Genome-wide and Interaction Linkage Scan for Nonsyndromic Cleft Lip with or without Cleft Palate in Two Multiplex Families in Shenyang, China

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Objectives To identify the loci involved in nonsyndromic cleft lip with or without cleft palate (NSCL/P) in Northern Chinese people in Shenyang by using genomewide and interaction linkage scan. **Methods** Two multiplex families in Shenyang from North China were ascertained through probands with NSCL/P. Blood of every member was drawn for DNA extraction and analysis. Genotypes were available for 382 autosomal short tandem repeat (STR) markers from the ABI Prism Linkage Mapping Set version 2.5. Linkage between markers and NSCL/P was assessed by 2-point parametric LOD scores, multipoint-heterogeneity parametric LOD scores (HLODs), and multipoint nonparametric linkage score (NPL). **Results** The initial scan suggested linkage on Chromosomes 1, 2, and 15. In subsequent fine mapping, 1q32-q42 showed a maximum multipoint LOD score of 1.9(empirical P=0.013) and an NPL score of 2.35 (empirical P=0.053). For 2p24-p25, the multipoint NPL increased to 2.94 (empirical P=0.007). 2-locus interaction analysis obtained a maximum NPL score of 3.73 (P=0.00078) and a maximum LOD score of 3 for Chromosome 1 (at 221 cM) and Chromosome 2 (at 29 cM). **Conclusion** Both parametric and nonparametric linkage scores greatly increased over the initial linkage scores on 1q32-q42, suggesting a susceptibility locus in this region. Nonparametric linkage gave a strong evidence for a candidate region on chromosome 2p24-p25. The superiority of 2-locus linkage scores compared to single-locus scores gave additional evidence for linkage on 1q32-q42 and 2p24-p25, and suggested that certain genes in the two regions may contribute to NCSL/P risks with interaction.

Key words: Nonsyndromic cleft lip with or without cleft palate; Parametric linkage; Nonparametric linkage; Interaction

INTRODUCTION

Orofacial clefts, particularly nonsyndromic cleft lip with or without cleft palate (NSCL/P) which makes up 75% of orofacial clefts, are the most common birth defects, which are always accompanied with serious physiological and psychological implications and great family burden[1-2]. The prevalence varies widely with nationality and geography with an average prevalence of 1/700 live birth[3]. Shenyang in North China has one of the highest reported prevalence rates of oral clefts in the world, and 1.94 per 1 000 births were ascertained from hospital-based surveillance in 2 000 as reported[4-5]. This rate is much higher than those reported from south China and from the Nordic countries where the rates are also high[6]. Despite the high birth prevalence in Shenyang, few studies were conducted on the population. Therefore, it is of great importance to explore the disease factors for oral clefts in Shenyang.

Previous studies showed that NSCL/P was caused by both genetic and environmental factors, and as a complex trait, multiple loci were involved in NSCL/P[7]. Various association and linkage studies of different populations have identified some candidate chromosome loci with evidence of linkage for oral cleft: 1p36[8], 1q32-q41[9-10], 2p13[11], 2q32[12], 3p21.2[13], 3q[14], 4p16.1[15], 4q21-q31[16], 6p24.3[17], 7q34[13], 8p11-23[18], 11p12-q14[8], 11q23-q24, 13q33.1-34[19], 18q21.1[20], 19q13 [21], and pathogenic mutations have been identified, for example: IRF6 on 1q32-q41, TP73L on 3q27, MSX1 on 4p16.1, and PVRL1 on 11q23-q24[22–25]. However, none of these play a major role in NSCL/P, and they are responsible for only a fraction of NSCL/P cases[26].

In 2000, Prescott NJ *et al*. firstly reported CL/P genome scan results for affected sib pairs in England[27]. In 2002, Marazita ML firstly and also as the only one to date reported the genomewide linkage

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of Chinese people through the study of 36 NSCL/P multiplex families in South China, which showed that susceptible fragments were located on Chromosomes 1, 2, 4, 6, 14, 17, and 19[14]. Since then, more than 10 additional genome scans have been performed for different populations, and many susceptible chromosome fragments and genes for NSCL/P were identified. However, the genomewide linkage results are not consistent among different populations; the etiological reasons for most cases of NSCL/P remain elusive and no susceptibility gene that plays a major role in this disorder has been identified.

To verify those genomic regions in Chinese people that Marazita ML first reported and to compare the results between southern and northern Chinese populations, two Multiplex Families with NSCL/P in Shenyang from Northern China were analyzed by genomewide and interaction linkage scan.

SUBJECTS AND METHODS

Subjects

Data from Birth Defects Surveillance and CL/P surgery records in Liaoning Province of North China formed the basis of our study. Probands and all their available relatives were recruited as subject families. For the present study, we choose two multiplex families with two or more affected individuals in each. All members of the two families were Chinese in the Han dialect group. Each affected member had "CL plus CP" (CLP) and was evaluated by an experienced dysmorphologist and a clinical geneticist, and no syndromic anomalies were observed in their pedigreefamilies by birth histories investigation. All subjects had signed the informed-consent forms and the study was approved by the Institutional Review Boards of Peking University Medical Ethics Committee. Family histories as well as environmental histories were taken into consideration, and blood samples were collected for DNA extraction and analysis.

The families studied were referred to as Family Ⅰ and Family Ⅱ, both with an apparent autosomal dominant mode of inheritance (Fig. 1). 8 family members with CLP were considered to be affected and the other 12 individuals were unaffected. The two families had no overlap.

POWER ESTIMATION

The power to detect linkage was calculated with the SLINK[28-29] program with 500 replicates. The pedigrees showed an expected maximum LOD (logarithm of the odds) score of 2.09, assuming an autosomal dominant model with a penetrance of 0.75 and a phenocopy rate of 0.00001. The powers to detect LOD scores greater than 1 and 2 were 66% and 10%, respectively.

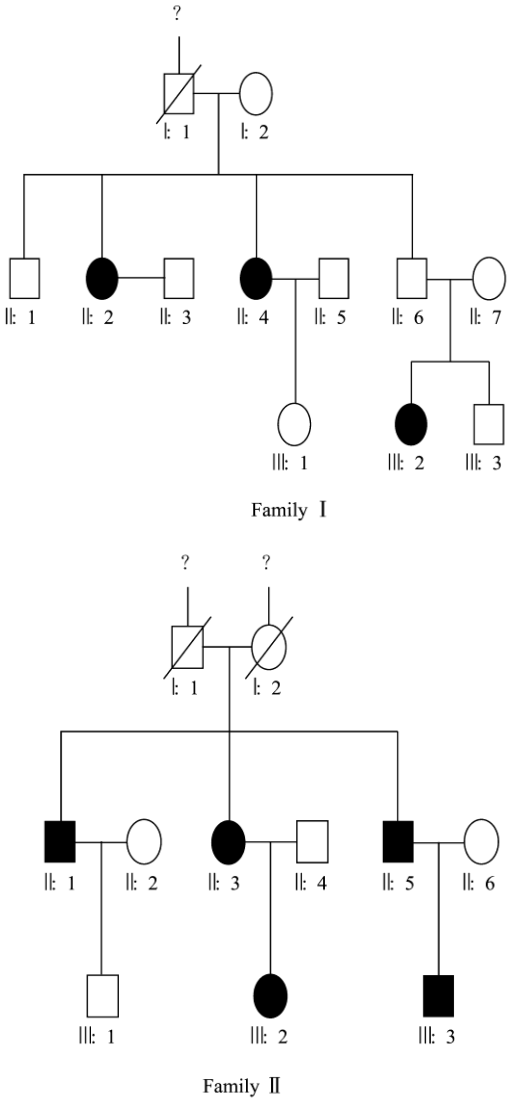

FIG. 1. Pedigrees of Family Ⅰ and Family Ⅱ with NSCL/P. Affected individuals are shown with blackened symbols. Deceased individuals with no available information on them are shown with a question mark.

GENOME SCAN

Genotyping

In the first stage, we genotyped the 8 affected individuals and the 12 unaffected relatives using 382 autosomal short tandem repeat (STR) markers for the ABI Prism Linkage Mapping Set version 2.5. This marker set has an average distance of 10 cm between STRs and an average heterozygosity of 0.75. In the

second stage of a high-density scan, eleven additional STR markers with an average heterozygosity of 0.78 were obtained from TSINGKE to refine the candidate linkage regions (Table 1). The average spacing between added markers was about 3 cM. PCR products were analyzed with ABI 3730 DNA analyzer; GeneMapper software version 3.0 was used to assign genotypes.

TABLE 1

Markers for High-Density Scan of 1q and 2p

Markers	Type	Primer	Cytogenetic	Heterozy -gosity	Physical Position Build36	Sex-averaged Map Position
D1S2668	STS	AFMA224XC1	1q32.1	0.78	202348076	210.48
D1S2692	STS	AFMA290XD1	1q32.2	0.88	206105496	215.4
D1S245	STS	AFM224XC1	1q32.2	0.85	207673041	218.66
D1S419	STS	AFM199WH2	1q32.3	0.91	212604505	224.1
D1S227	STS	AFM184YF6	1q41	0.7	215361061	229.03
D1S479	STS	AFM290WD1	1q42.12	0.8	224276287	234.87
D2S398	STS	AFMA127XB9	2p25.1	0.61	10460982	25.98
D2S2200	STS	AFMA232WF1	2p25.1	0.88	11899822	29.03
D2S2267	STS	AFMB283XG9	2p24.3	0.7	13653117	32.69
D2S272	STS	UT868	2p24.2	0.81	16730277	37.98
D2S2342	STS	D2S2342	2p24.1	0.69	20192641	42.06

LINKAGE ANALYSIS

Genotype error checking included checks for Mendelian inconsistencies performed with PEDCHECK[30] and unlikely double recombinants based on Merlin genotype error option. All genotype errors were removed before the linkage analysis. Rutgers Combined Linkage-Physical Map (Build 36) provided by MAP-O-MAT (http://compgen.rutgers.du/mapomat/) was used for the linkage analysis.

Both parametric and non-parametric linkage analysis were performed with the linkage software MERLIN[31]. A dominant model was assumed for all autosomal chromosomes, with a risk allele frequency of 0.0007, a penetrance of 0.75 for genotypes with 1 or 2 copies of the risk allele and a phenocopy rate of 0.00001. The maximum potential NPL score calculated for our pedigree structure by MERLIN was 3.28 (P=0.0005). Marker allele frequencies were estimated from the founders in the 2 families via MERLIN.

To reduce false positive signals, empirical p-values for the maximum LOD and NPL scores on Chromosomes 1 and 2 were calculated with 1 000 replicates generated with the gene dropping approach implemented in MERLIN. In each simulation, marker allele frequencies, genetic maps, pedigree structures, and missing data patterns were retained. The empirical significance level for an observed signal from the original dataset was determined as the proportion of simulations that exhibited equal or greater linkage scores.

INTERACTION ANALYSIS

To test whether an interaction occurs between two putative disease loci on Chromosomes 1 and 2 and acquire more accurate estimates of the disease-locus position, GENEHUNTER-TWO-LOCUS software was used to calculate 2-locus LOD and NPL scores as a function of both trait-locus positions[32]. The 2-trait-locus model contains allele frequencies for each trait locus, and a 3×3 (4×4 in the case of imprinting) matrix of penetrances. In our study, the single-locus model described above was used to construct 2-locus models through a spreadsheet One-Two for MS-Excel (written by *Manuel Mattheisen)*. Three classes of interaction models (heterogeneity, multiplicative, and additive) were tested according to Strauch *et al.*[32] In the heterogeneity model, it is assumed that a mutation at either locus can cause the phenotype by itself ("or" condition). In the multiplicative model, it is hypothesized that only mutations at both loci can cause the phenotype ("and" condition). As for the additive model, the penetrance of a single locus is simply added and rescaled to form 2-locus penetrances. Because the 2-locus analysis is

computationally-intense, compared to an analysis with 1-trait-locus, only the linkage candidate regions on Chromosomes 1 and 2 were selected for interaction tests. The results are presented by 3-dimensional plots.

RESULTS

Initial Scan

The initial genomewide scan revealed 3 regions with the multipoint NPL score >1.5 on Chromosomes 1, 2, and 15. Among the 3 regions, only the 1q region showed a multipoint parametric LOD score>1.5 (linked proportion of families α=1, means no heterogeneity). No other genomic regions had a signal stronger than an NPL or LOD score of 1.5. Refining efforts were focused on 1q32-q42 with the highest genomewide multipoint LOD score being 1.744 and on 2p24-p25 with the highest multipoint NPL score being 2.51 (Table 2).

FINE MAPPING

With 6 additional markers on Chromosome 1q32-q42 and 5 additional markers on Chromosom e 2p24-p25, an average spacing of about 3 cM was used for candidate region refining linkage. For Chromosome 1q32-q42, a maximum multipoint LOD score of 1.9(empirical *P*=0.013) and an NPL score of 2.35 (empirical *P*=0.053) were obtained, and the 2-point LOD for D1S245 was 1.74 (theta=0) (Fig. 2, Table 3). Compared with the initial linkage scores on 1q32-q42, both parametric and nonparametric linkage scores were obviously elevated, suggesting a susceptibility locus in this region. For Chromosome 2p24-p25, the multipoint NPL was elevated from the original 2.51 to 2.94 (empirical *P*=0.007), but the multipoint parametric LOD decreased to 0.12, and the 2-point LOD was less than 1 (Fig. 2) (Table 4). Although the parametric linkage results were not significant, nonparametric linkage gave a strong evidence for a candidate region on Chromosome 2p24-p25.

TABLE 2

Initial Genome Scan Results, Indicating Suggestive Evidence at Various Chromosomes Obtained with Multipoint Parametric and Nonparametric Linkage

STR	Position	Cytogenetic	Nonparametric		Parametric		
	cM		NPL	*P*	LOD	ALPHA	HLOD
D1S249	212.87	1q32.1	1.35	0.09	-1.350	0.479	0.793
D1S425	220.78	1q32.3	1.53	0.06	1.744	1	1.744
D2S168	27.70	2p25.1	1.23	0.11	-1.820	0.234	0.042
D2S305	40.50	2p24.2	2.51	0.006	0.882	1	0.882
D11S987	77.28	11q13.2	1.03	0.20	1.201	0	0
D15S205	87.45	15q25.2	1.83	0.03	-0.057	0	0
D15S120	123.45	15q26.3	1.13	0.13	-1.596	0	0

TABLE 3

Family-Specific Results with Combined Parametric and Nonparametric Linkage Scores for the Peak Region at Chromosome 1q32-q42

STR	Position	Cytogenetic	Nonparametric		Parametric			
	cM		NPL	*P*	2-LOD	LOD	ALPHA	HLOD
D1S2668	210.48	1q32.1	-0.64	0.70	0	-5.165	0	0
D1S2692	215.40	1q32.2	-1.20	0.90	0.08	-5.742	0	0
D1S245	218.66	1q32.2	2.21	0.013	1.74	1.859	1	1.859
D1S425	234.87	1q32.3	2.35	0.009	1.59	1.897	1	1.897
D1S419	224.10	1q32.3	2.31	0.01	1.20	1.886	1	1.886
D1S227	229.03	1q41	1.59	0.06	0.48	1.573	1	1.573
D1S479	234.87	1q42.12	0.95	0.20	0.33	-1.134	0.46	0.539

TABLE 4

Family-Specific Results with Combined Parametric and Nonparametric Linkage Scores for the Peak Region at Chromosome 2p24-p25

STR	Position cM	Cytogenetic	Nonparametric		Parametric			
			NPL	*P*	2-LOD	LOD	ALPHA	HLOD
D2S398	25.98	2p25.1	1.41	0.08	0	-1.686	0.273	0.065
D2S2200	29.03	2p25.1	2.94	0.002	0	0.120	0.624	0.176
D2S2267	32.69	2p24.3	2.92	0.002	0	0.116	0.621	0.172
D2S272	37.98	2p24.2	1.72	0.04	0	-0.063	0.451	0.106
D2S305	40.50	2p24.2	1.14	0.13	0	-0.444	0.265	0.040
D2S2342	42.06	2p24.1	-0.66	0.70	0	-5.427	0	0

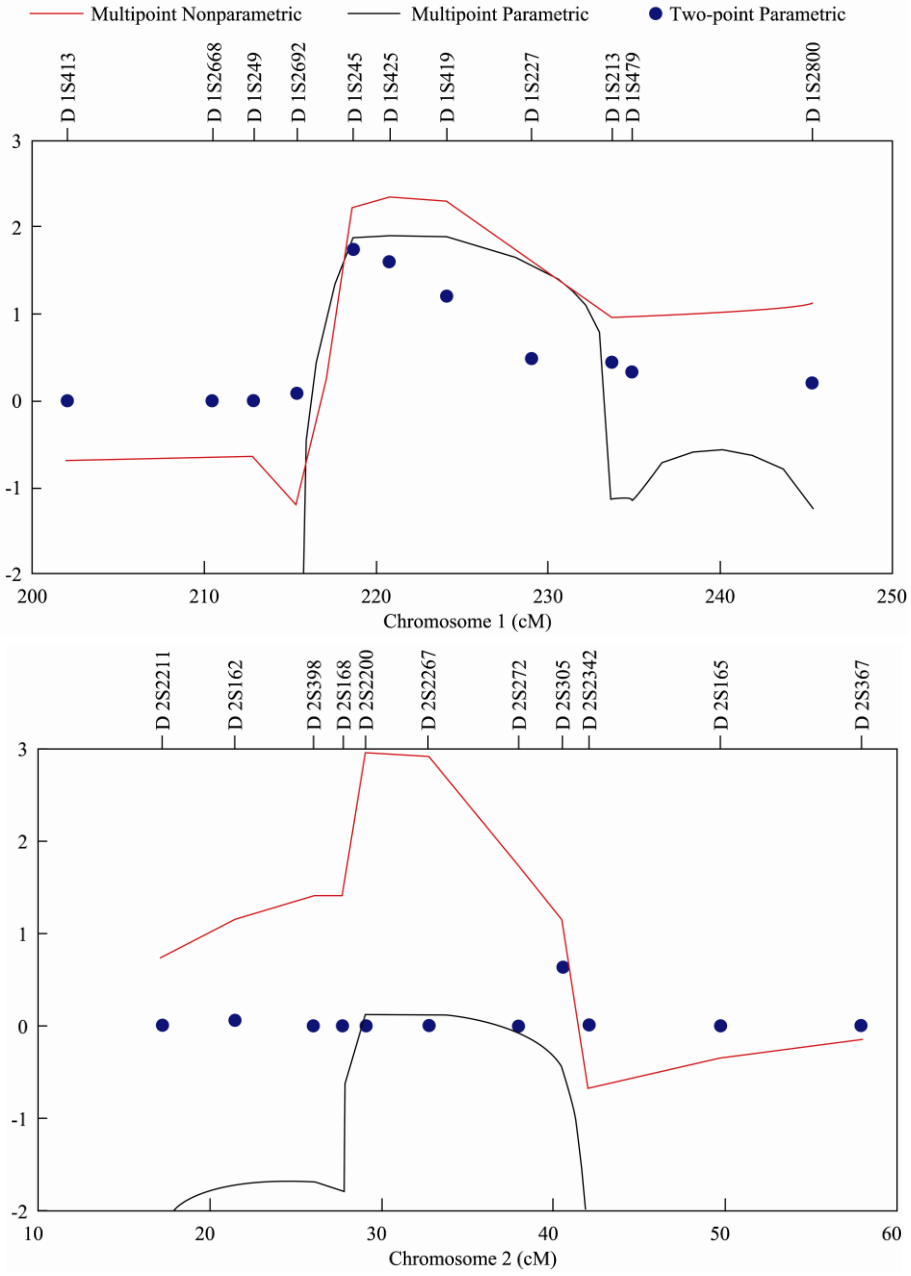

FIG. 2. Fine mapping linkage results for the two peak chromosomal regions. The multipoint parametric, nonparametric and 2-point parametric linkage scores are shown on the vertical axis, and the genetic distance (in cM) and markers are given on the horizontal axes.

INTERACTION ANALYSIS RESULTS

The 2-locus NPL and LOD scores on Chromosomes 1q and 2p were superior to both single-locus linkage scores. A maximum 2-locus NPL of 3.73 (P=0.00078) was achieved for Chromosome 1 (at 221 cM) and Chromosome 2 (at 29 cM) (Fig. 3), which was more significant than both single-locus NPL scores of 2.35 (P=0.009) for Chromosome 1q and 2.94 (P=0.006) for Chromosome 2p. The regions defined by 2-locus NPL P values<0.01 (NPL>2.72) were 219.5-233 cM on Chromosome 1 and 28-38.5 cM on Chromosome 2. The parametric analysis with 2 disease loci under the assumption of the multiplicative model achieved a maximum LOD score of 3.0 (Fig. 3) at the same coordinate for maximum 2-locus NPL, also superior to both single-locus LOD scores of 1.90 for Chromosome 1q and 0.12 for Chromosome 2p. While, the 2-locus LOD scores under heterogeneous and additive models were 1.42 and 1.47, respectively. Therefore, the multiplicative model gave the strongest evidence for interaction.

DISCUSSION

The etiology of nonsyndromic orofacial cleft is complex and is related to both genetic and environmental factors[33]. Although a number of studies have been undertaken to identify the genetic variations responsible for NSCL/P, the results have not been consistent among different populations, and no major susceptibility gene has been identified to date[7,34-37].

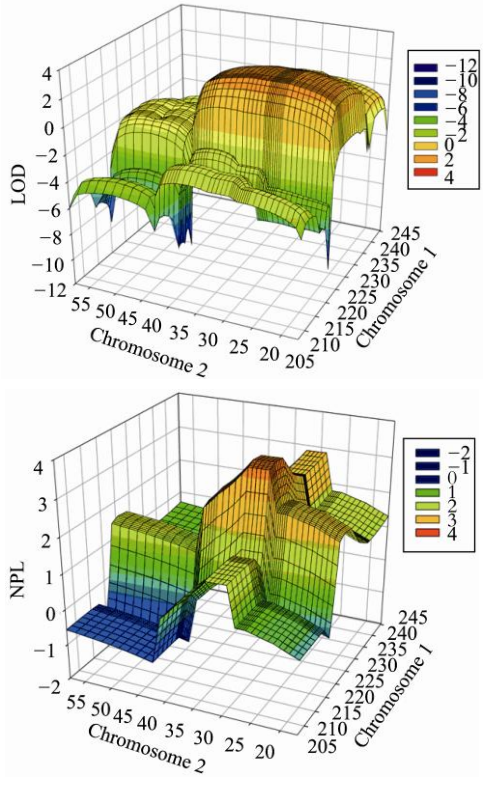

FIG. 3. An interaction analysis of the two putative loci on Chromosomes 1q and 2p.

In order to find out the susceptible genes of NSCL/P, several experimental methods were used, including breakpoint mapping, deletion mapping, direct sequencing of candidate genes/locus, linkage analysis, and linkage disequilibrium analysis[38]. Genome-wide linkage scans of complex traits would succeed when heterogeneity is minimized and sample sizes maximized.

In this study, two candidate regions, 1q32-q42 and 2p24-p25, were found based on a genomewide linkage scan with 382 polymorphic autosomal microsatellite markers with 2 NSCL/P families from North China. 1q32-q42 has been proven by previous studies of its susceptibility to NSCL/P through variation in an enhancer of the interferon regulatory factor-6 (IRF6) gene, but the other region 2p24-p25 has not been reported to be associated with NSCL/P.

On Chromosome 1q32-1q42, an LOD score of 1.74 was found in the scan. The 1q32 region is the location for IRF6 that was identified recently as the locus involved in van der Woude syndrome (VDWS[MIM 119300])[39]. Some previous work found a strong association between IRF6 and clefts[40], and isolated tooth agenesis[41]. However, we were not able to identify the functional genetic variant yet. The association with IRF6 was seen in markers in the gene and 100 kb downstream the gene (towards the centromere). In our results, another linkage signal could be seen for the clefts data at D1S245 (LOD score 1.74). This marker is upstream of IRF6, at 1q41-q42. These data add more complexity to this region and suggest that either the functional variant could be anywhere between D1S245 and D1S419, or more than one functional variant exists in the region. More recently, a large Danish family with cleft lip and palate was linked to a 6.5 Mb interval at 1q32.1-q32.3, which is inside the interval described above[42].

For Chomosome 2, our previous work suggested that the 2q32-q35 region could have a role in clefts, based on the meta-analysis approach (P=0.0004)[37], which contains the gene for the DNA-binding protein SATB2 (a.k.a. KIAA1034) that has been identified elsewhere through translocation-breakpoint analysis as a gene involved in cleft palate[43] and that also shows site and stage-specific expressions in murine palate development. The 2p13 region contains TGFA, the gene with the first reported association with CL/P[44] and numerous confirmatory reports[45]. TGFA has 40% sequence homology with epidermal

growth factor (EGF) and competes with EGF for binding to the EGF receptor. There are no obvious candidate genes for clefts lying in the 2p24 region. Considering the heterogeneity, we think that there may be a gene in the region which contributes to clefts.

Unfortunately, due to the few number of meioses in our sample set, the exact boundaries of the two candidate regions were hard to be defined. However, with the 2-locus linkage analysis, more accurate estimates of the disease-locus position can be acquired. The regions defined by 2-locus NPL *P* values<0.01 (NPL>2.72) were 219.5-233 cM on Chromosome 1 and 28-38.5 cM on Chromosome 2. And the interaction between the 2 loci on Chromosomes 1 and 2 was suggested by both the parametric and nonparametric 2-locus linkage analysis. Although it is hard to tell how significant this interaction is, the 2-locus linkage analysis was successfully used to analyze complex traits, such as familial combined hyperlipidemia[46], familial hypercholesterolemia[47] high factor VIII (FVIII) levels in blood (with imprinting)[48] and bipolar affective disorder[49]. In these studies, the superiority of 2-locus linkage scores compared to single-locus scores was considered as additional evidence for single-locus linkage and evidence for 2-locus interaction, and the higher the superiority, the stronger the evidence. Therefore, our results suggest that some genes in the two regions may contribute to clefts at risk as interaction.

Our study has some limitations. First, because of the family planning program launched in China in the 1980s, most families have only one or at most two children, which results in very small sibships. In addition, it was always difficult for us to examine all potential subjects in all families because of a number of reasons. For example, the intended participant had a job in another city and thus was not available at the time of data collection, or chose not to participate in the study. Second, this family dataset may not be big enough to represent Chinese people as a whole, maybe not even people living in North China or the Shenyang region, and a larger sample size is definitely needed for further study. Finally, besides Chromosomes 1 and 2, a previous linkage study conducted with 36 multiplex Chinese CL-P families showed evidence of linkage at Chromosomes 3, 4, 6, 18, and 21. However, in the present study, we did not observe any linkage in these regions.

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