCLO and NSCLP, the *P*-value in the NSCL/P group was smaller compared with that for the entire NSCL/P sample and also smaller compared with that for the NSCLO group. This is in contrast to the initial findings in the South American population, where the effect of rs2843159 was mainly driven by the NSCLO subgroup. This might be explained by the limited sample size (especially for the NSCLO subgroup) in the present study. Alternatively, it might reflect the variable phenotypic expressivity that has already been demonstrated in $Ski^{-/-}$ mice (5). Of note, a recent meta-analysis of GWAS data suggests that distinct genetic factors contribute to the NSCL/P subgroups NSCLO and NSCLP, further suggesting that subphenotyping is important in future genetic analysis in the field of orofacial clefting (3).

We also investigated whether common variants contribute to NSCL/P in other populations. A previous analysis of rs2843159 in a case–control sample of European ethnicity from Iowa yielded no positive findings (7). However, this study might have failed as a result of genetic heterogeneity at the *SKI* locus, if our hypothesis that other common variants within *SKI* are of etiological relevance to NSCL/P is correct. To address this question we performed a gene-based analysis of recent genome-wide data from European and Asian NSCL/P patients. The results from the European case–control data provided scant support for our hypothesis, as although the gene-based *P*-value approached nominal significance, it failed to reach the $P = 0.05$ criterion. The most strongly associated SNP in this data set, rs903916, which is located in intron 1 of *SKI*, showed a *P*-value of 0.00633, which did not withstand correction for multiple testing for the number of included SNPs. No additional support was provided from either the European or the Asian family-based data sets. In both of these analyses, the gene-based *P*-values were statistically non-significant and the most significant SNPs were located outside the genomic region of *SKI*. Although our results do not demonstrate a strong contribution of common variants within *SKI* in European and Asian samples, subtle effects of *SKI* SNPs cannot be completely ruled out. The lack of significance might be a result of the limited power of the samples. Alternatively, it might be that none of the SNPs present in the arrays is in strong linkage desequilibrium (LD) with a putative causal variant. In this case, the presence of some SNPs with nominal significance, either within or outside the *SKI* gene, might be explained by a different sample structure/population background in the analyzed GWAS sets.

Apart from the initial investigation by Vieira *et al.* (7), only one independent study, analyzing variants within *SKI* and NSCL/P, has been published to date (12). In that study, the authors first sequenced the *SKI* gene in 100 controls. One non-synonymous variant (rs28384811) was identified and this was subsequently genotyped in 148 NSCL/P cases and 147 controls. This analysis yielded a minor allele frequency of 10.5% in NSCL/P cases and of 17.7% in controls. Unfortunately, the only information provided concerning the genetic background of these individuals was that they were born in California (12), which renders interpretation of these findings difficult.

Accumulating evidence suggests that rare variants with high penetrance contribute to complex phenotypes such as orofacial clefting. Accordingly, Vieira *et al.* (7) attempted to identify such variants in subjects with NSCL/P by sequencing the *SKI* coding region, including exon–intron boundaries and untranslated regions. Within *SKI*, four non-synonymous variants were observed, one of which, A388V, was claimed by the authors to be of potential etiological relevance. However, this mutation was subsequently identified in nine of 1,064 European controls, and analysis of family members of the A288V index patient revealed that it had

been inherited from the unaffected mother. Although these data do not support the involvement of rare *SKI* variants in NSCL/P, *SKI* remains an interesting candidate gene in pedigrees with multiple affected family members, particularly those showing linkage to the *SKI* chromosomal region on 1p36.3. The fact that linkage of NSCL/P to a region between 1p36.1 and 1p36.3 has indeed already been reported (13) further supports the idea that private mutations within *SKI* might be responsible for the linkage findings in these families. Also, sequencing of *SKI* in patients with the 1p36 deletion syndrome might be envisaged, in particular in those 17% of patients with clefting anomalies (6). Rare, recessive alleles of *SKI* might be unmasked in these patients and could explain some of the facial anomalies (5).

In summary, the present data provide some limited support for the hypothesis that common *SKI* variants are susceptibility factors for NSCL/P, particularly in Native American populations. However, the phenotypic consequences of predisposing variants in any given individual are likely to be dependent on the respective genetic background. Thus, further studies in different ethnicities are warranted to elucidate the contribution of *SKI* variants to orofacial clefting.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Data S1.** Funding support: Additional details on dbGaP.

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