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Original Article

Gene-gene interactions between BMP4 and ARHGAP29 among non-syndromic cleft lip only (NSCLO) trios from western Han Chinese population

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Abstract: Background: Genome-wide association studies (GWAS) have found more than 20 genes associated with a risk of non-syndromic cleft lip with or without cleft palate (NSCL/P). However, the interactions between these risk genes have been rarely reported. Methods: Here we selected 47 Single Nucleotide Polymorphisms (SNP) from previous GWASs and tested for possible interactions among 302 NSCL/P case-parent trios from a western Han Chinese population to further explore the genetic etiology of NSCL/P. Conditional logistic regression models were performed including gene-gene (G×G) interaction. Results: Twenty pairwise interactions yielded significant *p*-values. Most of the signals of interaction between the SNPs were detected at the same gene including v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (MAFB), netrin 1 (NTN1), and single nucleotide polymorphic marker within interferon regulatory factor 6 (IRF6). We found evidence of the interaction between rs17563 (bone morphogenetic protein 4, BMP4) and rs560426 (subfamily A member 4/Rho GTPase activating protein 29, ARHGAP29) (P=0.00093) in NSCLO trios. Conclusions: Gene-gene interaction between markers in BMP4 and ARHGAP29 may influence the risk of NSCLO in western Han Chinese population, which might explain the missing heritability for NSCL/P.

Keywords: Case-parent trios, gene-gene interaction, non-syndromic cleft lip with or without cleft palate

Introduction

Orofacial clefts (OFCs) are the most common congenital malformations in the world, affecting approximately 1 in 700 newborns. People suffering from OFCs usually confront a series of difficulties, not only with defects in their appearance, speech and hearing, but also the adverse effect on their psychology and social integration. Although most OFCs can be cured by multidisciplinary approach to treatment, families and society have to bear a heavy financial burden.

OFCs can be divided into cleft lip with or without cleft palate (CL/P) and cleft palate (CP) based on differences in embryology and epidemiology. Furthermore, clefts are classified as non-syndromic or syndromic based on whether the child has other additional structural or cognitive abnormalities. Non-syndromic cleft lip with or without cleft palate (NSCL/P) is the most common form of OFC, but the birth prevalence rates vary in different populations and racial groups. The highest birth rates of NSCL/P are in Latin America and East Asia (especially in Japan and China), while blacks have the lowest rates [1].

Current evidence suggests multiple genetic and environmental factors drive the etiology of non-syndromic OFCs. To uncover the etiologic genes in NSCL/P, several genetic approaches such as linkage analysis, association studies, and whole-exon sequencing have been widely used. Although genome-wide association studies (GWAS) have reported multiple loci influencing the risk of NSCL/P [2-8], only 20% of the heritability can be explained [9]. This inability to account completely for the estimated heritabil-

Table 1. Cleft type and gender of samples from Western Han Chinese population

Cleft type	Male	Female	Unknown sex	Total
NSCL/P	178	111	13	302
NSCLP	109	54	10	173
NSCLO	69	57	3	129

Note: NSCL/P, non-syndromic cleft lip with or without cleft palate (NSCLO & NSCLP); NSCLP, non-syndromic cleft lip with cleft palate; NSCLO, non-syndromic cleft lip only.

ity of OFCs could be due to an undiscovered interaction between genes. To deepen our understanding of the genetic architecture of a complex trait like OFC, it is necessary to explore whether and how different risk loci may interact with one another in biologic and heterogeneous pathways.

Our research group replicated 47 loci reported by previous GWASs [2-6] and other studies [10, 11] to be associated with OFCs in the western Chinese population with NSCL/P, aiming to provide more powerful evidence for genes influencing risk to this population. Although gene-gene interaction research has been conducted in the past several years, the role of these interactions in the etiology of NSCL/P is uncertain. Therefore, we performed G×G analysis by conditional logistic regression models to analyze the interactions among these 47 SNPs to further evaluate their contribution in NSCL/P from a western Han Chinese population.

Methods

Samples

We recruited 302 complete NSCL/P case-parent trios from the Cleft Surgery Department of West China Hospital of Stomatology, Sichuan University (**Table 1**), including 129 families of infants with NSCLO and 173 families of infants with NSCLP. All cases were Han population from western China. They underwent clinical examination and received a diagnosis by oral surgeons. Syndromic forms of oral clefts were excluded by checking for other congenital anomalies or developmental delays. Informed consent was provided by each participant or their guardians. This study was approved by the Hospital Ethics Committee (HEC) of West China Hospital of Stomatology, Sichuan University, Chengdu, China (WCHSIRB-D-2015-057).

Genotyping

Venous blood samples were collected from all participants. Genomic DNA samples were isolated from peripheral blood by the protein precipitation method. All genotyping was done by the Genesky Bio-pharm Technology Company, using SNP scan technology.

Quality control

A quality control was conducted among SNPs selected from previews GWASs. The criteria of the quality control included: (1) Low minor allele frequency (MAF)>0.01; (2) Genotyping rate >0.95; (3) Hardy Weinberg equilibrium test (P>0.01). The qualified SNPs were used for formal G×G interaction tests.

Statistical analysis

Hardy-Weinberg Equilibrium (HWE) test and minor allele frequency (MAF) were calculated among unaffected parents using PLINK [12]. A single interaction between two SNPs was determined by R Package trio (v1.4.23) [13]. To generate a likelihood ratio test for G×G interactions, two conditional logistic regression models were fitted comparing the case genotype trios and the 15 matched pseudo-controls, one model consisting two coding variables for each of the two SNPs, and the other model additionally containing the four possible interactions of these variables. Then, the two models are compared by a likelihood ratio test, and the *p*-values are computed as a χ^2-distribution with four degrees of freedom [14]. We used a Bonferronni correction for 47 tests to determine a threshold for formal significance of P=0.0011.

Results

The NSCL/P samples were divided into 2 subgroups: 129 NSCLO cases (42.7%) and 173 NSCLP cases (57.3%), respectively. All 47 SNPs met quality control for this study. Their minor allele frequencies (MAF), and *P*-values of Hardy-Weinberg Equilibrium test are summarized in Supplementary Table 1.

To explore all potential interactions between the selected SNPs, we used two different methods to perform the analysis. From the results of likelihood ratio test for epistatic interactions based on genotypic TDTs in NSCL/P trios from

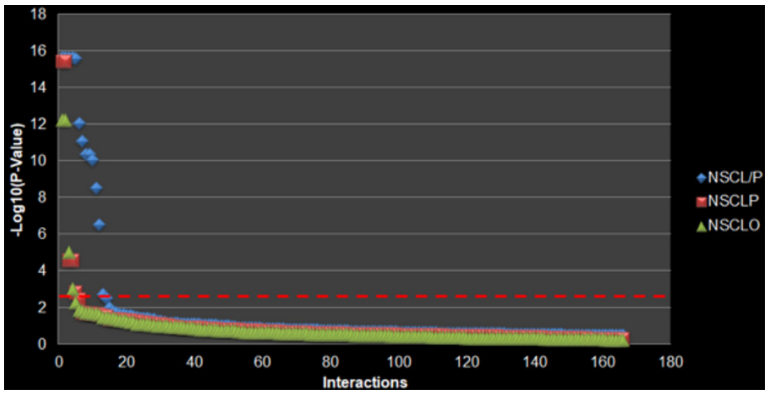

Figure 1. Interactions between SNPs by epistatic test among NSCLP.

Western Han Chinese, we found 20 pairs of SNP-SNP interactions significantly associated with NSCL/P after Bonferroni correction (*P*-value <0.0011): 12 pairs of interactions among NSCL/P, 4 pairs among NSCLP, and 4 pairs among NSCLO (**Figure 1** and **Table 2**).

Most of the significant interactions were between SNPs in or around the same gene, e.g. SNPs at MAFB, NTN1 and IRF6. At MAFB, we detected that rs11698025 and rs6102085 interacted with four SNPs: rs13041247, rs17820943, rs6065259 and rs6072081 respectively in the NSCL/P group (lowest P=2.20E-16), but there was no interaction between rs11698025 and rs6102085; rs6072081 and rs6102085 both interacted with rs13041247 and rs17870943 respectively in NSCLP group (Lowest P=3.64E-16); rs11698025 interacted with rs13041247 and rs1782943 respectively in NSCLO group (lowest P=5.65E-13). Besides, we also found interactions between SNPs at NTN1: rs4791774-rs9788972 (P=8.73E-12) and rs9788972-rs9915089 (P=2.96E-07) were significantly associated with NSCL/P; Interactions between SNPs in or around IRF6: rs2064163-rs2235371 (P=2.56E-16) and rs11119388-rs2235371 (P=9.09E-13) were significantly associated with NSCL/P; rs2064163-rs2235371 also showed significance in the NSCLO group (P=9.10E-06). Notably, the interaction between rs17563 (BMP4)-rs560426 (ARHGAP29) was specifically significantly associated with NSCLO (**Table 2**).

Discussion

NSCL/P is a common malformation with a complex and heterogeneous etiology. Previous genetic and epidemiologic studies have identified both genetic and environmental factors influencing the risk of this malformation. In the past decade, GWAS have successfully provided evidence for the genetic etiology of NSCL/P, but these rarely consider pairwise interactions between multiple genes and the effects of multiple independent rare variants. Therefore, G×G interactions studies may explain some of the "missing heritability" within a risk of non-syndromic OFCs, providing the potential biologic and biochemical pathways underpinning NSCL/P.

Our results first reported the interactions between rs560426 (ARHGAP29) and rs17563 (BMP4) for NSCLO in western Han Chinese population. rs560426 is located at the intron of ABCA4 gene which is a member of a superfamily of transmembrane proteins. Beaty et al. [2] first found an association between markers near and in ABCA4 (most significant SNP rs560426, $P=5.01\times10^{-12}$) among NSCL/P reaching genome-wide significance, especially in Asian populations. However, the expression of ABCA4 gene was restricted to the retina [2], which means ABCA4 is unlikely to be a primary etiologic gene for NSCL/P. ARHGAP29 is located in close proximity to ABCA4, which has been proposed to be a more plausible etiologic gene for clefting. This gene belongs to the family of guanine-activating proteins regulating the level of active RhoA-GTPase. In contrast to ABCA4, the expression of ARHGAP29 was observed in the craniofacial development, especially in the medial and lateral nasal processes [15] and was reduced in all epithelia of IRF6-deficient embryos which suggests it may act downstream of IRF6. After detecting genetic interactions between ARHGAP29 and PBX1, PBX2, P63, WNT3, and WNT9B [16], we linked these to a large network of genes implicated in craniofacial development. In addition, sequencing of ARHGAP29 in cases with NSCL/P identified potentially deleterious variants compared with controls. Leslie et al. [15] speculated that genome-wide significant associations for markers near ABCA4 were driven by rare variants located in adjacent LD blocks or that the SNP at ABCA4 is in LD with etiologic SNPs located in

Table 2. Gene-gene interaction based on conditional logistic regression models using cases and 15 pseudo controls in NSCL/P trios from Western Han Chinese

	SNP1	SNP2	LL with IAs	LL without IAs	Statistic	*P*-value
NSCL/P	rs11698025	rs13041247	-737.1	-796.1	117.9363	2.20E-16
	rs11698025	rs17820943	-737.1	-796.1	117.9363	2.20E-16
	rs11698025	rs6065259	-724.3	-765.2	81.8237	2.20E-16
	rs11698025	rs6072081	-743.6	-783.5	79.8997	2.20E-16
	rs2064163	rs2235371	-744.3	-783.9	79.2141	2.56E-16
	rs11119388	rs2235371	-745.6	-776.8	62.3964	9.09E-13
	rs4791774	rs9788972	-772.9	-801.7	57.7223	8.73E-12
	rs13041247	rs6102085	-737.5	-764.6	54.36	4.42E-11
	rs17820943	rs6102085	-737.5	-764.6	54.36	4.42E-11
	rs6065259	rs6102085	-716.1	-742.5	52.847	9.17E-11
	rs6072081	rs6102085	-743.1	-765.9	45.6273	2.94E-09
	rs9788972	rs9915089	-790.5	-808.5	35.9556	2.96E-07
NSCLP	rs13041247	rs6072081	-396.4	-435.7	78.48625	3.64E-16
	rs17820943	rs6072081	-396.4	-435.7	78.48625	3.64E-16
	rs13041247	rs6102085	-402.9	-416.1	26.39225	2.64E-05
	rs17820943	rs6102085	-402.9	-416.1	26.39225	2.64E-05
NSCLO	rs11698025	rs13041247	-300.5	-332.2	63.3773	5.65E-13
	rs11698025	rs17820943	-300.5	-332.2	63.3773	5.65E-13
	rs2064163	rs2235371	-318.3	-332.6	28.6754	9.10E-06
	rs17563	rs560426	-317.3	-326.6	18.6307	0.0009287

Note: NSCL/P, non-syndromic cleft lip with or without cleft palate (NSCLO & NSCLP); NSCLP, non-syndromic cleft lip with cleft palate; NSCLO, non-syndromic cleft lip only; MAF, Minor allele frequency; LL with IAs, likelihood ratio with interactions; LL without IAs, likelihood ratio without interactions. The table lists only the items with *p*-value less than 0.0011.

distant regulatory elements of ARHGAP29. Liu et al. [17] further confirmed that expression of ARHGAP29 was affected by rs560426 by CRISPR-Cas9-mediated homology-directed repair in oral epithelial cells. However, results of sequencing ARHGAP29 coding regions and exon-intron boundaries in 188 NSCL/P cases and 16 close relatives showed that heterozygous loss-of-function (LoF) variants in ARHGAP29 conferred a moderate effect on NSCL/P, while IRF6 (rs642961) and 1p22 (rs560426 and rs4147811) did not seem to contribute to the penetrance of the phenotype [18]. Although heterozygosity for LoF variants of ARHGAP29 has proven to increase the incidence and length of oral adhesions during orofacial development in vivo [19], the function of some SNPs at 1p22 hit in GWAS is still controversial. BMP4 located at chromosome 14q22-23 is functionally involved in craniofacial development. BMP4 is expressed in three midfacial processes (lateral nasal, medial nasal and maxillary processes), which form the lateral parts of the upper lip and the secondary palate [20]. A deficiency of BMP4 resulted in isolated cleft lip further revealed that BMP signaling has distinct roles in lip and palate fusion [21]. rs17563 is one of the important functional SNPs at BMP4. Many studies have focused on its effect on NSCL/P risk among humans. The C allele at rs17563 may increase the risk of NSCL/P in Asian and Caucasian populations, while it was found to exert a protective effect in a Brazilian population [22]. Hao et al. [23] found the rs17563 polymorphism was significantly associated with NSCLO but not with NSCPO or NSCLP and suggested that the C allele was a protective factor for NSCLO. The adverse results indicated that BMP4 rs17563 polymorphism contributed differently among different ethnicities and different forms of NSCL/P. Interestingly, in our study, the interaction between rs17563 with other SNPs was also detected only in NSCPO. We expanded the signal network of the interaction between BMP4 and ARHGAP29 and considered possible interactions that should be evaluated across populations.

Most of the SNP-SNP interactions were around the same gene, especially MAFB. MAFB was

first identified by the GWAS on case-parent trios from European and Asian populations [2]. It reported MAFB (most significant rs13041247) had strong evidence for association with the risk of NSCL/P in Asian and European families and showed that mRNA and protein were expressed during the development of the secondary palate in mice. Based on the previous GWAS [2], Li et al. [24] tested for G×G interaction involving the wingless-related integration site (WNT) signaling pathway and observed evidence of G×G interaction between markers in MAFB and WNT5B in both Asian and European groups (rs4765835: rs11696257 *P*-values =0.0076 among Asian trios; rs2807376: rs13041247 *P*-values =0.018 among European trios) through RF analyses followed by conditional logistic regression epistatic tests and the case-only analyses. Another study [25], using the sample from Leslie et al. [26], found significant G×G interaction between markers in MAFB and IRF6 (rs6029315: rs6681355, empirical $P=3.8\times10^{-8}$) in trios of European ancestry; however, no significant interaction was detected in trios of Asian ancestry perhaps due to the underrepresentation of key genotypes. Those results were encouraging and represented the beginning of a new interaction network for palatal development. Our team has detected evidence that rs6072081 significantly interacts with rs6102085 in NSCPO case-parent trios from western Han Chinese population, suggesting these two SNPs act in the same pathway in the etiology of CP [27]. In this study we observed significant interactions between these SNPs near MAFB in all groups (NSCLP, NSCLO and NSCL/P) further providing evidence of influence the risk of NSCL/P and indicating the potential correlation between those SNPs. The finding that these interactions were found in same gene explained why these related SNPs near MAFB probably participate in the same signal pathway, but they act differently in NSCLP and NSCLO in particular. In comparison of the whole group (NSCL/P) and two subgroups (NSCLP and NSCLO), we found rs17820943 and rs13041247 were involved in both types of NSCL/P, rs6072081 and rs6102085 was considered to be associated to cleft lip with cleft palate, while rs11698025 was associated for cleft lip only. This suggests a different etiology of these two types of cleft lip, further indicating a difference between cleft lip and cleft palate. By statistical significance, rs11698025 in several pairs showed the smallest *P*-value (P=2.20E-16), which may indicate rs11698025 plays a more important role in the risk of NSCL/P among all the 47 SNPs. Similarly, in the NSCLP group, it suggested that rs6072081 may have a greater association with cleft lip and palate than rs6102085 does.

In conclusion, even though our study is limited to a modest sample size, it provides further evidence for twenty pairs of statistically significant interactions between genes ARHGAP29 and BMP4, and SNPs (around MAFB, IRF6 and NTN1) influencing the risk of NSCL/P in a western Han Chinese population. This suggests a possible interaction network for palatal development and a different etiology of NSCLP versus NSCLO by the method of G×G interaction analysis. All these results deserve follow-up in additional studies of NSCL/P and suggest the importance of G×G interactions to enrich our understanding of the etiology of this complex genetic disease.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. Minor allele frequency and Hardy-Weinberg Equilibrium test of the SNPs for NSCL/P

CHR	GENE	SNP	BP (hg37)	A1	A2	NSCL/P		NSCLP		NSCLO	
						MAF	HWpval	MAF	HWpval	MAF	HWpval
1	*ABCA4*	rs560426	94553438	C	T	31.77%	0.04	28.40%	0.09	34.35%	0.19
1	*DIEXF*	rs126280	210019824	A	G	23.60%	0.91	25.20%	0.62	22.39%	0.43
1	*DIEXF*	rs2064163	210048819	T	G	39.90%	0.31	38.70%	1	40.86%	0.18
1	*DIEXF*	rs12063989	210049893	C	T	36.39%	0.29	34.20%	0.41	38.05%	0.56
1	*DIEXF*	rs4844913	210068117	G	A	47.58%	0.81	49.40%	0.46	46.18%	0.74
1	*IRF6*	rs2235371	209964080	T	C	33.50%	0.24	32.00%	0.12	34.64%	0.81
1	*IRF6*	rs642961	209989270	A	G	24.26%	0.91	27.00%	0.75	22.17%	0.88
1	*PAX7*	rs4920522	18940380	T	C	23.72%	0.07	24.60%	0.5	23.04%	0.09
1	*PAX7*	rs766325	18956458	A	G	21.45%	0.47	22.80%	0.6	20.43%	0.62
1	*PAX7*	rs6695765	18979320	C	T	36.12%	0.6	35.50%	0.79	36.58%	0.73
1	*PAX7*	rs742071	18979874	T	G	4.96%	0.65	5.00%	0.48	4.94%	0.58
1	*SLC25A24*	rs6677101	108699730	T	G	46.74%	0.87	44.80%	0.62	48.23%	0.91
1	*SYT14*	rs9429830	210110537	T	C	48.93%	0.01	48.00%	0.38	49.68%	0.01
1	*SYT14*	rs11119388	210174417	G	A	40.26%	0.61	38.30%	0.3	41.74%	0.83
1	*SYT14*	rs227178	210216946	C	T	44.00%	0.93	46.20%	0.8	42.35%	0.66
1	*SYT14*	rs2485893	210348155	G	A	47.94%	0.87	48.90%	0.62	47.25%	0.83
1	*ABCA4*	rs481931	94570016	T	G	32.61%	0.03	32.90%	0.21	32.40%	0.14
1	*ABCA4*	rs4147811	94575056	T	C	33.25%	0.02	33.50%	0.27	33.04%	0.04
2	*THADA*	rs7590268	43540125	G	T	3.22%	1	2.30%	1	3.92%	1
3	*EPHA3*	rs7632427	89534377	C	T	16.01%	0.1	13.80%	0.12	17.68%	0.46
4	*GRID2*	rs12506428	93830884	C	T	49.50%	0.37	48.30%	0.08	47.83%	0.75
8	*DCAF4L2*	rs6558002	27389542	C	T	16.39%	0.3	16.40%	0.65	16.42%	0.32
8	*EPHX2*	rs987525	129946154	A	C	8.09%	0.79	6.90%	1	8.99%	1
8	*LOC728724*	rs12543318	88868340	A	C	34.74%	0.86	32.20%	0.48	36.67%	0.42
9	*FOXE1*	rs894673	100612270	A	T	11.94%	0.33	11.00%	1	12.68%	0.22
9	*FOXE1*	rs3758249	100614140	T	C	12.23%	0.26	10.90%	1	13.23%	0.16
9	*FOXE1*	rs4460498	100620412	T	C	12.02%	0.18	11.00%	1	12.83%	0.14
10	*VAX1*	rs7078160	118827560	A	G	49.00%	0.37	48.50%	1	49.42%	0.24
10	*VAX1*	rs4752028	118834991	C	T	38.78%	0.93	38.70%	1	38.84%	0.91
13	*SPRY2*	rs9574565	80668874	T	C	12.62%	0.36	13.20%	0.42	12.17%	0.61
13	*SPRY2*	rs8001641	80692811	A	G	15.15%	1	15.30%	0.34	15.01%	0.67
14	*BMP4*	rs17563	54417522	G	A	31.67%	0.13	31.90%	0.25	31.47%	0.38
15	*FMN1*	rs1258763	33050423	T	C	7.65%	0.38	5.20%	0.51	9.53%	0.53
15	*TPM1*	rs7179658	63312695	C	T	15.84%	0.13	14.90%	0.81	16.52%	0.12
16	*Intergenic*	rs8049367	3930444	T	C	33.42%	0.47	34.50%	0.68	32.61%	0.14
17	*NTN1*	rs9788972	8919630	A	G	23.43%	0.31	24.90%	0.33	22.32%	0.02
17	*NTN1*	rs9915089	8952894	T	C	20.21%	0.61	22.00%	0.72	18.84%	0.22
17	*NTN1*	rs8081823	8965551	A	G	40.81%	1	40.80%	0.9	40.84%	0.82
17	*NOG*	rs17760296	54615617	G	T	1.65%	1	1.90%	1	1.45%	1
17	*NTN1*	rs4791774	8932119	G	A	23.31%	0.82	24.00%	1	22.75%	0.76
17	*NTN1*	rs8069536	8956285	T	G	3.55%	1	3.80%	1	3.33%	1
20	*MAFB*	rs13041247	39269074	C	T	40.26%	0.24	40.40%	0.1	40.14%	1
20	*MAFB*	rs6072081	39261054	G	A	40.59%	0.15	41.00%	0.03	40.29%	1
20	*MAFB*	rs6065259	39261979	A	G	38.05%	0.48	37.90%	0.23	38.17%	1
20	*MAFB*	rs17820943	39268516	T	C	40.26%	0.24	40.40%	0.1	40.14%	1
20	*MAFB*	rs11698025	39274083	A	G	32.20%	0.71	33.50%	0.58	31.20%	1
20	*MAFB*	rs6102085	39281629	A	G	43.57%	0.8	45.20%	0.71	42.33%	1

Note: NSCL/P, non-syndromic cleft lip with or without cleft palate (NSCLO & NSCLP); NSCLP, non-syndromic cleft lip with cleft palate; NSCLO, non-syndromic cleft lip only; CHR, chromosome; BP, position; A1, Minor allele; A2, Major allele; MAF, minor allele frequency; HWpval, *p*-values for Hardy-Weinberg Equilibrium test.