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Gene-gene interaction of single nucleotide polymorphisms in 16p13.3 may contribute to the risk of non-syndromic cleft lip with or without cleft palate in Chinese case-parent trios

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Abstract

Non-syndromic cleft lip with or without cleft palate (NSCL/P) is a common birth defect with a complex and heterogeneous etiology. A recent genome-wide association study (GWAS) among Chinese populations has identified a new region at 16p13.3 as being associated with NSCL/P, which requires further replication. Here, we attempted to replicate and further clarify the genetic association between this region and NSCL/P, as well as testing for potential gene-gene ($G \times G$) and gene-environment ($G \times E$) interactions. We conducted transmission disequilibrium tests on 69 single nucleotide polymorphisms (SNPs) mapping to 16p13.3 among 806 Chinese case-parent trios ascertained through an international consortium where a GWAS of oral clefts was conducted. $G \times G$, as well as $G \times E$ interactions involving maternal environmental tobacco smoke (ETS) and multivitamin supplementation, were explored using conditional logistic regression model. We applied Cordell's method as implemented in the R package TRIO to test for possible interactions. While no SNPs showed evidence of linkage and association with NSCL/P after Bonferroni correction, we found signals of $G \times G$ interactions between SNPs in 16p13.3. Nine pairs of SNP-SNP interactions attained significance after Bonferroni correction, among which the most significant interaction was found between rs2072346 (*ADCY9*) and rs11646137 (intergenic region, $P = 7.2 \times 10^{-5}$). Linkage disequilibrium (LD) analysis revealed only low level of LD between these SNPs. This study failed to confirm the significant association between SNPs within

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SUPPORTING INFORMATION

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16p13.3 and the risk of NSCL/P, but underlined the importance of taking into account potential $G \times G$ interactions for the genetic association analysis of NSCL/P.

Keywords

case-parent trios; gene-gene interaction; genome-wide association study; non-syndromic cleft lip with or without cleft palate

1 INTRODUCTION

Non-syndromic cleft lip with or without cleft palate (NSCL/P) is a common human birth defect involving multiple genetic and environmental risk factors (Dixon, Marazita, Beaty, & Murray, 2011). Despite advances in surgical treatments, NSCL/P still imposes a broad range of adverse implications on affected individuals and their families (Dixon et al., 2011). The prevalence rates are generally higher in Asian populations compared to others. Chinese populations have a relatively high birth prevalence rate of 1.2 NSCL/P case among 1,000 live births (Cooper, Ratay, & Marazita, 2006).

Identifying key genes responsible for genetic susceptibility to this disorder has been the subject of decades of research. Genome-wide association studies (GWAS) have provided major advances in our knowledge of the genetic architecture underlying NSCL/P, and at least a dozen genes and regions have been identified to have significant marginal effect in multiple populations (Beaty et al., 2010; Birnbaum et al., 2009; Grant et al., 2009; Mangold et al., 2010; Sun et al., 2015). In addition, gene-gene ($G \times G$) and gene-environment ($G \times E$) interactions have been increasingly considered essential in the etiology of NSCL/P (Beaty et al., 2013; Letra et al., 2012; Li et al., 2015; Sull et al., 2009). Several environmental exposures, such as environmental tobacco smoke (ETS), alcohol consumption, and folate supplementation have been suggested to interact with certain genes to influence the risk of NSCL/P (Beaty et al., 2011; Shi et al., 2007; Wu et al., 2012). Recently, Li et al. (2015) found evidence of $G \times G$ interactions between genes in WNT family and other genes identified by GWAS, using a combined approach of various statistical methods. However, the evidence for potential $G \times E$ and $G \times G$ interactions has been rather inconclusive to date, probably due to a lack of power and/or the intrinsically heterogeneous etiology for this common birth defect.

Recently, the first case-control GWAS specifically conducted among Chinese populations identified a novel risk region associated with NSCL/P: chromosome 16p13.3, where the most significant SNP was rs8049367 located between the *CREBBP* and *ADCY9* genes (estimated odds ratio = 0.74, $P = 8.98 \times 10^{-12}$) (Sun et al., 2015). This GWAS incorporated a three-stage case-control design with more than 5,000 subjects total, and is the first study of NSCL/P exclusively focused on the Chinese populations. The purpose of the present study was to replicate and further clarify the contribution of genetic variants in the 16p13.3 region on NSCL/P in Chinese populations while incorporating potential $G \times G$ and $G \times E$ interactions. A total of 69 single nucleotide polymorphisms (SNPs) were selected and examined in 806 Chinese trios drawn from an international consortium established for a

GWAS of non-syndromic oral clefts using case-parents trios of multiple ethnicities (Beaty et al., 2010).

2 METHODS

2.1 Sample description

We used data on 806 case-parent trios with Chinese ancestry from an international consortium (Beaty et al., 2010). Research protocols were reviewed and approved by institutional review boards (IRBs) at each institution of this consortium, including IRBs at The Johns Hopkins School of Public Health, University of Iowa, University of Pittsburgh, Utah State University, and all foreign collaborators. Written informed consent was obtained from parents. DNA for cases and their biological parents was obtained from whole blood, saliva, or a mouthwash sample. History of maternal exposures was collected through direct interview with the mothers. As expected for NSCL/P, there were more male cases (67%) compared to females. The exposure rates of maternal cigarette smoking and alcohol consumption were very low (<3%), so only environmental tobacco smoke (ETS) and multivitamin supplementation (exposure rate of 40.9% and 12.9%, respectively) were analyzed in the tests for GxE interaction. Detailed information on sample collection and exposure measurement was described previously (Beaty et al., 2010).

2.2 SNPs and genotyping

At the 16p13.3, we focused on a 391Kb region covering two protein-encoding genes—*CREBBP* and *ADCY9*—and the region between them (Figure 1), in which 100 SNPs were genotyped from the original consortium, where we selected 806 case-parent trios. The Center for Inherited Disease Research (CIDR) at the Johns Hopkins University genotyped DNA samples using Illumina Human610-Quad v.1_B Bead Chip (Beaty et al., 2010). Several quality control (QC) criteria were used to flag SNPs: (1) unacceptably high rates (>10%) of missing genotype calls; (2) low minor allele frequency (MAF) among founders (<0.1); (3) significant deviation ($P < 0.001$) from Hardy–Weinberg equilibrium (HWE) among parents and (4) high rates of Mendelian errors (>5%) between the observed genotypes of parents and the child. Plink v1.07 was used to filter all SNPs. This process excluded 31 SNPs all due to low MAF, thus leaving 69 SNPs available for analysis (shown in Table S1 Supplemental Material).

2.3 Statistical analysis

Minor allele frequencies and pairwise linkage disequilibrium (LD) were computed among founders (Table S1 and Figure S1 Supplemental Material). Pairwise LD was measured as r^2 and D' using Haploview (version 4.2) (Barrett, Fry, Maller, & Daly, 2005), and 16 haplotype blocks were identified (Figure S1 Supplemental Material). The allelic transmission disequilibrium test (TDT) of each SNP was performed to test for linkage and association as implemented in PLINK (version 1.07, <http://pngu.mgh.harvard.edu/~purcell/plink/data.shtml>). This allelic TDT measured the over-transmission of an allele from heterozygous parents to the affected offspring. Significance level was set as 7.25×10^{-4} ($0.05/69$) using Bonferroni correction.

We screened for possible $G \times E$ interaction between SNPs in 16p13.3 and two common maternal exposures followed a strategy described in (Kraft, Yen, Stram, Morrison, & Gauderman, 2007). Using this extension of conditional logistic regression, the 2 degrees of freedom (df) likelihood ratio test was conducted to investigate the combined effects of G and $G \times E$, then followed by a 1 df likelihood ratio test estimating effects of $G \times E$ interaction alone.

Pairwise $G \times G$ interactions were explored using Cordell's method, which fits a conditional logistic regression model (Cordell, 2002). The core idea behind case-parent trios in this logistic regression framework is to create "pseudo-controls" by considering all possible genotypes from any parental mating, thus yielding a matched case-control design where each trio generates the "case" and 15 "pseudo-controls." For two SNPs from different genes, this method compares the genotypes at these two SNPs in the observed case and 15 matched pseudo-controls. Only SNPs from different genes/regions were considered in this test for $G \times G$ interaction. The significance level was set at $P = 7.25 \times 10^{-4}$ after a Bonferroni correction (0.05/69), and we excluded all tests yielding $P < 10^{-12}$ to minimize false positive findings. In addition, we carried out permutation tests where "case" versus "pseudo-control" status was randomly shuffled 10,000 times within each trio to assess the empirical significance of potential interaction (among those that reached Bonferroni significance level). Empirical P values were determined as the proportion of all replicates yielding a more extreme test statistic than the observed data interaction analyses were performed using TRIO package (Version 3.8.0) in the open source environment R (Version 3.1.0), available at <http://www.bioconductor.org>.

3 RESULTS

The searching for marginal gene effects by the allelic TDT revealed four SNPs showed nominal significance of linkage and association ($P < 0.05$) with NSCL/P (Table S2 Supplemental Material), yet none of these associations remained significant after correction for multiple testing. Tests for gene-environment ($G \times E$) interaction between these 69 SNPs in 16p13 and ETS or multivitamin supplementation yielded only nominally significant signals (Table S3 Supplemental Material), which again did not remain significant after Bonferroni correction.

Although no SNP achieved the significance level when considered alone or together with environmental risk factors, the $G \times G$ interaction screening process yielded statistically significant signals within this region. A total of nine pairs of SNPs within 16p13.3 reached the adjusted significance level (Figure 2), among which the pair composed of rs2072346 (*ADCY9*) and rs11646137 (intergenic region) yielded the most significant signal ($P = 7.2 \times 10^{-5}$, Table 1). SNP rs2239318, rs11646137, and rs2531995 showed some significant evidence of $G \times G$ interactions with multiple other SNPs in Figure 2 (all significant SNP pairs are shown in Figure 2). In addition, linkage disequilibrium analysis revealed only low level of LD between all of these SNPs showing significant $G \times G$ interaction ($r^2 < 0.23$). All these pairwise SNP-SNP interactions remained significant in the permutation tests.

4 DISCUSSION

A novel risk region, chromosome 16p13.3, was identified to be associated with NSCL/P in a recently published GWAS among Chinese populations (Sun et al., 2015). To replicate this GWAS signal, we evaluated 69 SNPs within 16p13.3, including SNPs in and near the *CREBBP* and *ADCY9* genes, among 806 Chinese case-parent trios. No single SNPs achieved significance in the allelic TDT of linkage and association, and P values of $G \times E$ tests were only marginally significant. In the analysis explicitly considering $G \times G$ interaction, nine pairs of SNPs showed significant interactions after Bonferroni correction, and all of them were confirmed in permutation tests. These results suggest there may be $G \times G$ interactions between SNPs within 16p13.3 that might play an important role in the etiology of NSCL/P.

Two genes were examined in the present study. The cAMP response element binding protein (CREB)-binding protein gene (*CREBBP*) is ubiquitously expressed and is involved in the transcriptional coactivation of many different transcription factors. Mutations in *CREBBP* and its flanking region underlie Rubinstein-Taybi syndrome (Hennekam, 2006), a Mendelian malformation syndrome which is characterized by cognitive impairment and congenital anomalies, which can include oral clefts (Hennekam & Van Doorne, 1990). Structural aberrations of this chromosomal region (e.g., deletions or duplications) were also reported to be related to various congenital anomalies, with *CREBBP* being a key gene (Demeer et al., 2013; Sun et al., 2014). Some previously reported cases were also found to have deletions of Adenylate cyclase-9 (*ADCY9*) (Wojcik et al., 2010), but its function is poorly defined. Several studies suggest variants linked to syndromic forms of oral cleft showing a Mendelian mode of inheritance can also influence on non-syndromic clefts (Mossey, Little, Munger, Dixon, & Shaw, 2009). One typical example is *IRF6* which is the causal gene of van der Woude syndrome, the most common Mendelian syndrome where CL/P is a hallmark phenotype (Kondo et al., 2002). Given their function in syndromic forms of oral clefts, the genetic variation or gene-gene/gene-environment interactions between *CREBBP* and *ADCY9* may reasonably influence the risk of non-syndromic CL/P.

The present study failed to replicate the findings of rs8049367 (lying in the intergenic region) in the latest GWAS of NSCL/P (Sun et al., 2015). This may due to different study design or the limited sample size of our study, which was much smaller than that of Sun et al.'s GWAS. So far, there have been several studies evaluating the role of $G \times E$ interaction to gain further insight into the involvement of genetic markers in the development of NSCL/P (Beaty et al., 2011; Shi et al., 2007; Wu et al., 2012). Genes without large marginal effects could significantly influence the risk of diseases by interacting with certain environmental factors during early development (Beaty et al., 2011; Wu et al., 2014). Therefore, pure interactions (i.e., those with no apparent main effects) could be missed if the gene is examined in a statistical model ignoring $G \times E$ interaction. In the present study, formal tests for $G \times E$ interaction failed to show statistical signals for interaction between SNPs in 16p13.3 and two common maternal exposures.

Several studies have demonstrated gene-gene interaction may play an important role in etiologically complex disorders such as NSCL/P (Letra et al., 2012; Li et al., 2015).

Recently, Li et al. (2015) showed compelling evidence of $G \times G$ interactions between *WNT5B* and *MAFB* in both European and Asian trios, and also found some ancestry-specific interactions involved in NSCL/P (Li et al., 2015). In the present study, nine pairs of SNPs in 16p13.3 achieved significance after Bonferroni correction, and all of them were confirmed in permutation tests. Several SNPs showed significant interactions with more than one SNPs, which may provide further support that genetic elements in this region may interact with one another. SNPs involved in significant interactions did not show a high level of LD with rs8049367 (the top significant SNP in the GWAS conducted by Sun et al. (2015)). We also performed $G \times G$ analysis using imputed genotype data in this chromosomal region, and these analyses yielded additional significant interactions (Table S4 Supplemental Material). The significance level used in $G \times G$ analyses was 7.25×10^{-4} (0.05 divided by the number of SNPs included rather than the number of tests performed), since for etiologically heterogeneous phenotypes such as CL/P, overly strict definitions of “significance” could be improper and increase the likelihood of missing potential signals.

As shown in Table 1, some SNPs identified as showing significant GxG interaction are located in the non-coding interval between *CREBBP* and *ADCY9*. Although little is known about the function of these intergenic regions of the genome, it has been demonstrated that non-coding regions may have a crucial role in the regulation of coding regions (Nobrega, Ovcharenko, Afzal, & Rubin, 2003). A recent study using mouse model revealed that a “gene desert” region of chromosome 8q24 contains remote enhancers controlling *MYC* gene expression in the developing face (Uslu et al., 2014). We noticed that between *CREBBP* and *ADCY9* there exists a long non-coding RNA (lncRNA) RP11-462G12.1 with two exons. The lncRNA has been recently found to be pervasively transcribed in the genome, and they have been widely thought to play important regulatory roles (Amaral & Mattick, 2008; Guttman et al., 2009; Wilusz, Sunwoo, & Spector, 2009). It has been shown that mutations and dysregulations of lncRNAs can contribute to numerous human diseases including cancer, coronary diseases, and diabetes (Burd et al., 2010; Kim et al., 2014; Tufarelli et al., 2003; Wapinski & Chang, 2011). Therefore, lncRNA may provide a functional basis of disease-associated polymorphisms outside protein-coding regions, and also argue for an etiologic role of GxG interactions involving non-coding markers. We conducted further search of interaction between polymorphisms lying within lncRNA and the remaining intergenic region of chromosome 16p13.3, and additional six pairs of SNPs reached the adjusted significance level (Table S5 Supplemental Material). It is worth noting that SNP rs7197403, which interacted with the intergenic SNP rs12446940 ($P = 1.22 \times 10^{-7}$), was located in an exon of this lncRNA whereas the other five SNPs were located in lncRNA introns. SNPs that reached the significance level within *CREBBP* and *ADCY9* were all intronic polymorphisms and could potentially regulate gene expression. Altogether, these results may predict some biological interaction between lncRNA RP11-462G12.1 and its nearby genes and regions. Since little is known about the molecular mechanisms underlying this disease, additional mechanism studies are warranted to further elucidate these statistical interactions.

In summary, our study failed in replicating the significant marginal association between individual polymorphic variants in 16p13.3 and NSCL/P, as previously reported in a recent GWAS (Sun et al., 2015). However, we found SNPs mapped to this region may influence the

risk of this common birth defect by interacting with each other in Chinese populations. One limitation of the present study is its disregard of rare variants in the 16p13.3 region, whose effects should be carefully measured in future studies. Polymorphisms downstream of *CREBBP* and upstream of *ACDY9* may also influence risk, yet they were not considered in our study. Further validation of the involvement of genetic markers within 16p13.3 is still warranted, and the underlying molecular mechanisms of the interactions within this region require further clarification. Our findings underscored the need to explore gene–gene interaction in order to expand our understanding of complex disorders such as NSCL/P, and offered preliminary hints for further investigation into pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

The chromosome 16p13.3 region including *CREBBP* and *ADCY9*. Source from Ensembl genome browser based on build 36 (<http://may2009.archive.ensembl.org>). [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 2.

Significant GxG interactions within 16p13.3. Significant SNPs are shown along the *X*-axis ordered by their physical locations, and genes are shown as black bars along the *X*-axis (n.b. positions on the *X*-axis do not represent the actual locations on the chromosome). The *Y*-axis displays the $-\log_{10}P$ from Cordell's 4 degrees of freedom tests for $G \times G$ interactions. Solid lines connect pairs of interacting SNPs. The dashed line indicates the significance level adjusted for multiple tests by Bonferroni method. The inverted triangle marks the relative position of rs8049367 in this region. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1

Pairs of single nucleotide polymorphisms (SNPs) showing significant interactions after Bonferroni correction using Cordell's test among Chinese non-syndromic cleft lip with or without cleft palate trios

Gene1	SNP1	MAF1	Gene2	SNP2	MAF2	r ²	P value	Empiric p ^d
CREBBP	rs2239318	0.278	Intergenic region	rs2238419	0.431	0.03	7.70E-05	—
	rs11076787	0.159		rs2283491	0.237	0.02	1.01E-04	—
	rs2239318	0.278		rs11649508	0.493	0.05	1.39E-04	—
	rs886528	0.440		rs12446940	0.357	0.07	3.58E-04	0.0002
	rs2239318	0.278		rs11646233	0.448	0.05	4.66E-04	0.0006
ADCY9	rs2072346	0.458	Intergenic region	rs11646137	0.336	0.02	7.22E-05	0.0001
	rs2238438	0.416		rs11646137	0.336	0.22	4.67E-04	0.0002
	rs2531995	0.320		rs11646137	0.336	0.00	5.58E-04	0.0012
	rs2531995	0.320		rs11640300	0.235	0.02	6.58E-04	0.0005

MAF, minor allele frequency; r², linkage disequilibrium between SNP pairs.

^dEmpiric P-value from permutation tests. A “—” indicates no test statistic generated from permuted datasets exceeded the observed test statistic.