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Novel Cleft Susceptibility Genes in Chromosome 6q

ABSTRACT

Cleft lip/palate is a defect of craniofacial development. In previous reports, chromosome 6q has been suggested as a candidate region for cleft lip/palate. A multipoint posterior probability of linkage analysis of multiplex families from the Philippines attributed an 88% probability of harboring a cleft-susceptibility gene to a narrower region on bands 6q14.2-14.3. We genotyped 2732 individuals from families and unrelated individuals with and without clefts to investigate the existence of possible cleft-susceptibility genes in this region. We found association of *PRSS35* and *SNAP91* genes with cleft lip/palate in the case-control cohort and in Caucasian families. Haplotype analyses support the individual associations with *PRSS35.* We found *Prss35* expression in the head and palate of mouse embryos at critical stages for palatogenesis, whereas *Snap91* was expressed in the adult brain. We provide further evidence of the involvement of chromosome 6q in cleft lip/palate and suggest *PRSS35* as a novel candidate gene.

KEY WORDS: cleft lip/palate, fine mapping, chromosome 6q, polymorphism.

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INTRODUCTION

Cleft lip with or without cleft palate (cleft lip/palate) results from defects in growth and patterning of the facial primordia. Over 300 syndromes, including some that are either chromosomal or Mendelian, might present a cleft of the lip and/or the palate as a feature, and comprise about 30% of all cleft cases. The remaining 70% are attributed to isolated, non-syndromic clefts, without any associated structural anomaly (Gorlin *et al.*, 2001). In the United States, approximately 6800 children with cleft lip/palate are born each year, representing $101,000/each of lifetime costs (Centers for Disease Control and Prevention, 2006).

The etiology of non-syndromic cleft lip/palate is multifactorial, and from 3 to 14 genes may be involved in addition to environmental factors (Schliekelman and Slatkin, 2002). Several genes/loci have shown positive linkage and/or association results in cleft lip/palate, cleft palate only, or both. To date, the most remarkable finding was the association between *IRF6* gene variants and non-syndromic cleft lip/palate (Zucchero *et al.*, 2004), replicated in several other populations (reviewed by Vieira, 2008). Recent evidence attributed its effect to a point mutation in a *TFAP2A* binding site in an enhancer of the *IRF6* gene promoter (Rahimov *et al.*, 2008). Polymorphic variants or rare point mutations in other genes have also been found in individuals with clefts, including *MSX1, TGFB3, FOXE1, PVR, PVRL1,* and *FGFs* (reviewed by Vieira, 2008). Recently, a variant on the regulatory region of the *PDGF-C* gene has been shown to decrease transcriptional activity and to be associated with cleft lip/palate (Choi *et al.,* 2008).

Genome-wide linkage scans of anonymous markers have provided important clues to narrowing down the number of candidate genes for cleft lip/palate. A genome scan of Chinese multiplex families revealed positive linkage results (LOD = 1.41) for cleft lip/palate near marker D6S1031 on chromosome 6q14.2 (Marazita *et al.,* 2002). The imputed posterior probability of linkage (PPL) (Logue and Vieland, 2004) for multiplex families from the Philippines identified a peak at ~95cM on chromosome 6q14.2-14.3 to which a ~88% probability of harboring a cleft-susceptibility gene was attributed (Govil *et al*., unpublished observations). Interestingly, this linkage peak was located between D6S1031 and D6S1056, overlapping with the results of the Chinese genome scan (Marazita *et al.,* 2002). Taken together, these point toward chromosome 6q as having a likely role in human clefting.

To investigate the existence of possible cleft-susceptibility genes in the 6q14.2-14.3 region, we used densely spaced markers to genotype 2732 individuals from families and unrelated persons with and without clefts. We also investigated embryonic expression of associated genes during mouse embryonic development.

Table 1. Details of Family-based and Case-Control Cohorts Investigated in the Study

Regional Group	Ancestral Origin	Number of Families	Number of Individuals	Affected Individuals	Unaffected Individuals	Affected Individuals by		Families	
						Cleft Lip	CL/P[b]	CLO+CL,CLP (no CPO affecteds)[c]	CLO+CLP+CL, CLP (no CPO affecteds)[d]
Family-based Cohorts									
United States	Pittsburgh	68	387	101	286	31	70	35	51
	Saint Louis	21	104	33	71	6	27	10	19
Central America	Guatemala	77	514	93	421	20	73	22	77
South America	ECLAMC[a]	171	513	171	342	–	–	–	–
Europe	Spain	36	136	43	93	10	33	9	33
	Turkey	29	288	38	250	17	21	14	29
East Asia	China	60	180	60	120	14	46	13	60
Subtotal Families		462	2122	539	1583	98	270	103	269
Case-Control Cohort									
South America	Brazil	0	610	328	282	4	324	–	–
TOTAL		462	2732	867	1865	103	594	103	269

[a] ECLAMC, Estudio Colaborativo de Malformaciones Congenitas (Latin American Collaborative Study of Congenital Malformations).
[b] CLO+ CL,CLP (no CPO affecteds) = all families where all affecteds are CLO + all families where at least one affected is CLO, and one affected is CLP, excluding any family where an affected is CPO.
[c] CLO + CLP + CL,CLP (no CPO affecteds) = all families where all affecteds are CLO + all families where all affecteds are CLP + all families where at least one affected is CLO, and one affected is CLP, excluding any family where an affected is CPO.

STUDY POPULATION & METHODS

Study Population

The study population consisted of 2732 individuals (867 affected with cleft lip/palate) and unaffected family members and unrelated control individuals. Family-based samples came from multiplex families of the United States, Spain, Turkey, Guatemala, and China, and from family trios ascertained through the ECLAMC (Latin American Collaborative Study of Congenital Malformations) registry, a hospital-based birth defects registry with sites across South America (Castilla and Orioli, 2004). Case-control samples were all ethnically matched Caucasian individuals from Brazil (Table 1).

All cases had non-syndromic cleft lip with or without cleft palate. Non-syndromic status was determined according to patient records. Families were ascertained through probands, and additional relatives were recruited. Individuals presenting cleft palate only or unknown cleft types, and controls with family history of clefting were excluded. The study was approved by the local review boards and accredited by the University of Pittsburgh Institutional Review Board as an umbrella to the international collaboration efforts. Informed consent was obtained from participants and from parents/guardians of children under 15 yrs of age. Saliva samples were collected as the source of genomic DNA. DNA extraction followed established protocols.

Selection of Candidate Loci and Genetic Markers and Genotyping Procedures

Single-nucleotide polymorphisms spanning the chromosome 6q14.2-14.3 region were selected by means of the International HapMap Project database (http://www.hapmap.org). We generated a linkage disequilibrium plot of the candidate region where 5 genes and 528 polymorphisms were identified (Appendix). We used the function *"Download tag SNP data"* and selected 26 polymorphisms as representative of the polymorphisms in the region. We selected polymorphisms that maximally represent the linkage disequilibrium structure of a given region, to avoid redundant information (Carlson *et al.*, 2004). Preference was given to polymorphisms with high heterozygosity levels and different minor allele frequencies, to avoid intermarker linkage disequilibrium.

Genotyping was performed with Taqman chemistry (Ranade *et al.,* 2001) on an automatic sequence-detection instrument (ABI Prism 7900HT, Applied Biosystems, Foster City, CA, USA).

Details of the selected polymorphisms are available in the Appendix.

Statistical Analyses

Chi-square and Fisher exact tests were used for case-control comparisons and determination of fit to Hardy-Weinberg equilibrium. Genotype and allele frequencies of each polymorphism were compared between cases and controls. We used Bonferroni correction to adjust for multiple testing, considering a significance level of 0.05 divided by the number of independent tests (26) to give a corrected p-value ($\alpha = 0.002$).

For family-based analyses, we tested for linkage disequilibrium between marker alleles and cleft lip/palate using the Family Based Association Test (FBAT) (Horvath *et al.*, 2001). We examined the transmission of alleles from heterozygous parents to affected offspring. We tested each population individually and as a pooled Caucasian (US, Madrid, and Turkey) and total family data set (US, Guatemala, Spain, Turkey, and China). We further analyzed the families according to cleft subgroups: (1) families where all affecteds have cleft lip only plus

families where at least one affected has cleft lip only, and one affected has cleft lip and palate; and (2) families where all affecteds have cleft lip only plus families where all affecteds have cleft lip and palate plus families where at least one affected has cleft lip only, and one affected has cleft lip and palate.

We performed haplotype analyses using the function 'hbat' of the FBAT software. We created haplotypes using 2-, 3-, and 4-sliding windows.

Gene Expression Analyses

We used real-time PCR to investigate the expression of *Prss35* and *Snap91* during embryonic development. We used total, head, and palate mRNA of mouse embryos (Zyagen Laboratories, San Diego, CA, USA) at different stages of pregnancy [embryonic days (ED) 10-18]. As a positive control for *Prss35* expression, we used cDNA obtained from the ovaries of mice undergoing a stimulated estrous cycle (the period of peak *Prss35* expression) (Miyakoshi *et al.*, 2006). Brain cDNA (Clontech, Mountain View, CA, USA) served as a positive control for *Snap91*. β-*actin* (*Actb*) was used as endogenous control. [Primer sequences and reaction conditions are available in the Appendix.] Products were resolved in agarose gel electrophoresis stained with ethidium bromide. Images were captured by means of a GelDoc 2000 system and Quantity One software (BioRad Laboratories, Hercules, CA, USA). Products were purified and sequenced at the Oregon National Primate Research Center Molecular and Cell Biology Core Laboratory (Beaverton, OR, USA) for confirmation of the identity of the amplicons.

RESULTS

Association Analyses

We tested 26 single-nucleotide polymorphisms spanning the 5 genes present on chromosome 6q14.2-14.3 (*PRSS35, SNAP91, 401268, C6orf159*, and *CYB5R4*) for association with cleft lip/palate in a large sample cohort. After adjusting for multiple testing, we found significant associations between an intronic marker in *PRSS35* (rs7753918) with cleft lip/palate in the case-control cohort ($p = 0.00001$). In addition, 2 intergenic markers near *SNAP 91* (rs6454338 and rs10943957) showed significant association in the US ($p = 0.001$) and pooled Caucasian ($p = 0.002$) families. Borderline associations were also seen with rs10943957 in the US ($p = 0.007$) families, with *CYB5R4* (rs6940766) in the case-control ($p = 0.005$), and with *SNAP91* (rs217325) in the ECLAMC cohort ($p = 0.006$) (Table 2).

Under a nominal value of 0.05, markers in *PRSS35* (rs1171114 and rs512140) and markers in or flanking *SNAP91* (rs9294279, rs624076, rs10943957) were associated with families where all affecteds have cleft lip only plus families where at least one affected has cleft lip only, and one affected has cleft lip and palate in Chinese and Caucasian families. For families where all affecteds have cleft lip only plus families where all affecteds have cleft lip and palate plus families where at least one affected has cleft lip only, and one affected has cleft lip and palate, marker rs10943957 showed association in Caucasian families. [Detailed results are available in the Appendix.]

Haplotype analyses support the individual associations found for *PRSS35* and cleft lip/palate in Caucasian families ($p = 0.008$ and $p = 0.003$, for 3- and 4-window haplotypes, respectively; Table 3).

Expression Analyses

We used RT-PCR to determine whether *Prss35* and *Snap91* are expressed during the periods of mammalian craniofacial development. Expression of *Prss35* was evident at ED10, decreased at ED11, then increased and peaked at ED12 and ED13. Lower levels of expression were noted each day from ED14 through ED18 (Fig., A). In contrast, *Snap91* gene expression was undetectable in the embryonic material analyzed, but was significant in the adult brain (Fig., B). Limited levels of *Snap91*expression were noted only in embryo-derived cDNA samples after an additional 5 to 10 PCR cycles (data not shown).

We then used cDNA from mouse head and palate to investigate *Prss35* expression at periods critical for palate development (ED12-15). *Prss35* was expressed at all stages in the developing mouse head (Fig., C). In the palate, *Prss35* expression peaked at ED12 and ED13, then declined dramatically at ED14 and ED14.5. No expression was detected at ED15 (Fig., D, and Appendix).

DISCUSSION

Chromosome 6 has been long considered a candidate for the etiology of oral-facial clefts. Studies have reported the presence of cleft lip/palate in individuals with deletions and translocations affecting chromosome 6 (Hopkin *et al.,* 1997; Yu *et al.,* 2005). More specifically, chromosome 6q was identified in a previous genome scan as a cleft-susceptibility region in Chinese individuals (Marazita *et al.*, 2002), and a later meta-analysis supported these findings (Marazita *et al.,* 2004). Imputed multipoint posterior probabilities of linkage for Filipino multiplex families revealed a region at 6q14.2-14.3, between markers D6S1031 and D6S1056, presenting an ~88% chance of harboring a cleft gene (Govil *et al.,* unpublished observations). The posterior probability of linkage, a class of likelihood-based, model-free statistics, is designed for accumulation of evidence for or against linkage across multiple, heterogeneous sets of data (Vieland, 1998, 2006), and allows for measurement of the probability to have found true linkage.

To investigate the existence of possible cleft-susceptibility genes in the 6q14.2-14.3 region, we performed association tests with densely spaced single-nucleotide polymorphisms and 2732 individuals from eight different populations. We found association of a novel gene, *PRSS35*, with cleft lip/palate in a case-control cohort from Brazil. Studies with unrelated cases and controls always raise the question as to whether the individuals are appropriately matched, particularly when admixture is a feature of the population. To overcome possible confounding results of undetectable population stratification, we limited our case and control groups to include individuals of only Caucasian descent. We also found association of *PRSS35* with cleft subgroups in our family cohorts, more specifically in Guatemalan,

Table 2. Summary of Results for Association Tests with Markers in the Chromosome 6q14.2-14.3 Region and Cleft Lip/Palate in the Studied Populations

			Family-based[a]								
		Case-Control[a]	Individual Populations							Groups	
SNP	Gene	Brazil	Pittsburgh	St. Louis	Guatemala	Spain	Turkey	China	ECLAMC	Caucasian	Pooled[b]
rs10943944	*PRSS35*	0.01	0.86	0.65	0.97	1.00	1.00	0.35	0.18	1.00	0.65
rs7753918	*PRSS35*	**0.00001**	0.73	0.32	0.35	0.25	0.74	0.56	0.18	0.69	0.98
rs9449648	*PRSS35*	0.98	0.22	0.18	0.67	0.81	0.08	0.19	–	0.17	0.06
rs1171114	*PRSS35*	0.06	0.75	0.93	0.51	0.68	0.06	0.07	–	0.58	0.26
rs512140	*PRSS35*	0.17	0.04	0.33	0.35	0.68	0.44	0.49	–	0.09	0.04
rs1171105	—	0.10	0.36	0.39	0.80	0.85	0.69	0.32	–	0.34	0.30
rs2023238	*SNAP91*	0.55	0.22	0.93	0.55	0.48	0.41	0.11	–	0.41	0.57
rs9294279	*SNAP91*	0.12	0.18	0.69	0.23	0.85	0.35	0.16	–	0.25	0.35
rs3798867	*SNAP91*	0.23	0.55	0.74	0.74	0.14	0.03	0.02	–	0.22	0.03
rs217325	*SNAP91*	0.05	0.11	0.92	0.71	0.32	0.16	0.16	*0.006*	0.23	0.38
rs217308	*SNAP91*	0.60	0.55	0.74	0.54	0.18	0.06	0.02	–	0.42	0.04
rs755101	*SNAP91*	0.50	0.29	0.88	0.23	0.43	0.05	0.45	–	0.04	0.02
rs217290	*SNAP91*	0.65	0.55	0.74	0.64	0.26	0.03	0.02	–	0.28	0.03
rs217289	*SNAP91*	0.27	0.63	0.74	0.80	0.32	0.03	0.04	–	0.28	0.05
rs624076	—	0.18	0.02	0.32	0.16	0.51	0.82	0.45	–	0.12	0.03
rs614565	—	0.13	0.09	0.56	0.74	0.37	0.81	0.50	0.80	0.09	0.07
rs6454338	—	0.22	**0.001**	1.00	0.91	0.24	0.43	0.82	–	0.01	0.11
rs10943957	—	0.25	*0.007*	1.00	0.69	0.16	0.41	0.81	–	**0.002**	0.09
rs1325474	—	0.09	0.37	1.00	0.11	0.32	0.16	0.30	0.34	0.38	0.04
rs9350989	*C6orf159*	0.02	0.56	–	0.17	0.32	0.56	0.20	–	0.59	0.15
rs9353149	*CYB5R4*	0.24	0.32	0.56	0.08	0.53	–	0.90	–	0.32	0.14
rs2324482	*CYB5R4*	0.17	0.26	–	0.17	0.32	0.56	0.53	–	0.37	0.11
rs6940766	*CYB5R4*	*0.005*	0.28	0.41	0.30	0.16	0.02	0.32	0.74	0.45	0.47
rs1998742	*CYB5R4*	0.04	0.75	0.56	0.13	0.86	0.74	0.07	–	0.69	0.46
rs1325469	*CYB5R4*	0.12	0.28	0.32	0.16	0.26	0.08	0.39	–	0.48	0.08
rs7770749	*CYB5R4*	0.42	0.59	–	0.78	0.71	–	–	–	0.83	1.00

[a] Bold indicates statistically significant difference ($\alpha = 0.002$). Italic indicates borderline association.
[b] Comprises analysis of Pittsburgh, St. Louis, Guatemala, Spain, Turkey, and Beijing families pooled together. Cells with no numerical values represent untyped or uninformative markers in the respective population.

Table 3. Results of Haplotype Analyses for *PRSS35* Markers in Caucasian Families (US, Madrid, and Turkey)

	PRSS35 Markers					
Haplotype Window	rs10943944	rs7753918	rs9449648	rs1171114	rs512140	rs1171105
Window=2	0.62 TA	0.09 AA	0.03 AT	0.07 TA	0.48 AT	
Window=3	0.63 TAA	0.30 AAT	**0.008 ATA**	0.12 TAC		
Window=4	0.15 CGAC	0.37 AGTA	**0.003 GCGC**			

Chinese, and Caucasian populations. Analyses of *PRSS35* marker haplotypes in the Caucasian families further support the associations found in the case-control cohort. Although our target region does not completely overlap with the region previously described in Chinese families (Marazita *et al.,* 2002), the fact that both studies found positive results in different populations reinforces the possibility that cleft-susceptibility genes may be located on chromosome 6q.

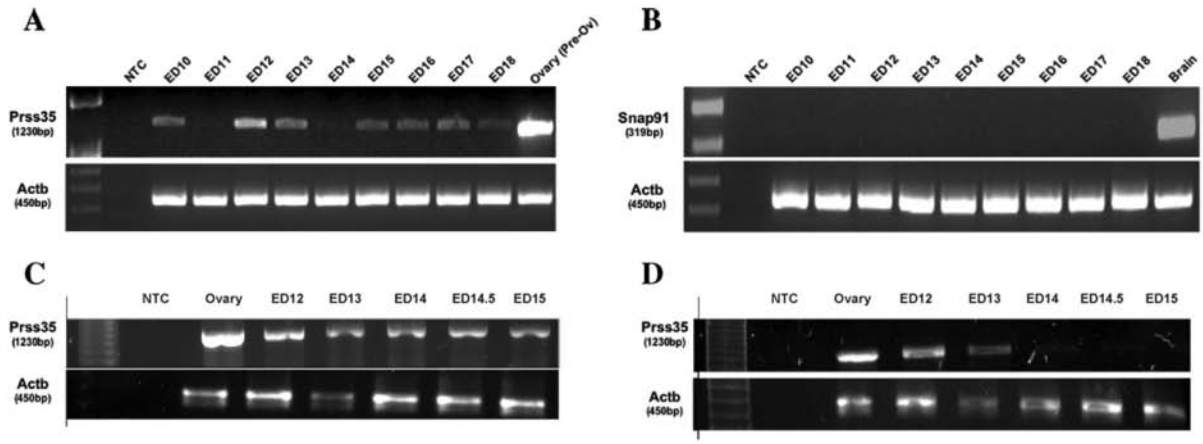

Figure. Analyses of *Prss35* and *Snap91* gene expression during craniofacial development in mice. *Prss35* **(A)** and *Snap91* **(B)** gene expression was performed with cDNA generated from whole embryos (ED 10 through ED 18). *Prss35* expression in the head **(C)** and palate **(D)** of mouse embryos at critical stages for palate development (ED12-E15). β-*Actin (Actb)* was used as a normalization control. NTC, no template control; ED, embryonic day.

We observed clearly detectable levels of *Prss35* expression in the head and palate of mouse embryos during the periods of craniofacial development, and these increased particularly at embryonic days 12 and 13. We speculate that *Prss35* may be involved in the early stages of palatogenesis, and that, if disturbed, it may impose a risk to proper elevation of the palatal shelves rather than palatal fusion. *PRSS35* codes for a serine protease, belonging to a group of structurally and functionally diverse proteins critical for essential biological processes. For instance, proteases are determinants of cellular proliferation and migration during embryonic development (including palate development) for their ability to remodel the extracellular matrix. Similarly, other proteases, such as matrix metalloproteinases, have been postulated to play critical roles during palate formation (Iamaroon *et al.*, 1996; Morris-Wiman *et al.*, 1999, 2000; Blavier *et al.*, 2001; Brown *et al.*, 2002). Moreover, variations in one MMP gene, *MMP3,* have been reported in association with cleft lip/palate (Letra *et al.*, 2007). Taken together, these observations warrant additional research to determine the role of a protease such as *PRSS35* in palate development.

We also found significant association of intergenic markers near the *SNAP91* gene with cleft lip/palate in the US and pooled Caucasian families. Although unlikely to be of functional significance with respect to a phenotype, intergenic polymorphisms may localize to regulatory regions such as gene promoters and enhancers and affect gene function through transcriptional or translational regulation and ultimately be associated with complex diseases (Mottagui-Tabar *et al*., 2005). Borderline association was also found for a marker in *SNAP91* and the ECLAMC data set. *SNAP91* (synaptosomal-associated protein) encodes a synapse-associated protein with highest expression detected in the brain (Ishikawa *et al.,* 1998). We did not detect *Snap91* expression in the embryonic periods analyzed in our study. Notwithstanding, the association with *SNAP91*, a central-nervous-system-associated gene, and cleft lip/palate raises intriguing questions. Brain abnormalities have been reported in patients with oral clefts as an additional phenotype. Individuals with clefts often present an increased incidence of brain structural anomalies that were further correlated to cognitive function and lower intelligence quotient (Nopoulos *et al.,* 2000, 2002, 2007). Problems with visual perceptual skills and higher incidence of reading disability among children with clefts have also been reported (Richman *et al.,* 1988). Although there may be a genetic link among *SNAP91*, brain abnormalities, and oral clefts, this remains to be solved.

In summary, the accumulated evidence for linkage of chromosomal region 6q14.2-14.3 with cleft lip/palate has driven us to pursue additional research within that particular region. Our results corroborate these findings. To our knowledge, this is the first report of an association of *PRSS35* and *SNAP91* with cleft lip/palate. The expression of *Prss35* mRNA at the time of palate formation further supports its role as a more suitable candidate gene for oral clefts at this time. Nonetheless, additional studies are necessary to explain the functional role of both genes in the susceptibility for human clefting.

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