Gene-Gene Interactions among *SPRY*s for Nonsyndromic Cleft Lip/Palate

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Abstract

Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is a common birth defect with a complex genetic architecture. Gene-gene interactions have been increasingly regarded as contributing to the etiology of NSCL/P. A recent genome-wide association study revealed that a novel single-nucleotide polymorphism at *SPRY1* in 4q28.1 showed a significant association with NSCL/P. In the current study, we explored the role of 3 *SPRY* genes in the etiology of NSCL/P by detecting gene-gene interactions: *SPRY1*, *SPRY2*, and *SPRY4*—with *SPRY3* excluded due to its special location on the X chromosome. We selected markers in 3 *SPRY* genes to test for gene-gene interactions using 1,908 case-parent trios recruited from an international consortium established for a genome-wide association study of nonsyndromic oral clefts. As the trios came from populations with different ancestries, subgroup analyses were conducted among Europeans and Asians. Cordell's method based on conditional logistic regression models was applied to test for potential gene-gene interactions via the statistical package TRIO in R software. Gene-gene interaction analyses yielded 10 pairs of SNPs in Europeans and 6 pairs in Asians that achieved significance after Bonferroni correction. The significant interactions were confirmed in the 10,000-permutation tests (empirical $P = 0.003$ for the most significant interaction). The study identified gene-gene interactions among *SPRY* genes among 1,908 NSCL/P trios, which revealed the importance of potential gene-gene interactions for understanding the genetic architecture of NSCL/P. The evidence of gene-gene interactions in this study also provided clues for future biological studies to further investigate the mechanism of how *SPRY* genes participate in the development of NSCL/P.

Keywords: genetic association study, cleft palate, congenital abnormalities, birth defects, single-nucleotide polymorphism, case-parent trios

Introduction

Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is a common birth defect around the world, with varied prevalence among geographic regions (Panamonta et al. 2015). American Indians, Japanese, and Chinese have a relatively higher prevalence than do Africans and Europeans (Panamonta et al. 2015). The development of NSCL/P involves various genetic and environmental factors (Leslie and Marazita 2013). To date, genome-wide association studies (GWASs) have identified multiple loci influencing the risk of NSCL/P (Birnbaum et al. 2009; Beaty et al. 2010; Mangold et al. 2010; Ludwig et al. 2012; Sun et al. 2015; Ludwig et al. 2016; Leslie, Carlson, et al. 2016; Leslie, Liu, et al. 2016; Yu et al. 2017; Leslie, Carlson, Shaffer, Butali, et al. 2017). Despite much progress in the exploration of genetic risk factors for NSCL/P, only about 20% of the heritability can be explained by those susceptibility loci identified in the previous studies (Leslie, Carlson, Shaffer, Buxo, et al. 2017). The “missing heritability” can derive from common variants with too small effect sizes to be identified, rare variants not captured in GWASs, gene-gene interactions, gene-environment interactions, and parents-of-origin effects (Eichler et al. 2010). Therefore, testing for gene-gene interactions in the context of GWASs is an important approach to further explore the etiology of NSCL/P (Frazer et al. 2009; Steen 2012). Multiple gene-gene interactions have been identified to

contribute to the risk of NSCL/P. For example, Song et al. (2013) identified 3 single-nucleotide polymorphisms (SNPs) (rs2073485, rs2235371, and rs2236909) in *IRF6* showing interactions with rs2235373 in *PAX9*. Liu et al. (2017) reported 9 pairs of SNP-SNP interactions within the region of 16p13.3 potentially important to the susceptibility of NSCL/P. Evidence also supported gene-gene for *TGFA-MTHFR* and *WNT5B-MAFB* (Jugessur et al. 2003; Li et al. 2015). However, Beaty et al. (2013) failed to identify any interaction among 8q24, *IRF6*, *ABCA4*, *MAFB*, *PAX7*, *THADA*, *COL8A1*, and *DCAF4L2*. Although not all studies successfully identified gene-gene interactions among genes of interest, testing for gene-gene interactions was still a practical approach to explore the etiology of NSCL/P.

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A supplemental appendix to this article is available online.

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Sprouty genes contain 4 members—*SPRY1*, *SPRY2*, *SPRY3*, and *SPRY4*—located on 4 chromosomes. Yu et al. (2017) first reported that an SNP at *SPRY1* in 4q28.1 showed a significant association with NSCL/P after Bonferroni correction, with 3,379 NSCL/P cases and 8,593 controls with Chinese ancestry. They further successfully replicated this variant with another 861 trios with Asian ancestry. This study also replicated a previously reported locus, 13q31.1 (*SPRY2*), in the Chinese population. Several studies further reported signals in *SPRY1* and *SPRY2* to be associated with NSCL/P among European populations (Ludwig et al. 2012; Jia et al. 2015; de Araujo et al. 2016). Although GWASs have provided some evidence for these loci in the etiology of NSCL/P, the identified associations were primarily located in noncoding regions, and the underlying mechanisms remained unknown. Therefore, exploring gene-gene interactions among *SPRY* genes was a possible approach to better understand the etiology of NSCL/P.

In the current study, we aimed to explore potential gene-gene interactions among 3 *SPRY* genes (*SPRY1*, *SPRY2*, *SPRY4*). As *SPRY3* locates on the X chromosome, we excluded it for gene-gene interaction analysis. This study used GWAS data of 1,908 case-parent trios who were recruited from an international consortium (Beaty et al. 2010). Considering the genetic heterogeneity among populations with different ancestries, we also analyzed the data stratified by European and Asian populations.

Materials and Methods

The report of this study followed the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines.

Study Population

A total of 1,908 NSCL/P case-parent trios with European or Asian ancestry were drawn from an international consortium that conducted a GWAS. Details of the sample were introduced by Beaty et al. (2010). Most were with European ancestry (825 trios) or Asian ancestry (1,038 trios). Clinicians examined each case to exclude the syndromic forms of cleft lip with or without cleft palate. The research protocol was reviewed and approved by the Institutional Review Board of each institution participating in this international consortium, including the Johns Hopkins School of Public Health, University of Pittsburgh, Utah State University, University of Iowa, among others. Written informed consent was obtained from each participant. DNA was extracted from whole blood, saliva, or a mouthwash sample for cases and parents.

Genotyping and SNP Selection

DNA samples were genotyped with Illumina Human610-Quad v.1_B Bead Chip at the Center for Inherited Disease Research, Johns Hopkins University. SNPs located in or near gene

regions were mapped to genes according to the National Center for Biotechnology Information human genome build 36.1. In the process of quality control, SNPs would be excluded if they met any of the following criteria: 1) missing genotype information >10%, 2) low minor allele frequency (MAF) among founders <0.1, 3) significant deviation of Hardy-Weinberg equilibrium among parents <0.001, and 4) Mendelian error >5%. Quality control was performed for all 1,908 trios by PLINK 1.07 and for European and Asian subgroup analysis, yielding 149 SNPs for all 1,908 trios, 206 SNPs for European trios, and 189 SNPs for Asian trios for analysis. Detailed information of the SNPs is included in Appendix Tables 1 to 3.

Statistical Methods

The Cordell (2002) method was used to detect gene-gene interactions, where 15 “pseudo-controls” were created for each case and a conditional logistic regression model was built to assess the SNP-SNP interaction under the additive model of inheritance. To consider 2-way interactions, the 15 pseudo-controls were generated on the basis of the parents’ genotypes at the 2 loci, where the genotype at the 2 loci for each pseudo-control consisted of 1 of the 2-locus genotypes that was possible but not transmitted to the case. The conditional logistic regression model under the additive model of inheritance (full model) was as follows:

$$\log\left(\frac{p_{ij}}{1-p_{ij}}\right) = a_1x_1 + d_1z_1 + a_2x_2 + d_2z_2 + i_{aa}x_1x_2 + i_{ad}x_1z_2 + i_{da}z_1x_2 + i_{dd}z_1z_2,$$

where x_i and z_i were dummy variables related to the underlying genotype at locus i ; the coefficients a_1 , d_1 , a_2 , and d_2 represented additive (a_i) and dominance (d_i) effects at the 2 loci; and i_{aa} , i_{ad} , i_{da} , and i_{dd} corresponded to the epistatic interaction effects (Cordell 2002). The lack of interactions could be inferred when 4 interaction coefficients equaled zero. Then, the model could be simplified as

$$\log\left(\frac{p_{ij}}{1-p_{ij}}\right) = a_1x_1 + d_1z_1 + a_2x_2 + d_2z_2 (\text{null model}).$$

Likelihood ratio tests were conducted to compare the full model and null model, providing an overall 4-*df* test of interaction. The significance levels were set as 2.4×10^{-4} (0.05/206) for the European population and 2.6×10^{-4} (0.05/189) for the Asian population after the Bonferroni correction. The permutation test was also conducted to minimize the possibility of obtaining false-positive results. The disease status of “cases” and “pseudo-controls” was randomly shuffled 10,000 times. The P value for the permutation test was the probability of obtaining a more extreme statistic than the observed data. Cordell’s method and permutation tests were both conducted in the TRIO package (version 3.16.0) in R software (version 3.4.3).

Table 1. Top 10 Pairs of Single-Nucleotide Polymorphisms for Gene-Gene Interactions among 1,908 Trios with Nonsyndromic Cleft Lip with or without Cleft Palate.

First Gene	Marker 1	MAF1 ^a	Second Gene	Marker 2	MAF2 ^a	P Value	Empirical P Value ^b
<i>SPRY1</i>	rs7697652	0.280	<i>SPRY2</i>	rs9601485	0.220	3.59E-05 ^c	<0.001
<i>SPRY1</i>	rs7697652	0.280	<i>SPRY2</i>	rs7320320	0.206	3.49E-04	
<i>SPRY1</i>	rs7697652	0.280	<i>SPRY2</i>	rs4267202	0.280	4.18E-04	
<i>SPRY2</i>	rs1478875	0.490	<i>SPRY4</i>	rs4244027	0.433	6.28E-04	
<i>SPRY1</i>	rs12505913	0.383	<i>SPRY2</i>	rs2096347	0.247	6.56E-04	
<i>SPRY2</i>	rs1010390	0.428	<i>SPRY4</i>	rs4244027	0.433	6.97E-04	
<i>SPRY2</i>	rs486467	0.292	<i>SPRY4</i>	rs9800206	0.464	8.51E-04	
<i>SPRY1</i>	rs12233855	0.438	<i>SPRY2</i>	rs2759243	0.410	1.02E-03	
<i>SPRY1</i>	rs12505913	0.383	<i>SPRY2</i>	rs2876759	0.385	1.08E-03	
<i>SPRY1</i>	rs12505913	0.383	<i>SPRY2</i>	rs1984294	0.386	1.10E-03	

^aMinor allele frequency.^bEmpirical *P* values from permutation test were calculated only for single-nucleotide polymorphism pairs remaining significant after Bonferroni correction.^cRemaining significant after Bonferroni correction.

To further check the consistency of genetic risk factors across different ancestries, subgroup analyses were conducted for the European and Asian populations, since these 2 populations together composed nearly 95% of all trios.

Results

A total of 1,908 NSCL/P trios were included in the analysis of gene-gene interactions, with subgroup analyses performed in the European trios and Asian trios. Quality controls of genotyped data for all 1,908 trios excluded 64 SNPs due to a failing Hardy-Weinberg equilibrium test and 46 SNPs due to low MAF, leaving 149 SNPs to be analyzed. In the quality control of subgroup analyses, 39 SNPs and 56 SNPs were filtered due to low MAF in European trios and Asian trios, respectively, leaving 206 SNPs and 189 SNPs for each subgroup.

In the test for gene-gene interactions among 1,908 trios, 1 pair of SNPs achieved significance after Bonferroni correction, rs7697652 (*SPRY1*) and rs9601485 (*SPRY2*), with a *P* value of 3.59×10^{-5} . The top 10 pairs of SNPs for gene-gene interactions among 1,908 trios are listed in Table 1.

To check the consistency across different ancestries, subgroup analyses were conducted among European trios and Asian trios. The analyses showed appealing results where another pair of genes, *SPRY2-SPRY4*, appeared in the stratified analyses based on European and Asian subgroups. Moreover, the pairs of genes involved in gene-gene interactions kept consistent across subgroups—namely, *SPRY1-SPRY2* and *SPRY2-SPRY4*. Among trios of European ancestry, 10 pairs of SNPs reached the corrected significance level, with rs2876761 (*SPRY2*) and rs11739594 (*SPRY4*) yielding the most significant signal ($P = 4.91 \times 10^{-5}$). Among them, 4 pairs of SNPs supported the interactions between *SPRY2* and *SPRY4*, as well as 6 pairs of SNPs indicating interactions between *SPRY1* and *SPRY2*. Among the trios of Asian ancestry, 6 pairs of SNPs showed statistically significant interactions after the Bonferroni correction for multiple tests, with rs2478223 (*SPRY2*) and rs9800206 (*SPRY4*) yielding the smallest *P* value, 2.81×10^{-5} .

The top 10 pairs of SNPs for gene-gene interactions in European and Asian population are listed in Table 2. Permutation tests were also performed to confirm the results. The pairwise gene-gene interactions discovered here remained significant in permutation tests. Empirical *P* values of permutation tests for those SNP pairs that achieved the corrected significance are also listed in Tables 1 and 2.

Discussion

Rs908822 in *SPRY1* (Yu et al. 2017) and rs8001641 as well as rs9545308 in *SPRY2* (Ludwig et al. 2012; Yu et al. 2017) were previously identified to be associated with the risk of NSCL/P in different populations. In the current study, we used 1,908 NSCL/P trios to explore potential gene-gene interactions among *SPRY* genes. To note, *SPRY3* was excluded due to its special location on the X chromosome. Although the single-SNP association analysis did not show any signal (data not shown), the gene-gene interaction analysis yielded 10 and 6 pairs of SNPs with significant interactions among European and Asian trios, respectively. The permutation tests further verified the significant findings.

SPRYs are sprouty receptor tyrosine kinase signaling antagonist genes that could play vital regulatory functions in biological processes (Hacohen et al. 1998; Gross et al. 2001; Impagnatiello et al. 2001). Yang et al. (2010) reported in their functional study that the conditional expression of *SPRY1* in neural crest cells causes craniofacial defects in mice. The overexpression or deficiency of *SPRY2* was reported to result in facial malformations and cleft palates in mice as well. *SPRY2* may also act on the development of cleft palate via, or independent of, fibroblast growth factor (FGF) signaling pathways (Goodnough et al. 2007; Matsumura et al. 2011). Additionally, *SPRY4* was reported to interact with *IRF6* in periderm function in transgenic mice (Kousa et al. 2017). Moreover, the proteins encoded by *SPRY* genes act as crucial regulatory factors of FGF signaling pathways (Thisse and Thisse 2005). To note, previous studies identified several candidate genes for NSCL/P

Table 2. Top 10 Pairs of Single-Nucleotide Polymorphisms for Gene-Gene Interactions among Trios with European or Asian Ancestry.

Ancestry: First Gene	Marker 1	MAF1 ^a	Second Gene	Marker 2	MAF2 ^a	P Value	Empirical P Value ^b
European							
SPRY2	rs2876761	0.324	SPRY4	rs11739594	0.331	4.91E-05 ^c	<0.001
SPRY1	rs3860074	0.301	SPRY2	rs11149181	0.217	1.39E-04 ^c	<0.001
SPRY1	rs3860074	0.301	SPRY2	rs12854744	0.216	1.47E-04 ^c	<0.001
SPRY1	rs7677413	0.188	SPRY2	rs7321702	0.489	1.66E-04 ^c	<0.001
SPRY1	rs3860074	0.301	SPRY2	rs11618325	0.212	1.82E-04 ^c	<0.001
SPRY1	rs3860074	0.301	SPRY2	rs1358977	0.217	1.87E-04 ^c	<0.001
SPRY1	rs12233855	0.457	SPRY2	rs9574697	0.212	2.01E-04 ^c	<0.001
SPRY2	rs4344612	0.240	SPRY4	rs4912846	0.257	2.06E-04 ^c	<0.001
SPRY2	rs4344612	0.240	SPRY4	rs11954937	0.287	2.27E-04 ^c	<0.001
SPRY2	rs12867395	0.192	SPRY4	rs4912846	0.257	2.31E-04 ^c	<0.001
Asian							
SPRY2	rs2478223	0.238	SPRY4	rs9800206	0.423	2.81E-05 ^c	0.003
SPRY2	rs2503378	0.241	SPRY4	rs9800206	0.423	3.53E-05 ^c	0.001
SPRY2	rs2759236	0.424	SPRY4	rs10061008	0.262	1.32E-04 ^c	0.003
SPRY2	rs2794251	0.425	SPRY4	rs10061008	0.262	1.58E-04 ^c	0.004
SPRY1	rs4833925	0.164	SPRY2	rs1287530	0.195	2.12E-04 ^c	<0.001
SPRY2	rs2759243	0.429	SPRY4	rs10061008	0.262	2.44E-04 ^c	0.002
SPRY2	rs7329886	0.456	SPRY4	rs9800206	0.423	3.45E-04	
SPRY2	rs1176270	0.429	SPRY4	rs10061008	0.262	4.46E-04	
SPRY1	rs7697652	0.224	SPRY2	rs9601485	0.206	4.72E-04	
SPRY1	rs7697652	0.224	SPRY2	rs7320320	0.205	5.05E-04	

^aMinor allele frequency.^bEmpirical P values from permutation test were calculated only for single-nucleotide polymorphism pairs remaining significant after Bonferroni correction.^cRemaining significant after Bonferroni correction.

in FGF signaling pathways, such as *FGF10*, *FGFR1*, and *FGF19* (Nikopoulou et al. 2011; Wang et al. 2013; Yu et al. 2017). The biological evidence explained here indicated that *SPRY* genes potentially contribute to the development of NSCL/P.

Several studies identified pairwise gene-gene interactions contributing to the etiology of NSCL/P, such as *PAX9-IRF6* (Song et al. 2013), *MAFB-IRF6* (Xiao et al. 2017), and *WNT-MAFB* (Li et al. 2015). In our study, 1 pair of SNPs in *SPRY* genes was identified in 1,908 NSCL/P trios from different ancestries, which involved *SPRY1* and *SPRY2*. In addition, in subgroup analyses, 7 pairs of SNPs involving *SPRY1* and *SPRY2* showed significant interactions among European and Asian subgroups, which enriched the evidence of the interaction between *SPRY1* and *SPRY2*. Of these, 6 pairs of SNPs were revealed in European trios and 1 pair in Asian trios. Moreover, subgroup analyses revealed another 9 pairs of interactions between *SPRY2* and *SPRY4*. Four pairs of SNPs in Europeans trios and 5 pairs of SNPs in Asians trios showed statistically significant interactions between *SPRY2* and *SPRY4*. Despite none of these SNP-SNP interactions showing significance simultaneously in the overall analysis and subgroup analyses, multiple signals involving *SPRY1-SPRY2* and *SPRY2-SPRY4* altogether demonstrated that gene-gene interactions among *SPRY* genes potentially influence the risk of NSCL/P.

In the current study, several SNPs showed significant interactions with >1 SNP in another gene. For example, rs3860074

(*SPRY1*) simultaneously interacted with rs11149181, rs12854744, rs11618325, and rs1358977 in *SPRY2*, which might further provide evidence that genetic segments in *SPRY1* and *SPRY2* could interact with each other. A similar situation was also seen between *SPRY2* and *SPRY4*. The SNPs involved in the gene-gene interactions in this study were mostly intron variants or located in intergenic regions. However, it was suggested that noncoding variants could regulate the expression of coding regions and play important roles in human traits and complex diseases (Zhang and Lupski 2015). In addition, 1 of the SNPs for gene-gene interaction, rs1358977 (*SPRY2*), belongs to non-coding transcript variants, which were recently considered functional genetic elements and risk factors of complex diseases (Kellis et al. 2014; Lai et al. 2015; Saghaeian Jazi et al. 2016). Thus, the noncoding SNPs identified by our interaction analysis may indirectly influence the transcription as well as the protein products. To note, proteins encoded by *SPRY* genes share a highly conserved ~110-residue cysteine-rich sequence in the C-terminal half, which is responsible for targeting the protein to cell membrane (Hacohen et al. 1998). The competition among *SPRY* proteins can be inferred according to the similarity of their binding domain membrane. If simultaneous variations of 2 *SPRY* genes led to abnormal affinity of their products to the membrane, the competitive inhibition could happen and then result in a nonadditive inhibitory effect of *SPRY* on downstream pathways, since the inhibitory effect differs among isoforms (Lao et al. 2006). *SPRY2* protein was found to have a distinctly stronger inhibition effect on

downstream pathways than other isoforms (Lao et al. 2006). This special character of *SPRY2* might be a possible explanation about why only variants in *SPRY2* showed interactions with *SPRY1* and *SPRY4* in this study, but none of variants in *SPRY1* and *SPRY4* showed interactions. Nevertheless, the biological mechanisms of how *SPRY* genes interacting with one another in the development of NSCL/P remain unclear. Further biological investigations are required to verify our findings.

Ludwig et al. (2012) conducted a meta-analysis of GWASs and reported a signal in *SPRY2*, rs8001641, located at chromosome 13:80118676. Jia et al. (2015) and Moreno Uribe et al. (2017) then replicated this signal among European populations yet failed to replicate it among Asian populations (Jia et al. 2015). Yu et al. (2017) reported rs9545308 in *SPRY2* to be associated with NSCL/P among Chinese people, as well as a novel signal in *SPRY1* (rs908822). These studies implied that *SPRY* genes potentially participated in the etiology of NSCL/P. However, the current study failed to replicate previous signals in *SPRY* genes (data not shown), which may have resulted from a limited sample size and an insufficient power.

Our study identified that gene-gene interactions among *SPRY* genes were associated with the risk of NSCL/P. This study enriched the evidence for the role of *SPRY* genes in NSCL/P and highlighted the importance of exploring gene-gene interactions in etiologic studies. Further studies are required to verify the findings of the current study, and the underlying mechanisms of these interactions need to be explored by more fundamental studies.

Author Contributions

R. Zhou, contributed to conception, design, data analysis, and interpretation, drafted and critically revised manuscript; M. Wang, contributed to conception and data analysis, critically revised the manuscript; W. Li, S. Wang, Z. Zhou, J. Li, contributed to data interpretation, critically revised the manuscript; T. Wu, T.H. Beaty, contributed to conception, design, and data acquisition, critically revised the manuscript; H. Zhu, contributed to conception, design, and data interpretation, critically revised manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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