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X- linked markers in *DMD* associated with oral clefts

Poorav J. Patel[1], **Terri H. Beaty**[1], **Ingo Ruczinski**[1], **Jeffrey C. Murray**[2], **Mary L. Marazita**[3], **Ronald G. Munger**[4], **Jacqueline B. Hetmanski**[1], **Tao Wu**[1], **Tanda Murray**[1], **Margaret Rose**[1], **Richard J. Redett**[1], **Shin C. Jin**[1], **Rolv T. Lie**[5], **Yah-Huei Wu-Chou**[6], **Hong Wang**[7], **Xiaoqian Ye**[8,9], **Vincent Yeow**[10], **Samuel Chong**[11], **Sun Ha Jee**[12], **Bing Shi**[13], and **Alan F. Scott**[1]

[1]Johns Hopkins University, Baltimore, MD

[2]University of Iowa, Iowa City, IA

[3]University of Pittsburgh, Pittsburgh, PA

[4]Utah State University, Logan, UT

[5]University of Bergen, Bergen, Norway

[6]Chang Gung Memorial Hospital, Linkou, Taiwan

[7]Peking University Health Science Center, Beijing, China

[8]Mount Sinai Medical School, New York, NY

[9]Wuhan University, Wuhan, China

[10]KK Women's & Children's Hospital, Singapore

[11]National University of Singapore, Singapore

[12]Yonsei University, Seoul, Korea

[13]Sichuan University, Chengdu, China

Abstract

As part of an international consortium, case-parent trios were collected for a genome wide association study of isolated, non-syndromic oral clefts, including cleft lip (CL), cleft palate (CP) and cleft lip and palate (CLP). Non-syndromic oral clefts have a complex and heterogeneous etiology. Risk is influenced by genes, environmental factors, and differs markedly by gender. Family based association tests (FBAT) were used on 14,486 SNPs spanning the X chromosome, stratified by type of cleft and racial group. Significant results even after multiple comparisons correction were obtained for the Duchene's muscular dystrophy (*DMD*) gene, the largest single gene in the human genome, among CL/P trios (both CL and CLP combined). When stratified into groups of European and Asian ancestry, stronger signals were obtained for Asians. Although conventional sliding window haplotype analysis showed no increase in significance, analysis selected combinations of the 25 most significant SNPs in *DMD* identified four SNPs together that attained genome-wide significance among Asian CL/P trios, raising the possibility of interaction between distant SNPs within *DMD*.

Dr. Terri H. Beaty, Department of Epidemiology, School of Public Health Johns Hopkins University, 615 N Wolfe St., Baltimore, MD 21205-2103, USA tbeaty@jhsph.edu.

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Keywords

oral clefts; case-parent trios; X-linked; family-based association; DMD

Oral clefts are the most common craniofacial defect in humans (1, 2) with a prevalence among live born infants around 1:700 worldwide, but there is substantial variation across populations and racial groups (3, 4). Although over 400 malformation syndromes can include an oral cleft as a hallmark feature, ~70% of all CL/P cases and ~50% of all CP cases are isolated and non-syndromic (2). Children with non-syndromic oral clefts may also suffer from cognitive deficits, reading difficulty, social challenges, and reduced life spans (5). While surgical repair is effective, treatment is an economic burden for affected individuals, their families, and the healthcare system (6, 7).

The etiology of oral clefts is complex and heterogeneous. Oral clefts are grouped into three anatomical categories: clefts of the lip (CL), clefts of only the palate (CP), and clefts of both the lip and palate (CLP). The CL and CLP groups are often combined into cleft lip with or without palate (CL/P), because they show similar embryologic and epidemiologic patterns (1). However, recent research indicates CL and CLP may differ in their genetic etiology (8). Males are more likely to have CL/P compared to females (roughly 2:1), while CP occurs more often in females. Families ascertained through one cleft type are generally not at increased risk for another type, which could also reflect distinct etiologies (3, 8).

Among the many candidate genes for oral clefts (2, 9), some are on the X chromosome. The most widely recognized candidate gene on the X chromosome is *TBX22* which is associated with CP (10-14). Multiple mutations in *TBX22* have been described among CP cases (including nonsense, splice site, frameshift and missense mutations) (10, 11). Recently, Jugessur et al. (15) studied 18 candidate genes on the X chromosome in a large collection of cleft case-parent trios from Scandinavia, and found suggestive evidence for *OFD1* on Xp22, however, their results were not consistent between populations as similar as Denmark and Norway. The current study was conducted to test for association between oral clefts and markers along the entire X chromosome using a family based study design.

Materials and Methods

Study sample

The study sample included case-parent trios from 9 countries and 13 recruitment sites (16, 17). Cases were excluded if the proband was diagnosed with a syndromic form of oral cleft, was reported to be exposed to known teratogens, or had multiple birth defects. As shown in Table 1, the sample consisted largely of case-parent trios of Asian and European ancestry, with ~5% of parents being of African descent or “mixed” categories (where parents were not in the same racial category). For some analyses, we stratified by racial group, which effectively limited the sample to families of European (including European Americans) and Asian ancestry.

Quality control

The Center for Inherited Disease Research (CIDR) genotyped 589,945 markers on the Illumina Human610-Quad platform, which included 14,486 markers on the X. As part of the quality control (QC) process, we conducted an identity-by-descent (IBD) analysis to confirm relationships between and within families. Individuals who appeared to be related in unexpected ways or had apparent inconsistencies were excluded (n=32). Principal component analysis (PCA) was conducted using randomly selected, independent, autosomal SNPs to document the observed genetic variation among founders. Founder individuals and

families whose reported race was different from results of PCA were initially flagged (but not excluded), and the recruitment site was re-contacted for confirmation. If needed, racial groups were re-assigned.

Tests of deviation from Hardy-Weinberg equilibrium (HWE) within racial group were conducted among mothers (18) but not the hemizygous fathers. A threshold for HWE of $p = 1\times10^{-5}$ was used to flag SNPs. When the Asian and European groups were analyzed separately, only 7 unique SNPs failed this threshold within groups, and there was no overlap in which SNPs were flagged across groups. Among the European group, 1,081 SNPs had MAF<0.01; in the Asian group, 2,713 SNPs fell below this MAF threshold. In the total sample, only 825 SNPs failed to meet this threshold and were flagged.

Missing SNP information for all X-linked markers was another QC flag. Eight individuals in the Asian group and 8 in the European group had >5% missing genotype calls, while in the total population, 17 individuals had >5% missing data and were dropped. When considered by SNP, the Asian sub-group had 38 different SNPs with >5% missing data, while 82 SNPs exceeded this threshold among Europeans. In the complete sample, 54 SNPs had >5% missing genotype data.

Statistical analysis

We used the FBAT program (http://www.biostat.harvard.edu/fbat) to test the composite null hypothesis of no linkage or association between each SNP and an unobserved causal gene (19, 20). FBAT tests if Mendelian segregation of marker alleles is strictly independent of the phenotype, and calculates a chi-square statistic under a pre-specified model of inheritance (here the log-additive model) (19). For markers on the X chromosome, all fathers become uninformative because there can be no variation in transmission of X-linked markers to the child (i.e. sons must get the Y chromosome and daughters must get the one X-linked marker carried by the father). SNPs with low MAF are always less informative because there are fewer heterozygous mothers. SNPs were tested in CL, CP, CLP, and CL/P trios separately, and again stratified into European and Asian groups (dropping trios of African or mixed ancestry). These family based tests of association are robust to confounding due to population stratification, but given the baseline differences in MAF it is often prudent to stratify. Plots of $-\log_{10}(p)$ against the physical position along the X identified regions of interest, and intensity plots of the most significant SNPs were checked to verify genotype quality. Haplotype analysis using sliding windows of adjacent SNPs was conducted for all markers showing evidence of linkage and association.

Combinations of SNPs showing evidence of linkage and association were also used in a ‘haplotype’ analysis using UNPHASED (21), although not all significant SNPs were adjacent to one another. UNPHASED provides an overall omnibus (or global) test of association over all haplotypes, testing the null hypothesis that haplotypes are also independent of phenotype. A likelihood ratio test (LRT) was computed as twice the difference in log-likelihoods between a reduced and a complete model, which follows a chi-square distribution with degrees of freedom (df) equal to the difference in the number of free parameters between models. We also used a permutation option in UNPHASED to generate empiric p-values, where transmission status of haplotypes was randomly permuted over 5000 replicates.

Results

Fig. 1 shows results of the FBAT analysis on all markers along the X chromosome for CL/P and CP trios in the total sample and the two major racial groups separately (European and Asian). Since we only examined markers on the X chromosome, the family-wise error rate

of 5% is protected via a strict Bonferroni correction using all 14,486 SNPs on the X chromosome, yielding a significance cut-off of $0.05/14{,}486=3.5*10^{-6}$. Several SNPs retained significance and were beyond this corrected critical value in the total sample, i.e. they fell above the line in the top panels of Fig. 1. As often occurs, some of the most significant SNPs had low MAF where relatively few trios are informative, and a few reversals in the observed transmission patterns would completely obviate such extreme test statistics. Three of the most significant SNPs involved markers that were effectively monomorphic in one group, and had low frequency in the other, reflecting the fragility of these 'most significant' findings.

Table 2 lists the top 25 SNPs in the total sample (along with corresponding results from stratified analysis). It is intriguing, however, that one SNP among these retained its significance after Bonferroni correction and five other among these top SNPs mapped to the *DMD* gene, the largest gene in the human genome (spanning 2.4 Mb). These SNPs were more significant among Asian CL/P trios (probably because their MAF was higher than among Europeans, generating more informative trios). SNP rs6631759 gave the smallest individual p-value in FBAT among Asian CL/P trios, and we conducted a conventional 'sliding windows' haplotype analysis using 14 adjacent SNPs flanking this one marker. None of the haplotypes of size 2-5 showed greater significance than this highly polymorphic marker.

Combinations of the most significant 25 SNPs in *DMD* were used in a 'haplotype' analysis in UNPHASED, where 'haplotypes' of 2-5 SNPs together were tested (even though not all were not adjacent to one another). As shown in Table 3, some of these SNP combinations yielded highly significant p-values. The most significant combination of 4 SNPs spanned 1.94Mb, almost the entire length of *DMD* (top row Table 3). For these 4 SNPs, the omnibus LRT was 88.95 (with 15 df), giving an asymptotic $p=1.55*10^{-12}$ among all CL/P trios; while among the 895 Asian CL/P trios, this LRT was 64.06 (with 15 df) yielding $p=4.99*10^{-8}$. Permutation analysis of this most significant combination of SNPs yielded an empiric $p=2*10^{-4}$. The large distance between these SNPs makes it difficult to interpret these results as any traditional haplotype, but raises the potential for epistatic interaction influencing risk to CL/P.

In addition to these signals in *DMD* among Asian CL/P trios, four SNPs (rs17252760, rs35884642, rs5980360, rs4843989) the region around *TMEM185A* in Xq28 gave suggestive evidence of linkage and association (i.e. $10^{-4}<p<10^{-5}$). We conducted a similar analysis of 'haplotypes' to further explore the region around *TMEM185A* among Asian CL/P trios. However, these analyses yielded little or no improvement in p-values (data not shown).

Discussion

Birth prevalence rates of CL/P and CP vary by gender, and previous studies have suggested some X linked genes as candidate genes for oral clefts. The best documented candidate gene on the X is *TBX22* (10-14), and this gene was covered by 51 markers in our panel. Analysis of all 469 CP trios showed very little evidence of linkage and association to SNPs in or near *TBX22*, and the most significant individual SNP was rs5912393 at position 79229785 (p=0.01). This could reflect the limited power of our relatively modest sample size for CP or it could reflect a larger role for multiple rare variants in *TBX22* that would be very poorly tagged by our polymorphic markers. However, we did see suggestive signals in the very large *DMD* gene on Xp21.2 among 895 Asian CL/P trios and in the total sample of 1,591 case-parent CL/P trios.

There are several problems in analyzing markers on the X chromosome, including mundane issues of implementation in available programs, the information content of markers, and sample size. For X linked markers, all hemizygous fathers become uninformative, and only heterozygous mothers provide information. Although this consortium sample represents the largest collection of oral clefts described to date, our sample size was reduced by stratifying by type of cleft and by racial group (European and Asian ancestry groups). BEATY et al. (16) documented considerable differences between European and Asian groups in the observed evidence for markers on 8q24 associated with risk to CL/P in this same sample. So in the current analysis, we stratified as a check for heterogeneity.

To some extent, haplotype analysis can address gaps in tagging provided by individual SNPs. Tagging SNPs may be in LD with one another, and therefore should also be in LD with an unobserved causal variant within a haplotype block. A sliding window approach to haplotype analysis guarantees coverage over a small chromosomal region, but combinations of more distant SNPs can also be treated as a 'haplotype'. Several 'haplotypes' in *DMD* approached genome-wide significance, and one combination of 4 SNPs attained it among Asian CL/P trios ($p=4.99*10^{-8}$). However, these 4 SNPs were effectively uncorrelated with one another, (i.e. none were in pairwise LD), however, and it becomes difficult to argue all 4 SNPs could be correlated with any one high-risk allele at one unobserved causal gene. This significant combination of 4 SNPs suggests a potential interaction between regions within the very large *DMD* gene. This study of markers on the X chromosome does illustrate a need to further investigate potential association between X linked genes and risk of oral clefts.

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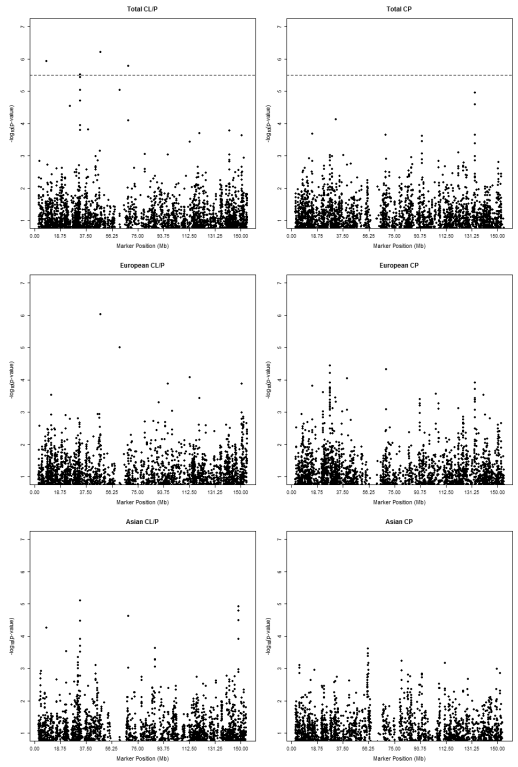

Figure 1.
Significance as measured by $-\log_{10}(p)$ of FBAT on individual SNPs under a log-additive model in a stratified analysis of European and Asian CL/P and CP trios separately. The line represents a Bonferroni corrected critical value.

Table 1

Complete case-parent trios by cleft type, gender, and ancestry

	European		Asian		Other		Total	
Cleft type	**male**	**female**	**male**	**female**	**male**	**female**	**male**	**female**
CL	137	98	118	97	5	2	260	197
CLP	288	145	464	216	11	10	763	371
CL/P	425	243	582	313	16	12	1023	568
CP	104	111	95	142	3	11	202	264
Unknown Cleft	0	0	2	1	0	0	2	1
Total by gender	529	354	679	456	19	23	1227	833
Total	**883**		**1135**		**42**		**2060**	

Table 2

Most significant SNPs from FBAT analysis under a log-additive model using the combined group of 1591 CL/P case-parent trios, 895 Asian and 668 European case-parent trios. Asymptotic p-values and minor allele frequencies are shown.

Marker	Position	Gene	# Inf. Fams Total	FBAT p-value in total	MAF in total sample	# Inf. Fams. Asians	FBAT p-value in in Asians	MAF in Asians	# Inf. Fams Europeans	FBAT p-value in in Europeams	MAF in Europeans
rs5906541	47851087	*SSX6*	88	0.0000006	0.040	--	--	--	87	0.000001	0.087
rs17269319 *	8427649	*KAL1*	82	0.0000012	0.034	67	0.0000550	0.054	--	--	--
rs5981162	68235478	*PJA1*	225	0.0000017	0.075	195	0.0000240	0.121	21	0.033006	0.015
rs5928207	33154050	*DMD*	682	0.0000031	0.355	478	0.0001220	0.488	189	0.016950	0.161
rs5928208	33163825	*DMD*	595	0.0000037	0.376	439	0.0000330	0.396	143	0.114597	0.117
rs6631759	33149274	*DMD*	560	0.0000093	0.279	471	0.0000078	0.470	78	0.735720	0.059
rs12558269 *	61916143	*LOC653588*	65	0.0000094	0.034	--	--	--	62	0.000010	0.071
rs5971698	33155155	*DMD*	627	0.0000200	0.357	462	0.0001980	0.436	150	0.074353	0.121
rs5986465	25665575	*LOC389842*	396	0.0000290	0.223	145	0.0061350	0.130	244	0.001607	0.337
rs5980788	68232663	*PJA1*	135	0.0000800	0.042	106	0.0009590	0.058	22	0.021810	0.015
rs5928214	33180372	*DMD*	603	0.0001120	0.385	431	0.0003030	0.389	158	0.205903	0.127
rs6610244	38967269	*MID1IP1*	664	0.0001530	0.475	394	0.0034780	0.299	252	0.017992	0.227
rs5972815	33243806	*DMD*	736	0.0001580	0.327	440	0.0016530	0.383	283	0.078127	0.271
rs5908459	141867688	*SPANXN4*	579	0.0001640	0.258	452	0.0047700	0.403	117	0.084119	0.086
rs1465731	119753400	*C1GALT1C1*	136	0.0002000	0.050	23	0.2971470	0.012	110	0.000372	0.092
rs933191	150847686	*MAGEA4*	168	0.0002320	0.059	14	0.5929800	0.007	149	0.000131	0.113
rs1117695	112868159	*LOC286528*	498	0.0003640	0.263	133	0.4351560	0.074	348	0.000085	0.461
rs7063555	47433961	*LOC643474*	343	0.0006940	0.145	86	0.0522600	0.045	247	0.002897	0.248
rs1954611	80264976	*NSBP1*	558	0.0008700	0.478	338	0.0644060	0.264	206	0.001949	0.191
rs5966762	96813207	*DIAPH2*	591	0.0009160	0.421	274	0.3337460	0.168	301	0.000131	0.285
rs5953801	141923672	*SPANXN4*	677	0.0009300	0.317	465	0.0025760	0.441	199	0.385389	0.167
rs5905410	44469799	*LOC644464*	627	0.0010260	0.363	255	0.0021510	0.163	357	0.068451	0.413
rs5925325	152092871	*MAGEA1*	790	0.0011430	0.379	442	0.0868330	0.416	331	0.002024	0.339
rs1569562	141847221	*SPANXN4*	806	0.0013580	0.371	449	0.0021580	0.406	343	0.443515	0.319

* SNP showed poor intensity plots upon examination

Table 3

Most significant 3- and 4-SNP haplotypes in the *DMD* gene using combinations of SNPs from the top 25 SNPs in 895 Asian CL/P trios evaluated by the UNPHASED program

SNP1	SNP2	SNP3	SNP4	SNP5	Span (kb)	p-value
rs2141757	rs5927788	rs2038354	rs6631759		1943	$4.99*10^{-08}$
rs5972354	rs4829227	rs4345727	rs6631759		1952	$8.71*10^{-08}$
rs1573952	rs5927788	rs4639663	rs6631759		1969	$9.09*10^{-08}$
	rs4829227	rs4345727	rs6631759		1650	$1.07*10^{-07}$
	rs4829227	rs4639663	rs6631759		1650	$1.22*10^{-07}$
rs2141757	rs5927788	rs5972437	rs6631759		1943	$1.43*10^{-07}$
rs2141757	rs2030002	rs2038354	rs6631759		1943	$1.54*10^{-07}$
	rs5927763	rs6631341	rs6631759	rs5971698	1958	$1.66*10^{-07}$
	rs5972354	rs4829227	rs6631759		1952	$1.71*10^{-07}$