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Original Article

Exon sequencing reveals that missense mutation of *PBX1* gene may increase the risk of non-syndromic cleft lip/palate

Jian Ma[1,2*], Bin Yin[1*], Jia-Yu Shi[3], Yan-Song Lin[1], Shi-Jun Duan[1], Bing Shi[1], Zhong-Lin Jia[1]

[1]*State Key Laboratory of Oral Diseases & National Clinical Research Center for Oral Diseases & Development of Cleft Lip and Palate, West China Hospital of Stomatology, Sichuan University, P.R. China;* [2]*Department of Oral and Maxillofacial Surgery, Hospital of Stomatology, The General Hospital of Ningxia Medical University, Ningxia Province, Yinchuan, 750004, P.R. China;* [3]*Division of Growth and Development and Section of Orthodontics, School of Dentistry, University of California, Los Angeles, USA.* [*]*Equal contributors.*

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Abstract: Objective: Non-syndromic oral cleft (NSOC) is one of the most common multifactorial birth defects. A previous animal study showed *PBX1* gene knockout mice consequently exhibited complete cleft lip/palate (CL/P). However, little is known about the association between *PBX1* and NSOC in humans. This study investigated the role of the *PBX1* gene in NSOC in the Han Chinese population. Methods: In all, 287 NSOCs were recruited for this study. First, exons in the *PBX1* gene were sequenced among 50 non-syndromic cleft lip and palate cases to screen for variations by the Sanger sequencing method. Then, we selected four SNPs to replicate among 237 NSOC trios and analyzed the data by using TDT and parent of origin effect methods. Results: Exon sequencing identified six variants of the *PBX1* gene. Among them, four variants were common variants. TDT analysis revealed allele G at rs2275558 and allele T at rs3835581 were over-transmitted in NSCL/P (P=0.039 and 0.038, respectively), which could increase the risk for NSCL/P. Parent of origin effect analysis indicated that allele G at rs2275558 was paternally over-transmitted for NSCL/P (P=0.0091). Conclusion: This is the first report that the *PBX1* gene is associated with NSCL/P, which indicates that it is a promising candidate gene for NSCL/P.

Keywords: Non-syndromic oral cleft, Sanger sequencing, single nucleotide polymorphism, *PBX1*

Introduction

Non-syndromic oral cleft (NSOC) is one of the most common birth defects and exerts a heavy economic burden on families and society [1]. Birth prevalence rates of NSOC are very different across populations and geographic locations. In general, China is considered one of the high incidence countries with a rate of about 1.67 per thousand according to the latest epidemiological survey [2]. NSOCs are generally divided into non-syndromic cleft palate (NSCP), and non-syndromic cleft lip with or without cleft palate (NSCL/P). As a multifactorial disease, a complex etiology including genetic and environmental factors influences the risk of NSOC. With the wide application of high throughput sequencing technology, increasing numbers of susceptibility genes have been discovered by several large genome-wide association studies (GWASs), but these can only explain a small part of the genetic effect. Furthermore, the heterogeneity of phenotypes and uncertain inheritance patterns further complicate the role of genetics in NSOC. Attractive GWAS findings from nine GWASs for CL/P [3-11], two GWASs for CPO [12, 13], and two GWAS meta-analyses [14, 15] have advanced our understanding of development of NSOC and provided new perspectives for future research. However, among several susceptibility genes found by GWASs that were statistically significant, the true biologic function is unknown. Multiple SNPs in *ABCA4* achieved genome-wide significance, but the subsequent whole mount in situ hybridization analysis of *ABCA4* and immunodetection of expressed *ABCA4* carried out in mice indicated that *ABCA4* was not positively expressed in the

Table 1. Characteristic of NSOCs patients

	NSCL/P	NSCP	NSOCs
Severity			
Complete cleft	137	43	180
Incomplete cleft	82	25	107
Sex			
Male	145	32	177
Female	74	36	110

Note: NSCL/P, Non-syndromic cleft lip with or without palate; NSCP, Non-syndromic cleft palate; NSOCs, Non-syndromic Oral clefts (NSCL/P&NSCP).

palate [3]. Before the GWAS era, research on candidate genes based on animal models was the main method to find novel disease-causing genes. Although the process was time-consuming, it did find some true CL/P susceptibility genes that were confirmed in subsequent GWAS studies, such as *IRF6* [4, 16] and *TP63* [15, 17]. Therefore, association studies between CL/P susceptibility genes and the population based on animal models are more convincing.

PBX1 (Pre-B cell leukemia transcription homeobox 1), a member of *PBX* gene family, encodes TALE homeodomain-containing transcription factors (TF) [18]. The current research on *PBX1* mainly focused on its role in metabolic abnormalities, cancer, and morphogenesis of the kidney and urinary tract [19-21]. Additionally, it was reported that loss of *PBX* genes (*PBX1*, *PBX2* and *PBX3*) in mice may lead to CL/P phenotype [22]. Especially, *PBX1* plays a more significant role in the formation of fully penetrant CL/P in comparison to the other two genes (*PBX2* and *PBX3*) [23]. Another functional study indicated that *PBX* proteins could regulate the expressions of *WNT, P63* and *IRF6,* subsequently control apoptosis, and finally affect the development of the midface [22]. Documented evidence showed that mutations of *WNT*, *P63*, and *IRF6*, downstream targets of *PBX1*, were associated with CL/P in both humans and mice [15, 24, 25]. Moreover, the *PBX1* gene interacts with the *ARHGAP29* gene which is a susceptibility gene for CL/P in Hispanic and non-Hispanic white (NHW) ethnicities [26]. Thus, we considered *PBX1* as a promising candidate gene for NSOC.

No population study on the associations between the *PBX1* gene and NSOC had been reported. The incidence of NSOC in the Western Han Chinese population is relatively high, and population mobility is quite low, making it is suitable for genetic research on NSOC. Thus, we first explored the association between the variations of *PBX1* gene and the occurrence of NSOC in a Han Chinese population in Western China.

Material and methods

Subjects

The samples included 237 NSOC cases, and 50 non-syndromic cleft lip and palate (NSCLP) cases (**Table 1**). All subjects were recruited between 2010 and 2013 from the Cleft Lip and Palate Surgery Department of the West China Hospital of Stomatology, Sichuan University. All patients recruited in this study were diagnosed with NSOC (without any other congenital malformation of the body or a family history of genetic disease) by a physician. All subjects were self-identified as Han Chinese and were asked about the history of oral clefts among their first- and second-degree relatives. Human subject study protocols were reviewed and approved by the institutional review board (IRB) of West China Hospital of Stomatology, Sichuan University in 2015 (WCHSIRB-D-2015-057). Informed consent was obtained from each participant prior to enrollment in the study.

DNA extraction and Sanger sequencing of exons in PBX1 gene

Genomic DNA was extracted from venous blood samples drawn from all participants by the phenol-chloroform extraction protocol. The exon sequences were downloaded from the UCSC database (http://genome.ucsc.edu/). The primers were designed to cover all nine exons of *PBX1* (**Table 2**). We performed PCR and sequencing by an ABI PRISM 3730 DNA Sequencer among fifty cases. The data were analyzed by the Sequence Scanner v1.0.

Genotyping

The four SNPs were genotyped by the ligase detection reaction method among 237 trios. We selected 10% of the samples at random to repeat the experiment. The genotypes were consistent with the previous ones.

Table 2. Exon sequencing primers of *PBX1* gene

Exon	Forward (5' to 3')	Reverse (5' to 3')	PCR Product (bp)
Exon 1	TGAAGACAAGCTTGAAGGATAAAA	GGCCGCTTTTGGATCAGT	600
Exon 2	TGCCACAGAGTTAGGGTTGG	ACAGTTTAGCACCCCCACAC	597
Exon 3	TTCTCTCTTTTTCCAGCCTTTC	GAATGCCTAGGTTTTTAACAGTTG	600
Exon 4	TTTGCACAAGTCTCTAGAAAAGC	AAGACGCAACTGTAAAAGAGGT	600
Exon 5	GCTTTTAGCGTTGGTTTTGG	ACACCTCACCCATTTGAAGC	596
Exon 6	TCGCATTTTATGTAGTTGTCCTTT	ATGCAAACCTCCAGACAACC	569
Exon 7	GTTCCCTTTCTTGGCTTGAA	ACTGAAAAGCCAGAGCCAAA	590
Exon 8	AGGGAAGAAAAATGGGGAGA	TGGCATGACCGATACAGAAA	585
Exon 9-1	CACTGGGAGGACCCAAACT	GAGGTTGAAGGGTTTCACGA	1213
Exon 9-2	TCACTCGAATCCCTCACTCC	CTGAGTGCTCCAGAGGTGGT	817
Exon 9-3	GGTCACTGACACAGAGAAGCA	TCCCCTGACTTCGCATTTAC	697
Exon 9-4	TTGGTGCCTCATTTTCTTCA	TCCAAGAGAACCCTTTTGTCTC	826
Exon 9-5	AAAGGCACTAGAAAGGTTGTGTC	AGAAATCCTGGGGTGCATCT	600

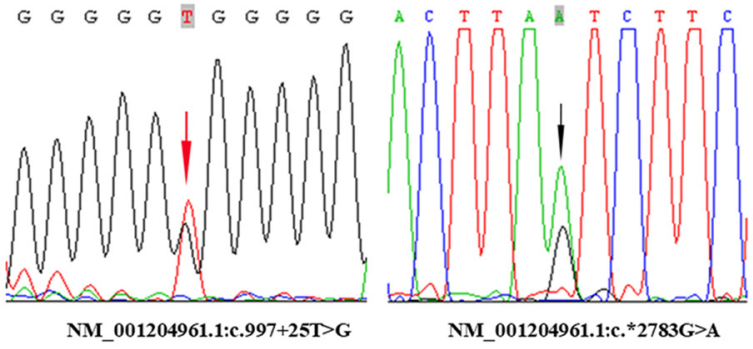

Figure 1. Sequence results of the two novel variants.

Table 3. *P*-values of Hardy-Weinberg equilibrium test in NSOC groups

SNP	Position (Hg19)	NSCL/P	NSCP	Control
rs2275558	164529120	0.36	1	0.46
rs3835581	164790567	1	0.35	0.9
rs41266618	164816415	1	1	0.74
rs3185695	164816956	0.75	0.83	0.073

Note: NSOCs, Non-syndromic oral clefts (NSCL/P&NSCP); SNP, Single Nucleotide Polymorphism; NSCL/P, Non-syndromic cleft lip with or without cleft palate; NSCP, Non-syndromic cleft palate.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) was assessed for all four SNPs among unaffected parents. TDT (transmission disequilibrium test) analysis and Pairwise LD (computed as both D' and r^2) for all SNPs were performed by the Haploview program. Parent-of-origin effect was assessed by PLINK to distinguish the parental preference of transmission.

Results

Exon sequencing of the *PBX1* gene identified six variants, including two novel variants (NM_001204961.1:c.997+25T>G and NM_001204961.1:c.*2783G>A) that are not listed in the public database (1000 Genome and ESP et al.), and four SNPs (rs2275558, rs3835581, rs41266618 and rs3185695). NM_001204961.1:c.997+25T>G, located in the intron of *PBX1* and NM_001204961.1:c.*2783G>A is in the 3' UTR of *PBX1* gene (**Figure 1**). This was detected only in two NSCLP cases.

To confirm whether *PBX1* gene is associated with NSOC, we selected four SNPs (rs2275558, rs3835581, rs41266618 and rs3185695) based on their minor allele frequency, and validated them among 237 complete trios of NSOC. All SNPs conformed to HWE among the unaffected parents (P>0.05) (**Table 3**).

Allelic TDT analysis on case-parent trios with heterozygous informative parents showed that allele G at rs2275558 and allele T at rs3835581 were over-transmitted for both of NSCL/P (P=0.039 and 0.038, respectively) and NSOC (P=0.036 and 0.014, respectively) (**Table 4**).

In view of the parental origin of the alleles, we performed parent-of-origin effect analysis to detect allelic transmission bias among parents.

Table 4. Allelic TDT Results for SNPs at *PBX1* among NSOCs Trios

SNP	Minor Allele	NSCL/P		NSCP		NSOCs	
		T/U	Chisq (*P*-value)	T/U	Chisq (*P*-value)	T/U	Chisq (*P*-value)
rs2275558	**G**	**99/72**	**4.26 (0.039)**	34/29	0.4 (0.53)	**133/101**	**4.38 (0.036)**
rs3835581	**T**	**104/77**	**4.03 (0.038)**	42/30	1.70 (0.19)	**146/107**	**6.01 (0.014)**
rs41266618	T	6/06	0 (1.04)	2/00	2 (0.16)	8/06	0.29 (0.59)
rs3185695	A	48/45	0.097 (0.76)	18/12	1.2 (0.27)	66/57	0.66 (0.42)

Note: SNP, Single Nucleotide Polymorphism; NSCL/P, Non-syndromic cleft lip with or without cleft palate; NSCP, Non-syndromic cleft palate; NSOCs, Non-syndromic oral clefts (NSCL/P&NSCP); T/U, transmitted/untransmitted; Chisq, Chi-Square; Bold characters indicate the items with *p*-value less than 0.05.

Table 5. Parent of origin effect of the SNPs at *PBX1* among NSOC trios

Cleft type	SNP	A1/A2	Paternal			Maternal			Z	P
			T/U	CHISQ	P	T/U	CHISQ	P		
NSCL/P	**rs2275558**	**G/A**	**58.5/33.5**	**6.79**	**0.0091**	38.5/39.5	0.013	0.91	1.86	0.063
	rs3835581	T/C	51.5/40.5	1.32	0.25	50.5/39.5	1.34	0.25	-0.018	0.99
	rs41266618	T/C	3.5/4.5	0.12	0.72	0.5/3.5	2.25	0.13	1.01	0.31
	rs3185695	A/G	28/22	0.72	0.4	20/24	0.36	0.55	1.02	0.31
NSCP	rs2275558	G/A	15/12	0.33	0.56	19/17	0.11	0.74	0.22	0.83
	rs3835581	T/C	16/16	0	1	24/15	2.08	0.15	-0.97	0.33
	rs41266618	T/C	1/00	1	0.32	1/00	1	0.32	NA	NA
	rs3185695	A/G	9/06	0.6	0.44	9/05	1.14	0.29	-0.24	0.81
NSOCs	**rs2275558**	**G/A**	**73.5/45.5**	**6.59**	**0.01**	57.5/56.5	0.009	0.93	1.74	0.082
	rs3835581	T/C	67.5/56.5	0.98	0.32	74.5/54.5	3.10	0.08	-0.53	0.60
	rs41266618	T/C	4.5/4.5	0	1	1.5/3.5	0.8	0.37	0.72	0.47
	rs3185695	A/G	37/28	1.25	0.26	29/29	0	1	0.77	0.44

Note: SNP, Single Nucleotide Polymorphism; A1, Minor allele; A2, Major allele; NSCL/P, Non-syndromic cleft lip with or without cleft palate; NSCP, Non-syndromic cleft palate; NSOC, Non-syndromic orofacial clefts (NSCL/P&NSCP); T/U, transmitted/untransmitted; CHISQ, Chi-Square; P, *p* value; Z, vector of the large sample Z statistic; Bold characters indicate the items with *p*-value less than 0.05.

No significant difference was observed for any subgroup of NSOC. Yet, allele G at rs2275558 did show a paternal over-transmission for NSCL/P (P=0.0091) and NSOC (P=0.01) (**Table 5**). There was no evidence of parental transmission bias in other SNPs.

To check whether the two associated SNPs travel together in the same LD block, we conducted pairwise linkage analysis, and the results showed very weak linkage between rs2275228 and rs3835581 (**Figure 2**), indicating that they were independent of each other.

Discussion

NSOC is a complex congenital malformation with strong heterogeneity [27]. Recently, genome wide association study (GWAS), the most effective technique, found more than 50 susceptibility loci for NSOC in distinct populations [28]. Although GWAS has made amazing achievements in the research of NSOC, unfortunately, most of the susceptibility loci found by various GWASs contributed minimally to the risk of the disease, explaining only about 10%-20% of the genetic effects [29]. Therefore, we have reason to believe that the current discoveries are just the tip of the iceberg, and that there are still many potential pathogenic genes not discovered.

The mechanism of NSOC is the failure of disappearance of embryonic epithelium from the frontonasal prominence (fnp) and paired maxillary prominence (mxp), ultimately leading to the obstruction of fusion in prominences. It was found that *PBX1* mRNA is extremely rich in epithelium, thus *PBX1* mutants may disrupt the normal process of epithelial disappearance by

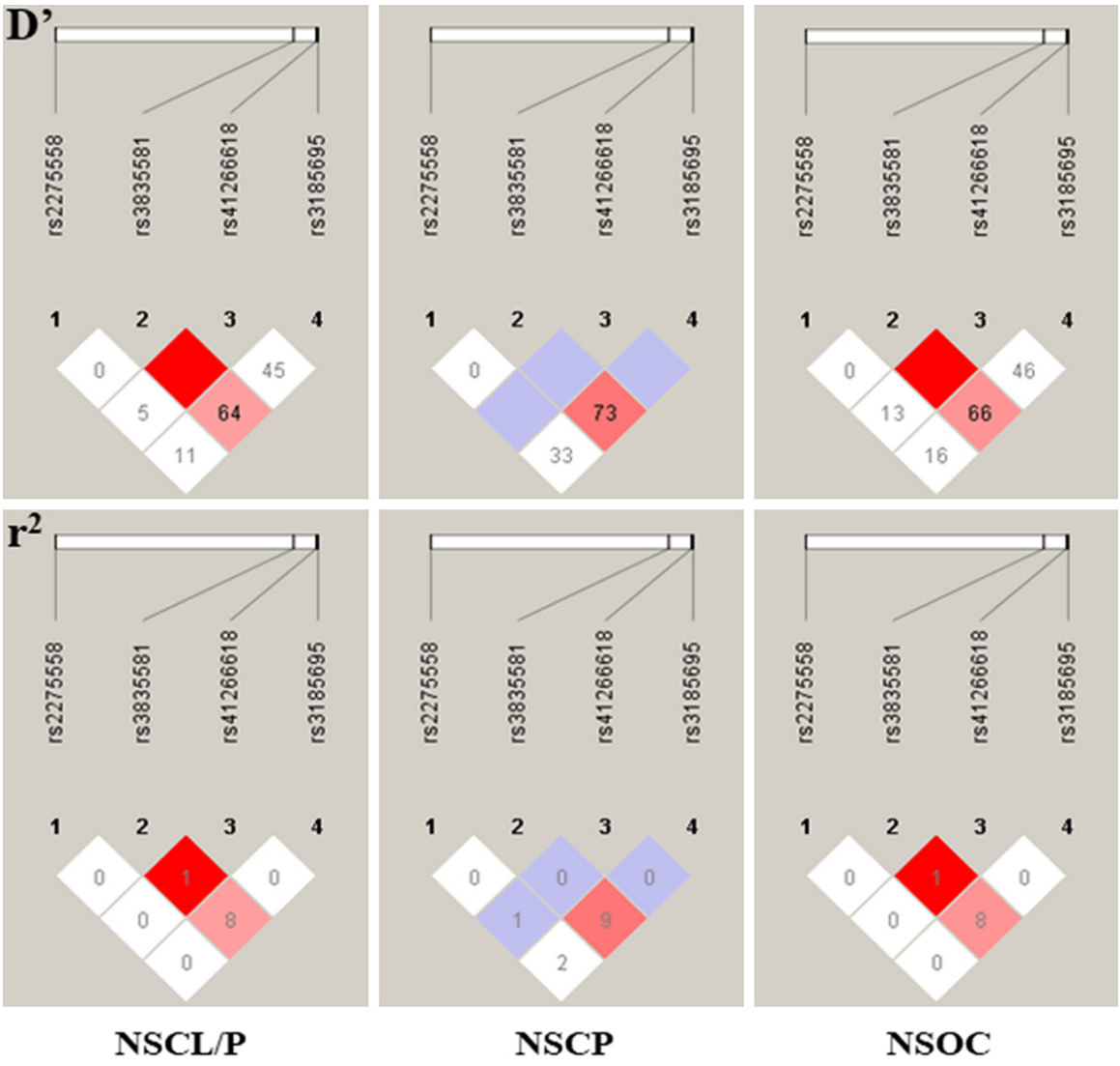

Figure 2. Linkage disequilibrium plots of the four SNPs of the *PBX1* gene.

two ways. One is through the interference of *PBX* epithelial apoptosis, and the second is the destruction of PBX-SNAIL1--dependent Epithelial-Mesenchymal-Transition (EMT) [22, 23]. The above evidence strongly suggested that *PBX1* indeed is involves in the occurrence of NSOC. However, the results of animal research may not directly apply to humans [30]. Human etiology studies are needed to validate the association between the *PBX1* gene and NSOC.

In this study, we first screened for variants in all exons adjacent to intronic regions, the 3' UTR, and the 5' UTR of *PBX1* gene among 50 NSCLP patients. A total of two novel variants and four SNPs were identified. Subsequently, four SNPs were selected for evaluation by conducting a family-based association study.

Considering the influence of population background difference on genetic analysis, we conducted TDT and parent-of-origin effect analysis based on case-parent trios. We did find that allele G at rs2275558 and allele T at rs3835581 were over-transmitted for NSCL/P (P=0.039 and 0.038, respectively) and NSOC (P=0.036 and 0.014, respectively) (**Table 4**). Similarly, no significant associations were found between all SNPs and NSCP. Parent-of-origin effects can reflect the influence of alleles from mother or father on phenotype [31]. This study showed that allele G at rs2275558 was significantly paternally over-transmitted among NSCL/P (**Table 5**). Statistical significance of the paternal transmission rather than of maternal transmission may be attributed to non-expression of the maternally derived alleles, which reflects

underlying imprinting [32]. In summary, epigenetic effects such as imprinting are gradually being recognized as a significant source of variations in complex traits [33].

Although rs2275558 and rs3835581 were identified as associated loci for NSCL/P, they were independent with each other with lower D' and r^2 (**Figure 2**). Compared to other SNPs, the missense variant rs2275558 (p.G21S) located in the coding region of *PBX1* which would change the structure of a protein, was previously reported to have an association with type 2 diabetes (T2DM) in a study on the Pima Indian population [34], but it was not associated with T2DM in Caucasians [35, 36]. The p.G21 residue is highly conserved among mammals including the mouse, rat, and chimpanzee, which indicates that it may play a important role. Although variation is conservative, the frequencies of alleles fluctuated across different populations. Therefore, multiracial populations need to be recruited to validate the association between rs2275558 and NSOC. Also, in vivo functional research of *PBX1* variants should be conducted in animal models in a future study.

In addition to this common variant, two novel heterozygous variants were detected in the *PBX1* of two sporadic patients with NSCLP, namely, a one-year-old boy and a three-year-old girl. These variants were absent in multiple online human gene variation databases. Although they were not in the coding region, variants in non-coding sequences have been found to affect the level and form of mRNA transcripts. Several studies have also confirmed that the 3' UTR of a gene has an important role in gene expression by binding with miRNA [37].

In sum, we confirmed the role of the *PBX1* gene in orofacial deformity from Western Han Chinese, which is consistent with a previous animal study. This work provides new evidence for the future study of the etiology of NSCL/P.

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Disclosure of conflict of interest

None.

Address correspondence to: Drs. Zhong-Lin Jia and Bing Shi, State Key Laboratory of Oral Diseases & National Clinical Research Center for Oral Diseases & Development of Cleft Lip and Palate, West China Hospital of Stomatology, Sichuan University, P.R. China. E-mail: zhonglinjia@sina.com (ZLJ); shibingcn@vip.sina.com (BS)

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