PVRL1 as a Candidate Gene for Nonsyndromic Cleft Lip With or Without Cleft Palate: No Evidence for the Involvement of Common or Rare Variants in Southern Han Chinese Patients

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The poliovirus receptor related-1 (*PVRL1*) gene encodes nectin-1, a cell–cell adhesion molecule (OMIM #600644), and is mutated in the cleft lip with or without cleft palate/ectodermal dysplasia-1 syndrome (CLPED1, OMIM #225000). In addition, *PVRL1* mutations have been associated with nonsyndromic cleft lip with or without a cleft palate (NSCL/P) in studies of multiethnic samples. To investigate the possible involvement of this gene in southern Han Chinese NSCL/P patients, we performed (i) a case–control association study, and (ii) a resequencing study. A set of 470 patients with NSCL/P and 693 controls were recruited, and a total of 45 tagging single-nucleotide polymorphisms (SNPs) were genotyped by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. In the resequencing study, the coding regions of the *PVRL1* α isoform were direct sequenced in 45 trios from multiply affected families. One (rs7128327) of the 45 tested SNPs showed a trend toward statistical significance in the genotypic-level chi-square test ($p=0.009567$). However, this result did not withstand correction for multiple testing. Likewise, sliding window haplotype analyses consisting of two, three, or four SNPs failed to detect any positive association. Resequencing analysis also failed to identify any novel rare sequence variants. In conclusion, the present study provided no support for the hypothesis that common or rare variants in *PVRL1* play a significant role in NSCL/P development in the southern Han Chinese population. This is the first study that has used tagging SNPs covering all the coding and noncoding regions to search for common NSCL/P-associated mutations of *PVRL1*.

Introduction

CLEFT LIP WITH or without a cleft palate (CL/P) is among the most common birth defects worldwide, and has been reported to affect 0.4 to 2.0 per 1000 infants born alive (Schutte and Murray, 1999). Approximately 70% of all CL/P cases occur as isolated, sporadic birth defects, known as nonsyndromic CL/P (NSCL/P), while the remaining 30% occur as a part of more than 300 different syndromes with the Mendelian inheritance pattern, in which CL/P is only one manifestation (Spritz, 2001). Many of the genes responsible for the Mendelian clefting syndromes have been identified, and research has implicated several of them in NSCL/P as well. These general CL/P predisposing genes include

IRF6, *p63*, *MSX1*, and *PVRL1* (Lidral *et al.*, 1998; Sozen *et al.*, 2001; Zuccherro *et al.*, 2004; Leoyklang *et al.*, 2006).

The poliovirus receptor related-1 (*PVRL1*) gene encodes nectin-1, a cell–cell adhesion molecule (OMIM #600644). Studies in mice have revealed that the mRNA of *PVRL1* is highly expressed in the medial edge epithelium of the developing palate (Suzuki *et al.*, 2000), suggesting that normal *PVRL1* function is involved in fusion of the palatal shelves during palatogenesis (Cobourne, 2004). However, the precise role of this gene in normal human development and the onset of CL/P remains to be defined.

Suzuki *et al.* (2000) published the first evidence that *PVRL1* mutations, especially the W185X homozygous loss-of-function coding mutation, result in a rare autosomal recessive

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syndrome CL/P-ectodermal dysplasia-1 (CLPED1, OMIM #225000). A follow-up study from the same research team identified heterozygosity of the nonsense W185X mutation as a genetic risk factor of NSCL/P in a northern Venezuelan population (Sozen *et al.*, 2001). These findings established *PVRL1* as a promising candidate gene of NSCL/P, and several subsequent investigations have been conducted on the coding regions of *PVRL1* (Item *et al.*, 2003; Scapoli *et al.*, 2004, 2006; Turhani *et al.*, 2005; Avila *et al.*, 2006; Ichikawa *et al.*, 2006; Tseng *et al.*, 2006; Sozen *et al.*, 2009; Zhao *et al.*, 2009; Shu *et al.*, 2011). Nevertheless, results from different ethnic groups have been conflicting (the findings of these studies are summarized in Table 1).

We recently analyzed a set of 100 Han Chinese NSCL/P patients to detect the presence of *PVRL1* missense mutations previously identified in Filipino NSCL/P cases (G361V in exon 6 of the β isoform [rs7940667], and S112T and T131A in exon 2 of the α isoform) (Avila *et al.*, 2006) and in Thai patients (V395M in exon 6 of the α isoform [rs141253617]) (Tongkobpetch *et al.*, 2008), and to further resequence the affected exons (2 and 5 of the α isoform). Surprisingly, neither of these mutations nor novel variants were detected in our Chinese study population (Shu *et al.*, 2011). These data demonstrated the complex etiology of NSCL/P, which may involve population-specific genetic risk factors.

The majority of NSCL/P-associated *PVRL1* investigations to date have been designed in an attempt to identify rare mutations that map directly to the coding regions. Such a strategy is based on the "rare variants-common diseases hypothesis," which proposes that a significant proportion of the inherited susceptibility to common human diseases may be due to the summation of the effects of a series of low-frequency dominantly and independently acting variants of a variety of different genes, each conferring a moderate but readily detectable increase in relative risk. The problem is that such rare variants will mostly be population specific because of founder effects resulting from genetic drift

(Bodmer and Bonilla, 2008). As a result, investigations of different ethnic groups will yield distinctive, and possibly conflicting, data. For example, although the *PVRL1* W185X nonsense mutation was identified as a genetic risk factor of NSCL/P in the northern Venezuelan population (Sozen *et al.*, 2001), a follow-up study conducted in Italians (Scapoli *et al.*, 2004), Iowans, Filipinos (Avila *et al.*, 2006), and Taiwanese (Tseng *et al.*, 2006) failed to replicate this association. Thus, an alternative hypothesis, named "common variants-common diseases," which considers common variants as tags of true disease loci, may be more suitable for assessing the common etiology of NSCL/P through populations. Therefore, we applied two strategies that perform a comprehensive gene association analysis of *PVRL1* in Han Chinese NSCL/P patients. First, we used a case-control study design that tests for a possible association with common variants using a set of 45 tagging single-nucleotide polymorphisms (SNPs), which overlapped ~50 kb at the 5'-end to 50 kb at the 3'-end of *PVRL1*. Second, we resequenced trios of affected child-normal parents with a positive NSCL/P family history to search for rare high-penetrance mutations. Using these approaches, both the rare- and common- hypotheses were tested in order to provide a more comprehensive view of the association between *PVRL1* and NSCL/P. To our knowledge, this is the first study of *PVRL1* to have used relatively more tagging SNPs covering all the coding regions along with promoters, introns, regulatory sequences, and splice sites, to search for common NSCL/P-associated mutations.

Materials and Methods

Samples

Study participants were recruited between 2008 and 2011 from the Second Affiliated Hospital of Shantou University Medical College. Enrollment was based on southern Han Chinese ethnicity (self-identification) and findings from a physical exam by a skilled plastic surgery team. Cases with congenital

TABLE 1. PUBLISHED *PVRL1* MUTATIONS IN NONSYNDROMIC CLEFT LIP WITH OR WITHOUT CLEFT PALATE PATIENTS FROM VARIOUS POPULATIONS

Publication year	Population (References)	Method used	Associated markers
2001	Cumana region (Venezuela) (Sozen <i>et al.</i> , 2001)	Sequencing of exon 3	W185X
2003	Austrian (Item <i>et al.</i> , 2003)	Sequencing of exons 1–6	442insE
2004	Italian (Scapoli <i>et al.</i> , 2004)	Genotyping the W185X mutation	None
2006	Iowan, Danish, and Filipino (Avila <i>et al.</i> , 2006)	Sequencing of entire coding regions	S112T, T131A ^a
2006	Japanese (Ichikawa <i>et al.</i> , 2006)	Genotyping three SNPs in/around <i>PVRL1</i>	G361V (rs7940667)
2006	Guatemalan (Neiswanger <i>et al.</i> , 2006)	Genotyping nine markers in/around <i>PVRL1</i>	None
2006	Italian (Scapoli <i>et al.</i> , 2006)	Genotyping two SNPs in/around <i>PVRL1</i>	rs3829260
2006	Taiwanese (Tseng <i>et al.</i> , 2006)	Sequencing of exons 1–6	R199Q, R210H, R212H
2008	Thai (Tongkobpetch <i>et al.</i> , 2008)	Sequencing of exons 3 and 5	None
2009	North America and Australian (Sozen <i>et al.</i> , 2009)	Sequencing of exons 1–6	V395M (rs141253617)
2009	Han Chinese (Zhao <i>et al.</i> , 2009)	Sequencing of entire coding regions	442insE, S447L ^a
2011	Han Chinese (Shu <i>et al.</i> , 2011)	Sequencing of exon 3	None
		Sequencing of exons 2 and 5	None
		Genotyping four SNPs in/around <i>PVRL1</i>	None

^aPrimary associated markers among a larger published set. SNP, single-nucleotide polymorphism.

TABLE 2. CHARACTERISTICS OF NONSYNDROMIC CLEFT LIP WITH OR WITHOUT CLEFT PALATE CASES AND CONTROLS/AFFECTED-PARENT TRIOS

<i>Case-control study (individuals)</i>			
	<i>Cases</i>	<i>Controls</i>	<i>p^a</i>
Gender			
Male	269	395	0.92
Female	201	298	
Sub-groups			
NSCL/P	385		
NSCLO	85		
NSCL/P total	470	693	
<i>Sequencing study (trios)</i>			
Gender			
Male	32		
Female	13		
Sub-groups			
NSCLP	40		
NSCLO	5		
NSCL/P total	45		

^aby 2×2 table χ^2 test.

NSCLP, nonsyndromic cleft lip with palate; NSCLO, nonsyndromic cleft lip only; NSCL/P, nonsyndromic cleft lip with/without cleft palate.

anomalies or developmental delays that could reflect a recognized malformation syndrome other than NSCL/P were excluded. Written informed consent was obtained from all participants or their guardians. In total, the study consisted of 470 patients with NSCL/P (age range: 1–45 years), 693 healthy blood donors (age range: 19–55 years) with a negative NSCL/P family history, and 45 index patients (with a positive family history) taken from the case group and both their parents. Study population characteristics are summarized in Table 2.

The protocol of this study was designed in compliance with the principles of the Declaration of Helsinki, and the study was approved by the Clinical Research Ethics Committee of the Shantou University Medical College.

Markers selection and genotyping

Forty-four tagging SNPs located in and around the *PVRL1* locus were selected using the tagger algorithm of the Haploview software version 4.1 (pair-wise tagging parameters: $r^2 \geq 0.8$; minor allele frequency [MAF] > 10%) and data from the HapMap Han Chinese of Beijing (HCB) release 24. The HapMap database included 155 SNPs with an MAF of > 0.1 in the HCB sample that mapped to the region 118,959,332–119,159,331 on chromosome 11 (from ~50 kb at the 5'-end to 50 kb at the 3'-end of *PVRL1*). All the 155 SNPs were completely captured by our tagging set of SNPs, with a mean r^2 of 0.957. Furthermore, rs3829260, which had been previously reported as being associated with nonsyndromic oral clefting in Guatemalans (Neiswanger *et al.*, 2006), was forced to be included.

Venous blood samples were drawn from participants, and DNA was extracted using a TIANamp Blood DNA Kit (Tiangen Biotech Co., Ltd.) according to the manufacturer's protocol. All genotyping experiments were performed by the Shanghai Benegene Biotechnology Co., Ltd. using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Sequenom, Inc.). To validate genotyping

identification, 10% of the samples were randomly repeated, and the results were found to be 100% concordant.

Of the total 45 SNPs included, we found that the mass signals of two were very low, and we could not acquire accurate genotype data; thus, these 2 SNPs were excluded from analysis. In addition, four other SNPs were excluded due to low genotyping performance (call rate < 95%). Finally, rs2368936 failed during the primer design stage, and was replaced with another SNP, rs4938718, which was in strong linkage disequilibrium (both D' and r^2 equaled 1; Hapmap release 24).

Sequencing

Polymerase chain reaction (PCR) amplification of the α isoform of *PVRL1* (GenBank accession number NM_002855), which includes exons 1–6, was performed on the 45 trios using primer sequences described by Tongkobpetch *et al.* (2008). Cycle sequencing was carried out by Shanghai Benegene Biotechnology Co., Ltd. using the ABI Prism™ BigDye™ Terminator Cycle Sequencing kit and the ABI Prism 3730 capillary sequencer (Applied Biosystems).

Statistical analysis

A total of 39 SNPs met the quality criteria and were carried through to the statistical analysis stage. The Hardy–Weinberg equilibrium (HWE) test was performed in the control group. The standard chi-square (χ^2) test and logistic regression analysis assuming an additive effect (dose-dependent genetic effect) of SNPs were carried out to compare allelic distributions between cases and controls. Further genotypic χ^2 testing with two degrees of freedom was utilized to assess the association of each genotype. Sliding window haplotypes consisting of two, three, and four SNPs were also tested. Bonferroni correction was used to adjust the results of multiple tests. A p -value of < 0.0012 (0.05/39 $\approx$ 0.0012) was regarded as being statistically significant. All the statistical analyses were carried out with Plink software (version 1.07) (Purcell *et al.*, 2007).

The power (for $\alpha = 0.05/39 \approx 0.0012$) of the present study was calculated for a marker with an MAF of 0.2 (de Assis *et al.*, 2011) and an additive effect, which has been observed for all proven NSCL/P loci to date (Birnbau *et al.*, 2009; Beaty *et al.*, 2010; Mangold *et al.*, 2010). The Quanto statistical software package version 1.2.4 (Gauderman, 2002) was used to calculate statistical power.

Results

All SNPs were in HWE, except rs11217365 and rs7933848 (p -values of 0.03659 and 0.001725, respectively), which were excluded from subsequent statistical analysis (Table 3).

Two of the 39 tested SNPs, rs12797352 and rs4409845, had p -values that were near the significance threshold (p -values of 0.03339 and 0.0424, respectively) in the allelic test. Another two SNPs, rs7129848 and rs7128327, had slightly lower p -values (0.0176 and 0.009567, respectively) in the genotypic test. However, neither of these results withstood correction of multiple testing (Table 3). Likewise, the sliding window haplotype analyses of two, three, or four SNPs detected no association between the marker sets and NSCL/P (data not shown). The power to detect the association with a marker having a MAF of 0.2 was determined to be nearly 80% (by assuming a range of odds ratios [ORs] of 1.3–6.0).

TABLE 3. ASSOCIATION BETWEEN ALLELE/GENOTYPE DISTRIBUTION WITH NONSYNDROMIC CLEFT LIP WITH OR WITHOUT CLEFT PALATE IN THE CASE-CONTROL STUDY

SNP	BP	p _{HWE}	A1	A2	AFF	UNAFF	F _A	F _U	p _{chi}	p _{add}	p _{geno}
rs10892411	118963038	0.70400	C	G	108/230/131	154/351/188	0.4755	0.4755	0.9996	0.9996	0.8646
rs2271249	118984601	0.86970	C	T	83/217/170	92/322/270	0.4074	0.3699	0.0685	0.0707	0.1287
rs4354701	118994022	0.17130	A	G	109/251/110	177/328/188	0.4989	0.4921	0.7449	0.7455	0.1212
rs4514434	118997484	0.70320	C	T	105/233/132	154/340/199	0.4713	0.4675	0.8591	0.8598	0.9725
rs10790324	119003687	1.00000	A	G	9/124/337	17/187/489	0.1511	0.1595	0.5844	0.5816	0.7978
rs4582984	119004224	0.88590	C	A	10/125/335	16/186/491	0.1543	0.1573	0.8433	0.8421	0.9731
rs11217365 ^a	119006295	0.03659	C	T	88/232/147	146/311/229	0.4368	0.4395	—	—	—
rs12291064	119007913	0.09289	T	C	41/157/272	55/249/389	0.2543	0.2590	0.7964	0.8045	0.6469
rs12273351	119008612	0.05172	T	G	91/206/173	136/313/244	0.4128	0.4221	0.6550	0.6677	0.8514
rs12279718	119008880	0.16390	A	C	94/212/164	138/322/233	0.4255	0.4315	0.7769	0.7834	0.8841
rs4412764	119013135	0.28630	A	G	35/138/261	43/233/396	0.2396	0.2374	0.9022	0.9057	0.4164
rs1022081	119014956	0.76330	G	C	29/175/266	46/259/388	0.2479	0.2532	0.7693	0.7701	0.9442
rs12797352	119023216	0.58190	G	A	63/215/190	119/327/246	0.3643	0.4082	0.0334	0.0353	0.1065
rs7103685	119028391	0.55400	C	T	72/182/208	84/302/299	0.3528	0.3431	0.6305	0.6417	0.1498
rs11217383	119042234	0.31470	T	C	3/105/356	15/150/515	0.1196	0.1324	0.3691	0.3684	0.1143
rs10790329	119042893	0.56550	C	T	39/174/257	48/282/363	0.2681	0.2727	0.8047	0.8054	0.3782
rs4459318	119046988	1.00000	T	C	98/231/133	153/334/183	0.4621	0.4776	0.4680	0.4677	0.7636
rs906830	119055035	0.49200	T	C	104/219/136	150/354/186	0.4651	0.4739	0.6799	0.6798	0.4674
rs7933848 ^a	119066572	0.00173	C	T	95/198/177	139/296/258	0.4128	0.4141	—	—	—
rs11217408	119069693	0.59040	A	C	12/110/348	8/150/535	0.1426	0.1198	0.1077	0.1091	0.1393
rs4938711	119071394	0.68970	C	T	61/226/181	108/324/260	0.3718	0.3902	0.3716	0.3709	0.4759
rs7129848	119071438	0.06259	T	C	29/145/296	21/244/428	0.216	0.2063	0.5768	0.5755	0.0176
rs4938713	119074204	1.00000	T	C	42/170/258	58/285/345	0.2702	0.2914	0.2657	0.2733	0.1965
rs4938717	119082121	0.75990	T	C	99/216/155	151/340/202	0.4404	0.4632	0.2789	0.2875	0.3731
rs12577411	119083752	0.87660	A	G	17/109/344	13/170/510	0.1521	0.1414	0.4724	0.4805	0.1732
rs4409845	119088042	0.09679	G	A	42/200/221	57/249/372	0.3067	0.2677	0.0424	0.0460	0.0561
rs11217426	119096088	0.08417	T	C	36/154/280	52/242/399	0.2404	0.2496	0.6126	0.6264	0.7473
rs3829260	119103939	0.81860	C	G	102/231/136	159/344/179	0.4638	0.4853	0.3083	0.3081	0.5635
rs4938718	119106707	0.76120	C	G	109/219/142	168/342/183	0.4649	0.4892	0.2500	0.2580	0.3638
rs10892444	119110064	0.22060	G	A	104/223/136	167/325/191	0.4654	0.4824	0.4242	0.4337	0.7143
rs10892445	119113143	0.16760	G	A	73/213/184	105/307/281	0.3819	0.3730	0.6638	0.6711	0.8920
rs11217460	119127372	0.75640	G	C	81/229/160	128/335/230	0.4160	0.4264	0.6166	0.6177	0.8575
rs4317995	119127991	0.35480	A	G	95/223/152	139/329/225	0.4394	0.4380	0.9464	0.9473	0.9976
rs11217466	119130203	0.08772	A	T	83/224/154	149/313/216	0.4230	0.4506	0.1927	0.2024	0.2632
rs7128327	119136491	0.48090	G	A	31/127/312	25/231/437	0.2011	0.2027	0.9212	0.9229	0.0096
rs10892453	119141577	0.92160	T	C	29/180/261	48/266/379	0.2532	0.2612	0.6655	0.6653	0.8700
rs9633947	119149570	0.55010	A	G	11/126/326	13/180/490	0.1598	0.1508	0.5575	0.5532	0.8028
rs11217496	119154166	0.68620	T	G	79/224/167	96/332/265	0.4064	0.3781	0.1693	0.1685	0.3358
rs11217503	119158057	0.68690	G	T	29/176/265	46/257/390	0.2489	0.2518	0.8756	0.8761	0.9487

Bold font indicates significant p -values ($p < 0.05$ of the HWE test; $p < 0.00012$ of the associated test).

^ars11217365 and rs7933848 were excluded from analysis based on deviation of HWE.

BP, base pair location based on Hapmap data release 24; p_{HWE} , p -value by HWE test; A1, minor allele name (based on whole sample); A2, major allele name; AFF, genotypes in cases, minor allele homozygous counts/heterozygous counts/major allele homozygous counts; UNAFF, genotypes in controls, minor allele homozygous counts/heterozygous counts/major allele homozygous counts; F_A, frequency of this allele in cases; F_U, frequency of this allele in controls; p_{chi} , p -value by basic allelic chi-square test (1 degree of freedom); p_{add} , p -value for t -statistic by conditional logistic regression model assuming an additive effects of minor allele dosage; p_{geno} , p -value by genotypic χ^2 test (2 degrees of freedom); HWE, Hardy-Weinberg equilibrium.

The entire coding sequence of the *PVRL1* α isoform was analyzed by PCR and direct sequencing for the entire set of 45 trios. We failed to detect any novel rare sequence variants in this set of study participants. In all trios, however, the known SNPs that were detected were homozygous for the common alleles (data not shown).

Discussion

In the present study, 470 patients with NSCL/P and 693 healthy controls were evaluated to verify whether common mutations of the *PVRL1* gene constitute a genetic risk for NSCL/P within the southern Han Chinese population. Al-

though no strong evidence of an associated marker was identified in our current dataset, one SNP (rs7128327, located 30 kb upstream of *PVRL1*) showed a trend toward statistical significance (p -value of 0.009567 by the χ^2 test at the genotypic level, but the adjusted p -value was not significant at $0.009567 \times 39 \approx 0.37$). The interpretation of this type of nominal significance should be carried out very carefully. First, it is possible that the marker may be etiologic or tagging another true etiologic locus; in this way, the current sample size may be too small to generate a strong enough p -value that can withstand multiple-testing correction. To evaluate this presumption, we determined that the present study has 80% power to detect a risk marker with an effect

size comparable to those of previous genome-wide association studies (GWASs) (OR of the previously identified NSCL/P-associated SNPs ranged from 1.3 to 6.0) (Birnbaum *et al.*, 2009; Beaty *et al.*, 2010; Mangold *et al.*, 2010). This suggests that some other markers may exist with moderate effects that are below the OR range of 1.3–6.0; such markers would be undetectable or not significant enough based on our sample size. A second possibility is that false positives may have been generated from the population stratification. To accept this presumption, it is first necessary to determine whether a pre-existing population stratification was present in the area from where the samples were obtained. Although enrollment in the present study was based on a seemingly homozygous genetic background of the southern Han ethnicity, evidence of sub-population structures were recently reported. Chen *et al.* (2009) demonstrated that distinct sub-populations existed within the Guangdong Province, the region from which subjects had been obtained in the present study. Therefore, in the absence of accurate methods that control the potential confounding of such intricate sub-population structures (such as principal component analysis or the transmission disequilibrium test used in the trio study), the population stratification may have biased our study's results. The third possibility to explain the nominal significance observed in this study relates to possible errors in the genotyping assay or enrollments; these types of confounding factors are not unexpected, given the large number of markers tested (de Assis *et al.*, 2011). Considering our data collectively and conservatively, we, thus, speculate that the SNP rs7128327 may not have a significant influence on NSCL/P susceptibility in patients from the Guangdong Province of China, but further studies with methods that control potential confounding effects are warranted.

Thus, the hypothesis that common variants in *PVRL1* play a significant role in the development of NSCL/P in the Han Chinese population was disproven. Interestingly, a recent GWAS aimed at searching for common NSCL/P susceptibility genes scanned a mixture of genomic samples of Asian origin (including Chinese samples), but failed to identify any genome-wide associated signal for the chromosomal 11q23 region harboring the *PVRL1* gene (Beaty *et al.*, 2010). This finding agrees with the negative results of our targeted investigation of the Han Chinese population.

It is possible that rare, private variants with a relatively high penetrance that map within *PVRL1* may be the causative mutation of NSCL/P in the Han Chinese populations (i.e., the rare variants-common diseases hypothesis). However, in our evaluation of exons 1–6 of the *PVRL1* α isoform in 45 trios of southern Han Chinese descent, no novel or previously associated mutations were identified. This finding was consistent with results from previous studies in Japanese (Ichikawa *et al.*, 2006), Taiwanese (Tseng *et al.*, 2006), and Chinese populations (Zhao *et al.*, 2009; Shu *et al.*, 2011). However, other studies of NSCL/P patients from Filipino (Avila *et al.*, 2006) and Thai (Tongkobpetch *et al.*, 2008) Asian origins identified several rare causative mutations. Given the complicated heterogeneous nature of NSCL/P and the number of potentially confounding factors (Carinci *et al.*, 2003), this level of discordant results among various ethnicities of a larger race classification is not unexpected (Scapoli *et al.*, 2005). For example, our research team previously investigated an NSCL/P SNP, rs987525 of 8q24, which had

been identified as being strongly associated in Caucasians, but we did not detect any such association in our Han Chinese dataset (Xu *et al.*, 2011). Thus, our study's findings further emphasize the importance of recognizing pathogenetic differences between ethnic groups to better understand the NSCL/P disease process and develop more effective targeted molecular therapies.

In conclusion, the present study found no support for the hypothesis that common or rare variants in *PVRL1* play a significant role in the development of NSCL/P in the southern Han Chinese population. However, some limitations exist in the present study that should be considered when interpreting our findings. First, this was a hospital-based association study, and selection bias might exist, owing to the potential population stratification just discussed. Future studies of genetic association with NSCL/P will likely benefit from including components such as case-parents trio study to improve the sensitivity of their analysis and accuracy of their results. Second, a small number of affected child-parent trios ($n=45$) were evaluated in the resequencing phase of our study, and only the α spliced isoform of the *PVRL1* gene mRNA was assayed. The α isoform was selected as the exclusive focus, as it encodes the cell-surface transmembrane receptor nectin-1, which mediates the cell-cell adhesion function. Thus, the presence of rare high penetrance mutations in other familial cases or other spliced isoforms (β and γ) of *PVRL1* cannot be absolutely excluded on the basis of our results.

Conclusion

The present study provided no support for the hypothesis that common or rare variants in *PVRL1* play a significant role in NSCL/P development in the southern Han Chinese population.

Acknowledgments

The authors are grateful to all the participants who donated their time and samples for this study. They would also like to express their gratitude to Dr. Jennifer C. van Velkinburgh for help in revising the article, to Drs. Yunpu He and Tingying Liu for sample and data collection, and to the Shanghai Benegene Biotechnology Co., Ltd. (China) for performing the genotyping and sequencing experiment. They thank Medjaden Bioscience Limited for assisting in the preparation of this article. This study was supported by a grant from the National Science Foundation for Young Scientists of China (No. 81001284).

Disclosure Statement

The authors declare that they have no competing financial interests.

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Received for publication December 1, 2011; received in revised form February 25, 2012; accepted February 26, 2012.