

ARTICLE

Association between genes on chromosome 4p16 and non-syndromic oral clefts in four populations

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Isolated cleft lip with or without cleft palate and cleft palate are among the most common human birth defects. Several candidate gene studies on *MSX1* have shown significant association between markers in *MSX1* and risk of oral clefts, and re-sequencing studies have identified multiple mutations in *MSX1* in a small minority of cases, which may account for 1–2% of all isolated oral clefts cases. We explored the 2-Mb region around *MSX1*, using a marker map of 393 single nucleotide polymorphisms (SNPs) in 297 cleft lip, with or without cleft palate, case–parent trios and 84 cleft palate trios from Maryland, Taiwan, Singapore, and Korea. Both individual markers and haplotypes of two to five SNPs showed several regions yielding statistical evidence for linkage and disequilibrium. Two genes (*STK32B* and *EVC*) yielded consistent evidence from cleft lip, with or without cleft palate, trios in all four populations. These two genes plus *EVC2* also yielded suggestive evidence for linkage and disequilibrium among cleft palate trios. This analysis suggests that several genes, not just *MSX1*, in this region may influence risk of oral clefts.

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INTRODUCTION

Oral clefts are among the most common human birth defects, and seem to be controlled by several genes. Conventionally, isolated cleft palate (CP) is considered distinct from isolated cleft lip with or without cleft palate (CL/P) and several genes may influence both, for example, for CL/P, at least six regions of linkage have been identified in genome-wide studies.¹ Birth prevalence rates differ significantly among populations and vary with gender.² Prevalence among live-born infants ranges from 1 per 500 to 1 per 1000 births, and is highest in Native Americans and lowest in populations of African descent.³ Classification of CL/P and CP cannot assure etiologic homogeneity, however, non-syndromic oral clefts seem to be influenced by multiple genes and environmental risk factors⁴ that may differ between CL/P and CP.⁵ Both linkage and candidate gene studies have found several genes influencing risk,⁶ furthermore, private mutations⁷ (i.e. those seen only in one family) and microdeletions⁸ have been identified. However, the exact number of causal genes and their mechanism of action remain unclear.⁷

The *MSX1* gene (Entrez GeneID: 4487)⁹ is a small muscle segment homeobox gene on chromosome 4p16, consisting of two exons and one intron spanning less than 4300 bp. An antisense transcript of the second exon has been reported.¹⁰ Build 129 of

dbSNP¹¹ reports 78 known SNPs in or near this gene, six in the coding sequence.

A linkage study of a single, large Dutch family segregating for both tooth agenesis and oral clefts identified a specific causal mutation in *MSX1*.¹² Multiple candidate gene studies in largely European-derived populations have shown consistent association between *MSX1* and CL/P, although studies in Asians were less consistent.¹³ Park *et al.*,¹⁴ Tongkobpetch *et al.*,¹⁵ and Otero *et al.*,¹⁶ have recently reported associations in Korean, Thai, and Colombian populations, respectively, adding two Asian and one admixed South American population to those giving evidence of association. We previously analyzed several SNPs, along with a common, intronic CA-repeat polymorphism, and found significant evidence of association.¹⁷ Jezewski *et al.*¹⁸ reported complete sequencing of *MSX1* in over 900 individuals from different ethnic backgrounds, and identified several new SNPs that may be directly causal, as well as identified three intronic SNPs showing association in either Asian or Caucasian populations. They estimated about 2% of oral clefts could be attributed to *MSX1*.¹⁸ As markers in this region have shown association with oral clefts, yet obvious causal mutations in *MSX1* are extremely rare, we examined a 2-Mb region around *MSX1* using a map of 393 SNPs in an international case–parent trio study. This region includes 11 known genes and

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six putative genes (Supplemental Figure 1). The function of most of these genes is unknown, but mutations in two genes (*EVC* and *EVC2*) cause Ellis-Van Creveld Syndrome (MIM 22550) or Weyers acrofacial dysostosis (MIM 193530); *CRMP1* has been shown to be a suppressor of tumor cell invasion controlling neural development and axonal growth.¹⁹

MATERIALS AND METHODS

Sample description

We collected data on case–parent trios registered at treatment centers in Maryland (MD; Johns Hopkins and the University of Maryland), Taiwan (Chang Gung Memorial Hospital), Singapore (KK Women's and Children's Hospital), and Korea (Yonsei University in Seoul). Research protocols were reviewed and approved by the Institutional Review Board (IRB) of each institution. Table 1 lists gender and the type of oral cleft for all trios examined in this study. The racial/ethnic backgrounds of the MD probands were 92.1% European American, 6.6% African American and 1.3% other. The cases from Korea and Taiwan were exclusively Asian, as were 94.2% of cases from Singapore. All Singaporean (SP) parents were unaffected, but five parents (four CL/P and one CP) among the 103 MD trios and two parents among the 172 Taiwanese (TW) trios were affected (two affected brothers from a single multiplex CP family, one of whom fathered two affected children (one with no DNA available) and the other had a single CP child; these cases are first cousins). All probands were given a clinical genetics evaluation to check for congenital anomalies or major developmental delays, and all were classified as having an isolated, non-syndromic oral cleft.

SNP selection, DNA and genotyping

Single nucleotide polymorphisms were selected for fine mapping in a 2-Mb region surrounding *MSX1* on chromosome 4p16 from position 4 000 000–6 000 000 of NCBI Build 35. When these SNPs were selected, the HapMap project was still in its genotyping phase, so dbSNP contained little allele frequency information on Caucasians and even less on Asians. Thus, we could not identify tagging SNPs, but SNPs were selected with a goal of having one SNP per 5 kb of physical distance. Variants with Illumina (San Diego, CA, USA) scores >0.6, dbSNP validation by submitters on multiple platforms, and high heterozygosity levels (particularly in multiple populations) were ranked higher in the selection process. In total, 429 unique SNPs were selected (see Supplemental Table 1 for positions and minor allele frequency (MAF) in each population), but not all met Hardy–Weinberg expectations in each population.

Genomic DNA samples were prepared as described previously.²⁰ DNA concentration was determined using the PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene OR, USA) and all DNA samples were stored at –20°C. A 4-μg aliquot of each genomic DNA sample was dispensed onto a bar-coded 96-well microtiter plate at a concentration of 100 ng/μl and genotyped for SNP markers using the Illumina Golden-Gate chemistry with Sentrix Array Matrices²¹ at the SNP Center, Johns Hopkins School of Medicine. Two cross-plate duplicates and four CEPH controls were included on each plate

to evaluate genotyping consistency within and between plates. Genotypes were generated on a BeadLab 1000 system.²² Of the 429 SNPs attempted, 393 were successfully genotyped.

Statistical methods

Single SNP and two to five SNP haplotypes were analyzed using a family-based association test (FBAT) program separately for CL/P and CP trios.^{23,24} Trios from all populations were combined for analysis of individual SNPs, but each population was analyzed separately when haplotypes were considered. Haplotype analysis assumes that parents are drawn from a single population, but single SNP analysis can be implemented on combined samples because the case–parent trio design compares alleles transmitted to the case against those present in the mating type but not transmitted.

We screened all markers for parent-of-origin effects using Clayton's extension of the TDT test in Stata 8.2,²⁵ and the transmission asymmetry test (TAT) suggested by Weinberg,²⁶ which is similar to the TDT but excludes matings between two heterozygotes (in which transmission can be ambiguous). When evidence of parent-specific over- or under-transmission of alleles at several SNPs was observed, haplotypes were examined for transmission distortion using the FAMHAP program that calculates maximum likelihood estimates (MLEs) of haplotype frequencies using the expectation-maximization algorithm and can handle missing data.^{27,28} Haplotype-based tests were used in the case in which the transmitted/non-transmitted status was permuted in each replicate, and the typical χ^2 statistic for marker combinations was replaced with the maximum χ^2 over all individual haplotypes (maximum TDT statistic). This approach gives an empiric *P*-value, corrected for the multiple haplotypes in each analysis, separately for maternal and paternal transmission.²⁹

RESULTS

The SNPs (393) were grouped in one of the known/putative genes in this region or in one of 12 intergenic regions (labeled 'No Locus #') beginning at position 4 Mb and ending at 6 Mb (Supplemental Table 2). Among these 393 SNPs, 62 had an MAF score between 0.5 and 5% in at least one population, whereas 54 SNPs were effectively monomorphic (MAF ≤ 0.005) in one or more populations, and 19 were monomorphic in all populations. One SNP was not in Hardy–Weinberg equilibrium in one population. In total, 352 SNPs were analyzed in the MD trios, 311 in the TW trios, 324 in the SP trios, and 314 in the KR trios. Among these SNPs, 308 are included in the HapMap II data and they picked up 48% of the linkage disequilibrium (LD) across this region in CEU samples and 49% in CHB/JPT samples. Patterns of LD across the entire region were calculated for each population using Haploview³⁰ (Supplemental Figure 2), and are comparable with only minor differences between populations.

Genetic distance between populations (CL/P and CP trios combined) was measured using eight polymorphic SNPs in and around *MSX1* in all populations (rs12651676, rs3821949, rs3116581, rs4075, rs12532, rs13104352, rs4473606, and rs4689955). As expected, the vast majority of the variation in haplotype frequencies occurred within populations (97.7%), whereas the among-population variation was only 2.3%. Pairwise *F_{ST}* values between these four populations revealed anticipated patterns of genetic distance: genetic distances (*F_{ST}*) among Asian populations were an order of magnitude smaller than those between MD and any of the three Asian populations (data not shown).

Individual SNP markers were used to test the composite null hypothesis of no linkage or no LD, and their empiric *P*-values are summarized in Figure 1, for the combined sample of 297 CL/P case–parent trios and for the 84 CP trios. Among CL/P trios, only one SNP (rs6446666) approached the 1% significance level. However, rs10937624 (*P*=0.01, in *LOC389145*) and rs12648800 (*P*=0.0054, in *EVC2*) at opposites ends of this 2-Mb region reached the 1% threshold among CP trios. Pooled analysis of all populations is valid for

Table 1 Proband gender and type of oral cleft according to recruitment site

| # Trios (# incomplete) ^a | CL/P | | | CP | | | |
|-------------------------------------|----------|--------|-------|------|--------|-------|----|
| | Male | Female | Total | Male | Female | Total | |
| Maryland | 102 | 44 | 32 | 76 | 13 | 13 | 26 |
| Korea ^b | 42 (2) | 22 | 18 | 40 | 2 | 0 | 2 |
| Singapore | 66 (13) | 24 | 11 | 35 | 12 | 19 | 31 |
| Taiwan | 171 | 95 | 51 | 146 | 6 | 19 | 25 |
| Total: | 381 (15) | 185 | 112 | 297 | 33 | 51 | 84 |

^aOnly one parent contributed DNA.

^bData on Korean CP families were not analyzed separately (due to small numbers), but they were included in any analysis of combined populations.

individual markers because the case–parent trio design is robust to population heterogeneity. This approach, however, is less suitable for haplotypes because estimation of haplotype frequencies typically assumes that samples were drawn from a single population. Therefore, we stratified on the basis of population for haplotype analyses.

Analysis of CL/P trios

Graphical Assessment of Sliding Windows (GrASP)³¹ was used to summarize statistical evidence of linkage and LD for individual SNPs and sliding windows of two to five SNPs (see Supplemental Figure 3). Markers in *MSX1* reached the 5% significance level in CL/P trios from three of four populations (MD, TW, and KR). Among MD CL/P trios, four SNPs (rs6826372, rs10937678, rs2279252, and rs1031919) in *EVC*, included in several haplotypes of different sizes, gave P -value <0.001 . These were found at the 3' end of a region of 16 SNPs in which several haplotypes had P -values <0.05 (Supplemental Figure 3 and Supplemental Table 3). The SNP rs1031919 had a P -value=0.0495 in single SNP analysis in SP trios, and the first three SNPs were part of a five-SNP haplotype in the TW samples yielding $P=0.0098$, indicating some consistency of signal for these SNPs. The MD samples also had five other regions showing $0.001 < P < 0.01$ (Supplemental Table 3).

The SP trios had one five-SNP haplotype near the 3' end of *STK32B* (rs6856163, rs12511127, rs7434429, rs7672067, and rs7440723), which

gave $P=0.00087$. The first four of these SNPs were involved in a four-SNP haplotype in the MD samples giving $P=0.029$, but none was significant in any other population, nor was there much signal around this haplotype in SP samples (Supplemental Figure 3).

Although there were no haplotypes with $P < 0.001$ in the TW samples, two fell between 0.01 and 0.001. A five-SNP haplotype in the intergenic region between *ZNF509* and *D4S234E* (consisting of rs7692312, rs7698300, rs4689340, rs1212085, and rs4318596) gave $P=0.00263$. This same haplotype was also significant in MD trios ($P=0.00498$), and overlapped the last two SNPs of another significant five-SNP haplotype ($P=0.00801$, Supplemental Table 3). None of these SNPs, however, were significant in either SP or KR trios. Among the KR CL/P trios, one five-SNP haplotype (rs4450871, rs4522817, rs13117014, rs12171324, and rs4689973) reached $P < 0.001$. This haplotype is just upstream from C17. The last three of these SNPs were included in a five-SNP haplotype yielding $P=0.01775$ among the TW trios (Supplemental Figure 3), but these SNPs were not significant in MD or SP trios.

Figure 2 summarizes evidence from analysis of individual SNPs and haplotypes for *STX18*, *MSX1* and the intergenic region between them. Here $-\log_{10}(P\text{-value})$ is plotted against physical location for 99 SNPs separately for each population. In these plots, vertical lines represent the strength of evidence from individual SNPs and horizontal lines represent the strength of evidence from haplotypes of two to five SNPs. Of these 99 SNPs, four had MAF value <0.05 in all four populations and therefore contributed little information (Supplemental Table 1). Among 95 SNPs polymorphic in one or more populations, the number of fully polymorphic SNPs (MAF >0.05) varied on the basis of population: MD had 94 SNPs, TW 74, SP 80, and KR had 76 SNPs. Among CL/P trios (Figure 2), MD and TW showed a consistent signal in *STX18*. In MD trios, haplotypes near the 5' end of this gene showed the strongest signal, whereas among TW trios, individual SNPs yielded fairly consistent signals but haplotypes did not provide additional evidence due to strong LD. All four populations showed some signal in the intergenic region, but its location was not consistent across populations. Three populations (MD, TW, and KR) gave statistical evidence just upstream of *MSX1*, but none within the gene itself.

A total of 68 SNPs were successfully genotyped in *STK32B*, but four were effectively monomorphic (MAF <0.05 ; Supplemental Table 1). Again in this gene, the number of SNPs with a MAF >0.05 varied by population: MD had 59; TW 62; SP 63; and KR 62. As seen in Figure 3, CL/P trios had at least one region in *STK32B*, yielding evidence of linkage and association at the 5% significance level from all populations although the location of signal varied. The MD CL/P trios had the strongest signal, located in and around exon 4.

The *EVC2–EVC–CRMP1* gene cluster included 100 polymorphic SNPs (of 106 tested, Supplemental Table 1) and showed evidence of linkage and association, though once again the location of statistical signals varied on the basis of population (Figure 4). The number of polymorphic SNPs varied by population: MD had 92, TW 85, SP 85, and KR 81. The MD CL/P trios gave the strongest signal near the 3' end of *EVC*. Interestingly, there was also a strong signal at the junction between the 3' end of *EVC* and the 3' end of *CRMP1* among both SP and KR CL/P trios. Among SP CL/P trios, the strongest signal was in *EVC2*. Within this gene cluster, screening individual SNPs revealed an intriguing pattern of stronger transmission distortion when alleles were inherited from fathers as opposed to mothers. Supplemental Table 4 shows allelic TAT analysis separately for maternal and paternal transmission at 41 informative SNPs in all CL/P trios. A total of 15 SNPs in *EVC2* showed statistically significant results

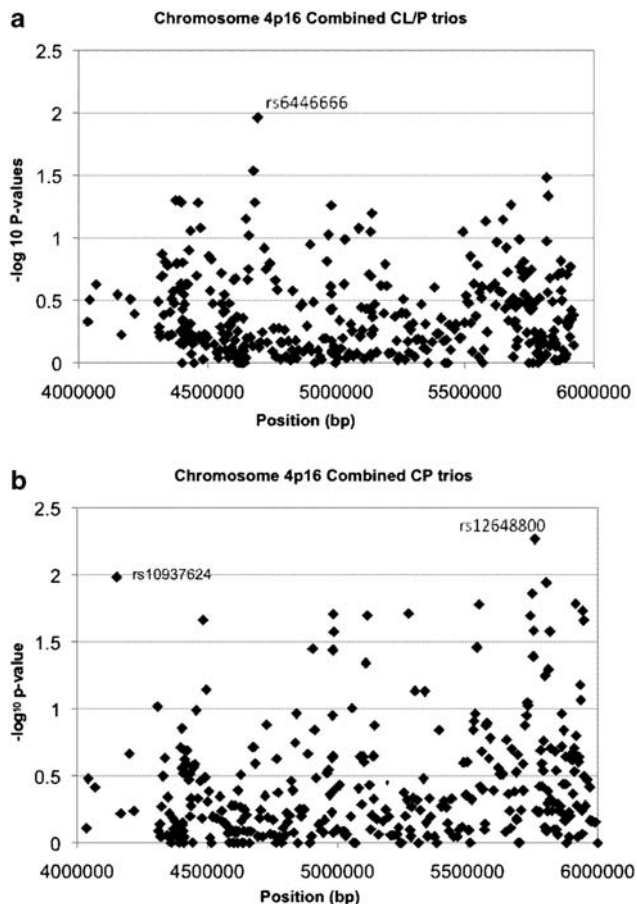


Figure 1 Empirical P -values from a family-based test for linkage in the presence of disequilibrium for 393 individual SNPs from a 2-Mb region on chromosome 4p16. (a) CL/P case–parent trios (297) from four populations (Maryland, Taiwan, Singapore, Korea) and (b) 84 CP case–parent trios from four populations (Maryland, Taiwan, Singapore and Korea)

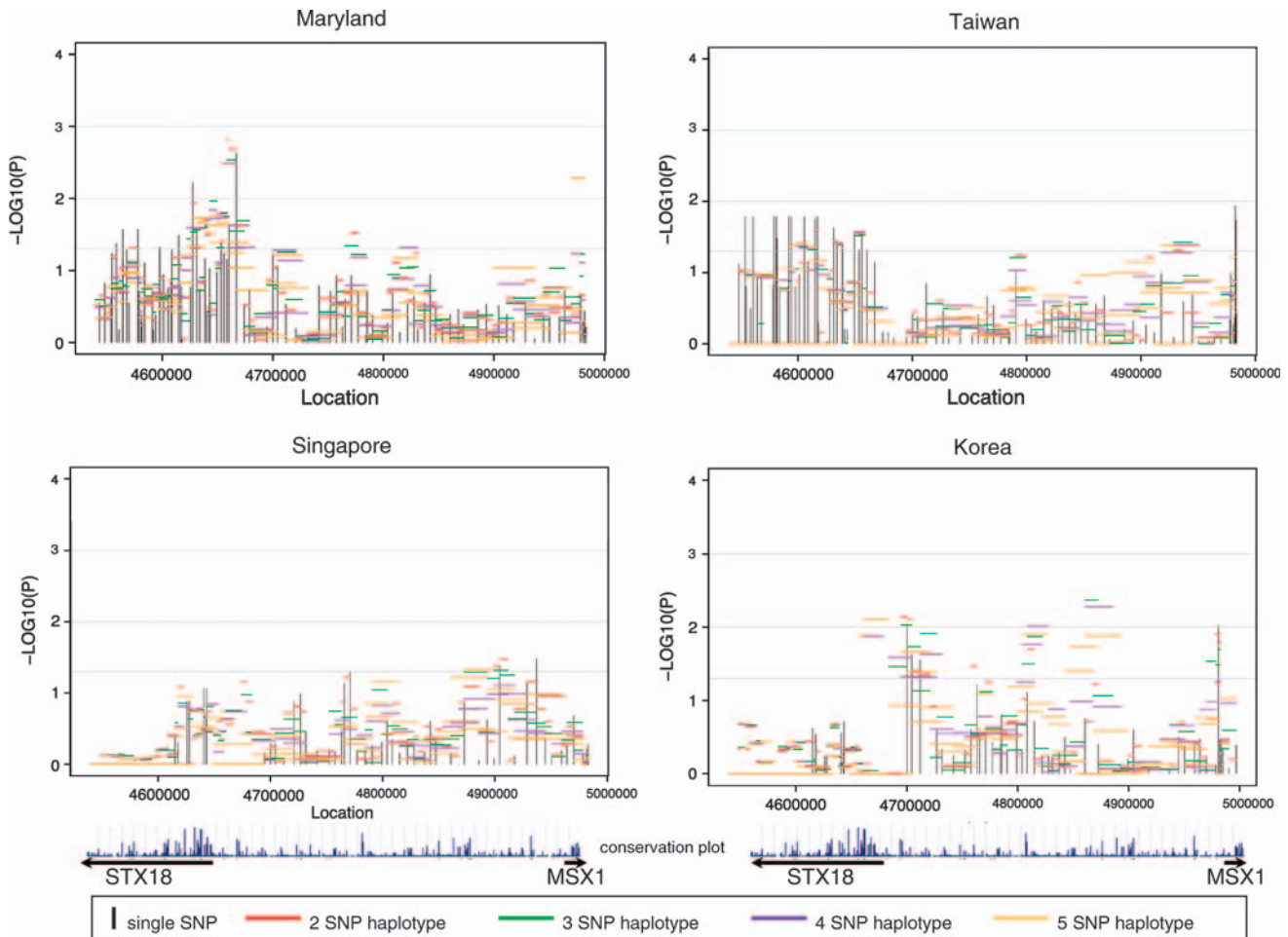


Figure 2 Significance of individual SNPs and sliding window haplotypes in *STX18* and *MSX1* for CL/P trios from four populations. Vertical lines represent $-\log_{10}(P\text{-value})$ for individual SNPs and horizontal lines represent $-\log_{10}(P\text{-value})$ for haplotypes of two to five SNPs over the entire region. The conservation plot indicates percent conservation of the sequence across vertebrates, as calculated by the UCSC genome browser website²⁹. Arrows below indicate the direction of transcription of each gene.

for the TAT when paternally transmitted, but only two among the maternally inherited alleles. Further analysis of haplotypes in *EVC2* using the FAMHAP program showed consistent evidence of over-transmission from fathers but not from mothers, with greater evidence provided among Asian trios. For example, considering two SNP haplotypes, rs6446384 and rs4689273 (which were not in the same LD block in the Asian samples, but are in the Caucasian samples), showed significant over-transmission of paternally derived haplotypes (maximum TDT=6.821, empiric $P\text{-value}$ =0.0145 among all Asian trios and maximum TDT=1.78, empiric $P\text{-value}$ =0.389 among MD trios). As this distortion of transmission patterns was seen among paternally derived alleles and haplotypes, it raises the possibility of imprinting at this gene.

Analysis of CP trios

Although the number of CP trios was smaller (Table 1), similar analyses yielded some evidence of linkage and LD. The MD CP trios gave evidence in *MSX1* and at the 5' end of *STK32B*, as well as near the 3' end of *EVC* (data not shown). The SP trios showed some signal in *STK32B*, near both ends of *EVC* and in the intergenic region between *EVC* and *EVC2* (data not shown). The TW trios, however, showed strong signal in the region from *LYAR* through *ZNF509* and

the intergenic region between *STX18* and *MSX1*. There was also some signal near *MSX1* and *EVC2* (data not shown).

DISCUSSION

On the basis of multiple reports on association between markers in *MSX1* and oral clefts, and few reports on causal mutations identified in this gene, we examined the region around *MSX1* for evidence of linkage and disequilibrium using case–parent trios identified through a case with an isolated, non-syndromic oral clefts recruited in an international study. Here we report results from a fine mapping panel of 393 SNPs extending from physical position 4–6 Mb on chromosome 4p16, covering 1 Mb on each side of *MSX1*.

We found evidence for linkage in the presence of disequilibrium in several genes and intergenic regions, raising the possibility multiple genes in this region may influence risk of oral clefts. There are three genetic regions that are of particular interest because of their consistent evidence in case–parent trios from four populations for both CL/P and CP: *STK32B*, the *EVC–EVC2–CRMP1* region, and the *STX18–MSX1* region.

The *EVC* (Entrez gene ID 2121)⁹ and *EVC2* (Entrez gene ID 132884)⁹ are new and intriguing candidate genes for oral clefts. Mutations in either of these genes can lead to Ellis-Van Creveld

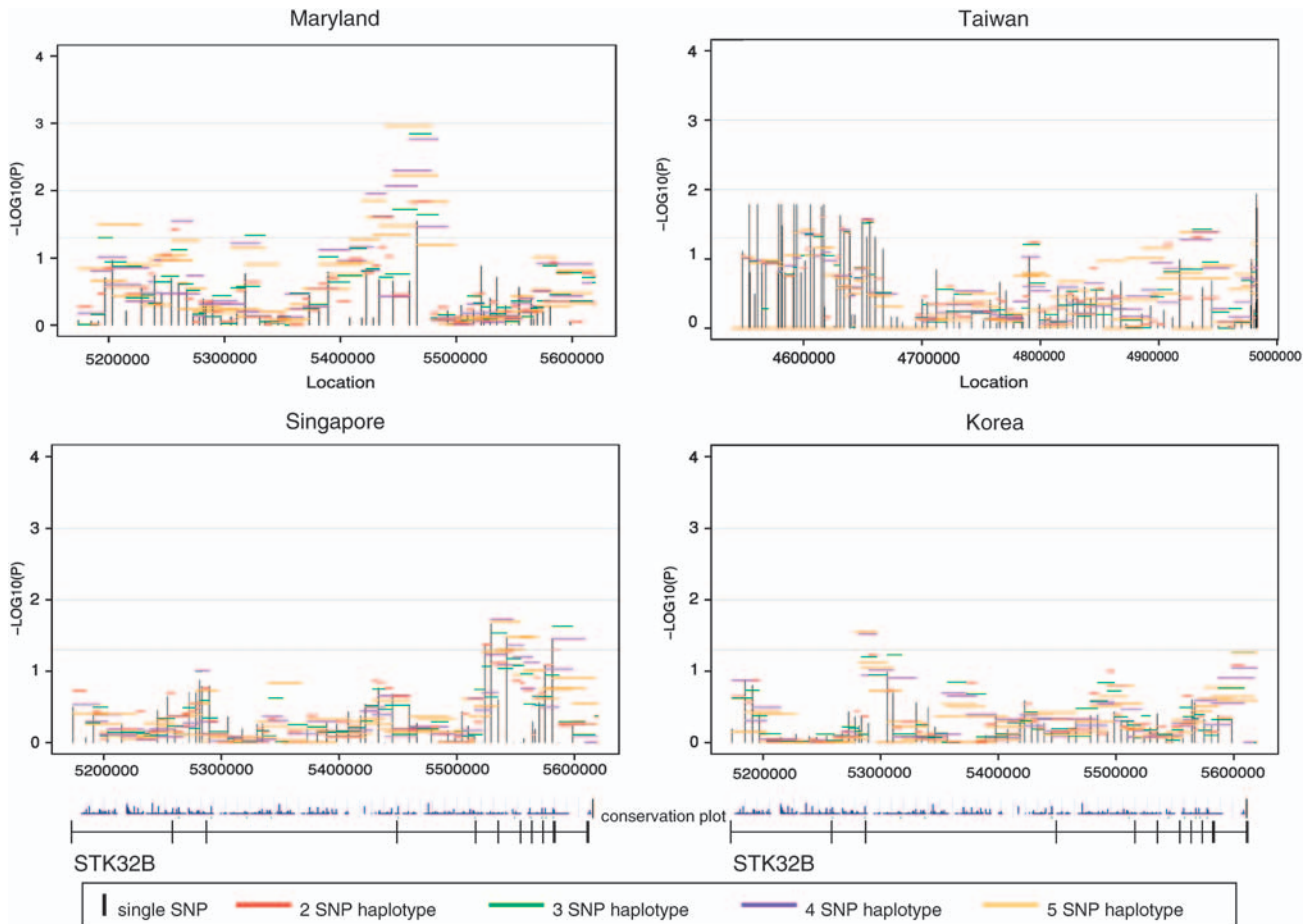


Figure 3 Significance of individual SNPs and sliding window haplotypes in *STK32B* in CL/P trios from four populations. Vertical lines represent $-\log_{10}(P)$ -value for individual SNPs and horizontal lines represent $-\log_{10}(P)$ -value for haplotypes of two to five SNPs over the gene. The gene is transcribed from left to right in these figures. The conservation plot indicates percent conservation of the sequence across vertebrates, as calculated by the UCSC genome browser website.²⁹ Exons are indicated by vertical lines on the line below the plots.

syndrome or Weyers acrofacial dysostosis. Ellis-Van Creveld syndrome is an autosomal recessive Mendelian syndrome not usually associated with oral clefts, but there have been reports of cases with 'partial hare-lip',³² fully erupted teeth at birth,³² tooth agenesis,³³ or dental abnormalities.³⁴ Weyers acrofacial dysostosis is associated with anomalies of the lower jaw, dentition and oral vestibule.³⁵ The *EVC* and *EVC2* genes are arranged head to head on the chromosome, separated by less than 3 kb distance, and seem to have a common promoter sequence. The *EVC* gene encodes a protein containing a leucine-zipper and a transmembrane domain. The *EVC2* gene also has a transmembrane domain, but is not homologous to *EVC*. These genes are less than 1 Mb away from marker D4S431, the locus at which Hecht *et al.*³⁶ found evidence suggestive of linkage. Both are expressed in the skeleton, but their functions are unknown. As these genes may influence the development of the mouth and jaw, and it seems reasonable to look for possible causal mutations, given the consistency of signal and their proximity to *MSX1*. Several SNPs in *EVC2* (and their haplotypes) showed intriguing evidence for paternal transmission distortion, raising the possibility of imprinting in this region.

The STX18 protein (Entrez gene ID 15942)⁹ is a SNAP receptor protein, which functions in the endoplasmic reticulum, intermediate

compartment and *cis*-Golgi vesicle trafficking.³⁷ It has been implicated in cell apoptosis through its binding of BNIP1.³⁸ Downregulation of STX18 expression is also associated with growth of human breast cancer cells.³⁹ Cell growth regulation could be important in the etiology of oral clefts, as apoptosis must occur in the embryonic nasal fin for the developing lip to form properly.⁴⁰ Programmed cell death also occurs at several other stages in palate development.⁴¹ Improper timing and/or failure of apoptosis at any of these developmental stages could create an oral cleft. A recent analysis of higher rates of cancer among families ascertained through an isolated oral cleft has been documented,⁴² and the tumor suppressor gene *TP63* is also a candidate gene for clefting⁴³, which makes the association between *STX18* and both cancer and clefting intriguing.

The *STK32B* protein (Entrez gene ID 55351)⁹ is a member of the serine/threonine kinase family. This family is involved in phosphorylation and ATP binding. Its specific function in the cell is currently unknown, making it difficult to speculate on its potential role in clefting. Interestingly, *MSX1* is the only one of these genes well conserved among vertebrates⁴⁴ (see Figures 2–4). Our statistical evidence of linkage and disequilibrium did not correlate with regions of conservation.

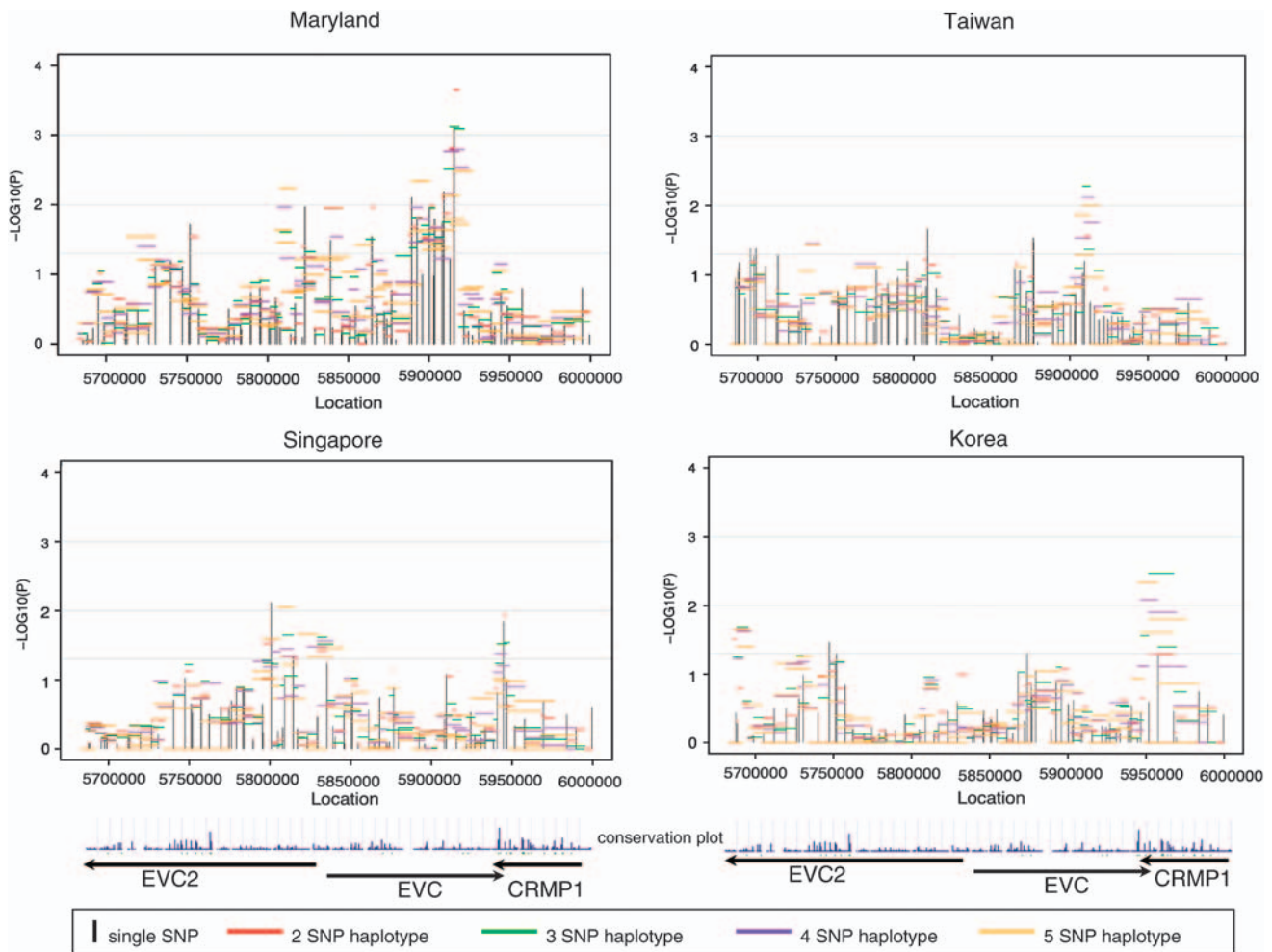


Figure 4 Significance of individual SNPs and sliding window haplotypes in *EVC2*, *EVC*, and *CRMP1* in CL/P trios from four populations. Vertical lines represent $-\log_{10}(P\text{-value})$ for individual SNPs and horizontal lines represent $-\log_{10}(P\text{-value})$ for haplotypes of two to five SNPs over the entire region. Arrows indicate direction of transcription. The conservation plot indicates percent conservation of the sequence across vertebrates, as calculated by the UCSC genome browser website²⁹.

The issue of multiple comparisons is never simple when analyzing multiple SNPs and haplotypes in a small physical region. As SNPs in strong LD generate highly correlated P -values, adjusting significance levels using a Bonferroni correction would be overly conservative. In our analysis, we used empirical P -values whenever possible, and relied on replication across the four populations to build valid inferences. We acknowledge confirmation from additional studies is still needed, and any study design based on LD between markers and unobserved causal variant(s) will have limited power to detect multiple rare variants that could also be important. Nonetheless, this analysis contributes to the evidence that genes in the 4p16 region influence risk of oral clefts, and suggests that *MSX1* may not be the only causal gene. As none of the new genes yielding intriguing evidence of linkage and disequilibrium (*STK32B*, *EVC*, *EVC2*, and *STX18*) have been tested for association with oral clefts before, it is critical to confirm this evidence of association in future studies.

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