Association of PRKCB Polymorphisms with Non-syndromic Orofacial Cleft among a Chinese Population

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Objective: *To investigate the association of PRKCB polymorphisms with non-syndromic orofacial cleft (NSOC) among a Chinese population.*

Methods: *A total of 2,724 patients with NSOC and 1,263 healthy controls of Han ethnicity were recruited. Clinical subtypes of NSOC were classified in detail, and 945 single nucleotide polymorphisms (SNPs) in the PRKCB gene were initially extracted from prior genome-wide association studies (GWAS) data. SNPs were selected based on minor allele frequency, Hardy-Weinberg equilibrium, call rate and linkage criteria, then genotyped using SNPscan. Statistical analyses included Hardy-Weinberg equilibrium testing, allelic/genotypic association analyses and linkage disequilibrium analysis. Functional annotation was conducted using RegulomeDB, 3DSNP and HaploReg databases.*

Results: *Two PRKCB SNPs (rs198198 and rs174828) demonstrated significant or borderline associations with multiple NSOC subtypes. Notably, the A allele of rs198198 presented a risk for NSCL/P (P = 0.02) and NSCLP (P = 0.03) and C allele of rs174828 presented a risk for NSCL/P (P = 0.03) in allelic association analysis. Only rs174828 exhibited significant genotypic associations with NSOC (P = 0.02), NSCL/P (P = 0.02) and NSCPO (P < 0.01). Functional annotation suggested both SNPs could modify transcription factor binding and enhancer activity.*

Conclusion: *These results demonstrate that rs198198 and rs174828 in PRKCB are associated with specific NSOC sub-phenotypes. Phenotype stratification and bioinformatics analyses highlight the role of PRKCB in NSOC susceptibility, highlighting the importance of non-canonical Wnt/Ca^{2+}-PKC signalling and emphasising the genetic heterogeneity within NSOC.*

Keywords: *association analysis, non-syndromic orofacial cleft, PRKCB, single nucleotide polymorphism*

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Orofacial cleft represents one of the most common craniofacial congenital malformations, imposing a substantial medical, psychological and financial burden on affected individuals and their families due to the requirement for long-term multidisciplinary interventions.[1,2] In China, the incidence of orofacial cleft is relatively high at 1.42 per 1,000 live births compared to other populations.[3] Around 70% of patients are classified as having non-syndromic orofacial cleft (NSOC), occurring without other congenital abnormalities. NSOC can be anatomically categorised into three main subtypes: non-syndromic cleft lip only (NSCLO), non-syndromic cleft lip and palate (NSCLP) and non-syndromic cleft palate only (NSCPO).[4] Unlike monogenic diseases, the

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aetiology of NSOC involves both genetic and environmental factors as well as their complex interactions; therefore, thorough exploration of NSOC aetiology is both significant and methodologically challenging.[5]

Recent advances in genome-wide association studies (GWAS) have led to the identification of more than 40 susceptibility genes and loci associated with NSOC.[6-9] However, the biological functions of these statistically significant loci and their precise roles in NSOC pathogenesis remain largely unexplored.[10,11] To address this gap, we turned our attention to biological function and selected candidate gene within the Wnt signalling pathway,[12] which is critically involved in embryonic development[13,14] and the pathogenesis of various craniofacial disorders, particularly orofacial cleft.[12,15-17]

Animal models with mutations in Wnt signalling genes exhibit orofacial development defects including cleft formation,[18-20] and the association between Wnt signalling genes and NSOC has been confirmed across multiple populations of different ethnicities.[21-23] Although previous investigations have predominantly focused on the canonical Wnt pathway, the role of non-canonical downstream effectors, such as the Wnt/Ca^{2+}-protein kinase C (PKC) axis, remains insufficiently characterised. Notably, there exists substantial interactive feedback and regulation between different Wnt signalling sub-pathways.[24] The non-canonical Wnt/Ca^{2+}-PKC signalling axis in particular appears to be intimately connected to embryonic and palatal development.[25] Within this pathway, PKC represents a group of protein kinases that play crucial regulatory roles in palatal morphogenesis.[25-27]

Notably, members of the PKC family, and particularly the PKC beta isoform (PRKCB), have shown dynamic spatiotemporal expression changes during mouse palatal development and susceptibility to teratogenic inhibition. The expression of conventional PKC (cPKC) is especially observed during the rapid growth phase of mouse palate development.[28] *PKC beta* (*PRKCB*), a member of the cPKC family, exhibits homogeneous distribution during normal palatal development, with expression changes corresponding to peaks in palatal interstitial proliferation. Secalonic acid D, a classic teratogen that induces cleft palate in mice, can functionally inhibit *PRKCB* both in vitro and in vivo,[25,28] suggesting that *PRKCB* might exert an antagonistic effect on proliferation in palatal mesenchymal cells. Therefore, exploring genetic variation in *PRKCB* offers a unique opportunity to elucidate novel mechanisms underlying NSOC pathogenesis that extend beyond the canonical Wnt pathway focus of most genetic studies thus far.

In this study, the present authors selected *PRKCB* as the candidate gene and conducted a comprehensive gene-phenotype association analysis in a Chinese Han population. Single nucleotide polymorphisms (SNPs) potentially related to NSOC pathogenesis were screened based on the present authors' previous GWAS and validated in a larger NSOC case-control cohort. Additionally, detailed phenotypic classification of NSOC was performed according to clinical presentation to explore potential associations between *PRKCB* variants and NSOC subtypes. This investigation aims to provide a foundation to further elucidate the role of the Wnt/Ca^{2+} pathway in the pathogenic mechanisms underlying orofacial cleft formation.

Materials and methods

Samples and materials

A total of 2,724 patients with NSOC and 1,263 healthy controls were enrolled in this study (Supplementary Table 1). The patient cohort was stratified into three distinct phenotypic subgroups: NSCLO (n = 1,190, 43.69%), NSCLP (n = 539, 19.79%) and NSCPO (n = 995, 36.53%). All patients were recruited from West China Hospital of Stomatology, Sichuan University, and study participants were selected based on several rigorous inclusion criteria. All subjects were of Han Chinese ethnicity from western China. NSOC diagnosis was confirmed independently by at least two physicians in the present authors' department according to established diagnostic criteria. Patients with other congenital anomalies or malformations (e.g., congenital heart disease or syndactyly) and those with a family history of congenital disorders were excluded. The control group consisted exclusively of western Han Chinese individuals with no personal or family history of congenital disorders.

This study was conducted in accordance with ethical research standards and received approval from the Ethics Committee of West China Hospital of Stomatology, Sichuan University (approval number WCHSIRB-D-2022-237-R1). Prior to peripheral blood collection, written informed consent was obtained from all the adult patients themselves or from the guardians of patients aged below 18 years.

SNP selection, genotyping and statistical analysis

For initial screening, the present authors extracted the genotyping data of 945 SNPs within the PRKCB gene from two previous GWAS in which their team partici-

Table 1 Significant PRKCB SNP associated with NSOC and subtypes.

Group	SNP	BP	A1	P	OR (95% CI)
NSOC	rs174828	24128365	C	5.70E-05	1.23 (1.11–1.36)
NSCL/P	rs174828	24128365	C	2.33E-06	1.30 (1.16–1.44)
	rs198198	24128397	T	9.21E-05	0.81 (0.73–0.90)
NSCLO	rs174828	24127109	A	9.26E-05	1.46 (1.21–1.77)

A1, minor allele.

pated.[7,29] The 945 SNPs were filtered based on the following criteria: minor allele frequency (MAF) > 0.05 in controls; Hardy-Weinberg equilibrium (HWE) $P > 1 \times 10^{-5}$ in controls; call rate > 90%; and pairwise linkage $r^2 < 0.8$.

SNPs meeting both the initial screening threshold ($P < 10^{-4}$) and the validation criteria (HWE equilibrium $P > 0.05$ and MAF > 0.01) in the replication groups were selected to perform association analyses in the validation stage.

SNP scan genotyping was performed for selected SNPs. The HWE, allelic and genotypic frequencies for each SNP were calculated using PLINK software (http://zzz.bwh.harvard.edu/plink). Genotypic relative risk (GRR) was calculated using the R package (version 4.5.2) of PLINK software. Differences between patients and controls were assessed using a chi-square test. Linkage disequilibrium (LD) analysis was conducted with Haploview 4.2 (www.broad.mit.edu/haploview), with D' and r^2 values calculated for each SNP pair.

Functional annotation

To investigate the potential regulatory mechanisms of the identified SNPs, bioinformatics analyses were conducted using three complementary SNP annotation databases: RegulomeDB v2.2 (https://regulomedb.org/regulome-search/), 3DSNP v2.0 (https://omic.tech/3dsnpv2/) and HaploReg v4.2 (http://pubs.broadinstitute.org/mammals/haploreg/haploreg.php).[30,31] These databases provide comprehensive annotations of regulatory functions for human noncoding variants and help determine potential causal relationships with clinical phenotypes. In the present analysis, SNPs with a 3DSNP score greater than 5 were considered likely to exert effects on gene regulation.[32]

Results

SNP selection

Two SNPs in the *PRKCB* gene, rs198198 and rs174828, met the selection criteria. Based on GWAS data, these SNPs showed statistically significant associations with NSOC or subtypes ($P < 10^{-4}$) (Table 1). Additionally, they were in accordance with HWE in controls ($P > 0.05$) and exhibited MAFs exceeding 0.01 (Supplementary Table 2, provided on request).

Allelic association analysis

In the allelic association analysis under the additive model, the present authors found that the A allele of rs198198 was significantly associated with NSCL/P ($P = 0.02$, odds ratio [OR] 1.12, 95% confidence interval [C] 1.02 to 1.25) and NSCLP ($P = 0.03$, OR 1.18, 95% CI 1.02 to 1.37), indicating a risk effect against NSOC subtypes (Fig 1a). The C allele of rs174828 also demonstrated an association with NSCL/P ($P = 0.03$, OR 1.12, 95% CI 1.01 to 1.25).

Genotypic association analysis and genotypic relative risk

Interestingly, genotypic association analysis revealed more robust relationships, with rs174828 showing significant associations with overall NSOC ($P = 0.01$), NSCL/P ($P = 0.02$) and NSCPO ($P < 0.01$) (Fig 1b). These findings suggest potential genotype-specific effects of rs174828 on NSOC susceptibility. rs198198 may have a marginal association with NSCL/P ($P = 0.06$) but does not meet the conventional threshold for significance.

Regarding GRR, individuals carrying the AA genotype of rs174828 have a significantly reduced risk of NSCPO ($P = 0.01$, OR 0.75, 95% CI 0.61 to 0.93). No significant association was observed for rs198198 (all GRR P values > 0.05) (Table 1).

Functional prediction

Since both rs198198 and rs174828 are located in intronic regions of the *PRKCB* gene, the present authors employed bioinformatics tools to investigate whether their effects on NSOC pathogenesis might be mediated through transcriptional regulation. RegulomeDB analysis assigned ranks of 4 and 2b to rs198198 and rs174828, respectively (Table 2), with the lower score for rs174828 suggesting stronger evidence for regulatory function.

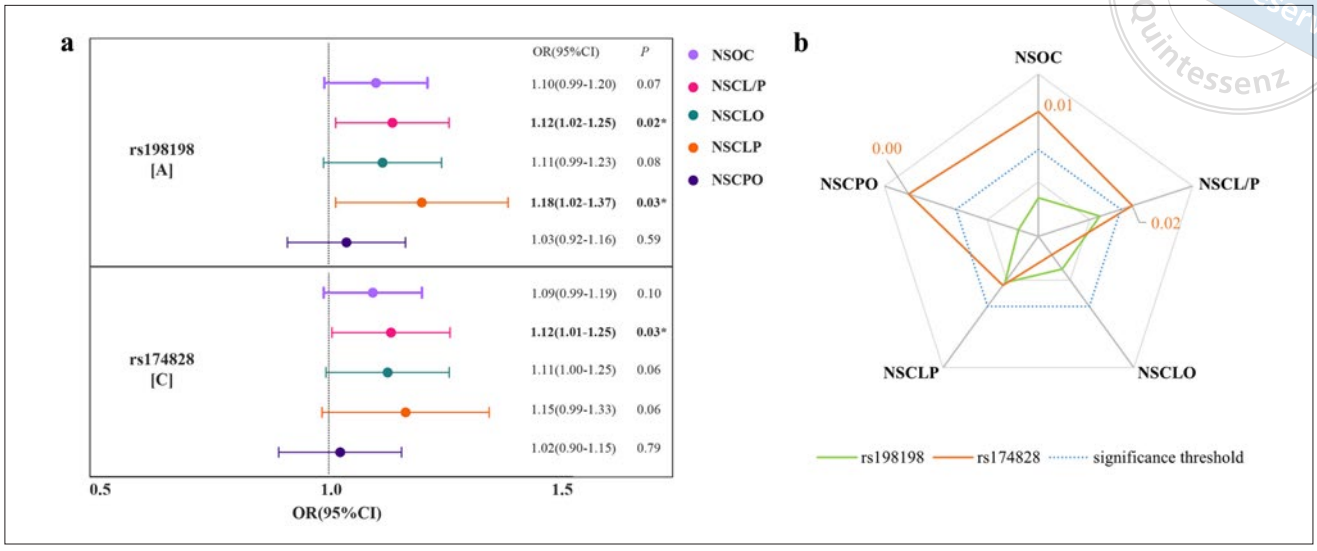

Fig 1 Fig 1a and b Association analysis of the selected SNPs at PRKCB in cases and controls. Allelic association analysis of rs198198 and rs174828 with NSOC subtypes (a). Genotypic association analysis of rs198198 and rs174828 with NSOC (b). NSOC, non-syndromic orofacial cleft; NSCL/P, non-syndromic cleft lip with or without cleft palate; NSCLO, non-syndromic cleft lip only; NSCLP, non-syndromic cleft lip and palate; NSCPO, non-syndromic cleft palate only.

Table 2 GRR of rs198198 and rs174828.

SNP	Risk allele	Non-risk allele	Group	Case		Control		*P* value	OR (95% CI)
				Heterzygote (risk)	Homozygote (non-risk)	Heterzygote (risk)	Homozygote (non-risk)		
rs198198	A	T	NSOC	1266	389	603	199	0.48	1.07 (0.88–1.31)
			NSCL/P	819	226	603	199	0.11	1.20 (0.96–1.49)
			NSCLP	249	68	603	199	0.23	1.21 (0.88–1.65)
			NSCLO	570	158	603	199	0.15	1.92 (0.94–1.51)
			NSCPO	447	163	603	199	0.42	0.91 (0.71–1.15)
rs174828	C	A	NSOC	1286	599	660	271	0.15	0.88 (0.74–1.05)
			NSCL/P	837	354	660	271	0.76	0.97 (0.80–1.17)
			NSCLP	262	107	660	271	0.97	1.01 (0.77–1.31)
			NSCLO	575	247	660	271	0.67	0.96 (0.78–1.17)
			NSCPO	449	245	660	271	0.01	0.75 (0.61–0.93)

3DSNP v2.0 annotation revealed that both SNPs obtained the highest scores in the "enhancer" category and were located within regions with enhancer status across 19 different cell types (Fig 2). This finding suggests potential roles in gene expression regulation through enhancer activity.

Motif analysis using the HaploReg database demonstrated that both SNPs could affect the binding affinity of multiple transcription factors. The T allele of rs198198 was predicted to alter 10 transcription factor binding sites (TFBSs), with transcription factors JUND, JUNB and TAL1 predicted to bind at this site by both 3DSNP and HaploReg databases. Similarly, the A allele of rs174828 was predicted to modify three : CDP, Mef2 and Pax-6. These findings provide potential mechanistic insights into how these intronic variants might influence PRKCB expression and subsequently affect NSOC susceptibility.

Discussion

PKC represents a group of evolutionarily conserved structurally related serine-/threonine-specific protein kinases within the Wnt/Ca^{2+} signalling pathway. These kinases mediate the actions of growth factors and diverse ligands by activating transcription factor networks that regulate cell proliferation, differentiation, extracellular matrix synthesis and apoptosis processes crucial for

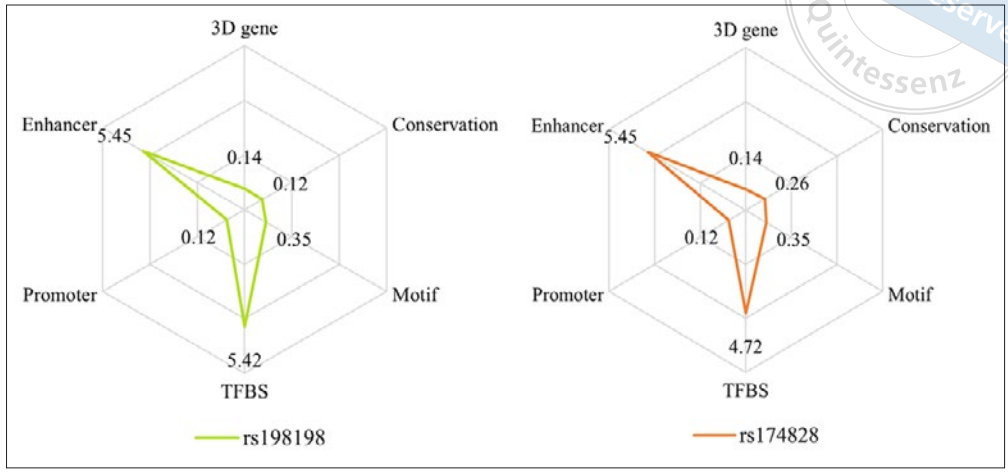

Fig 2 Annotation for rs198198 and rs174828 by 3-dimensional SNP. TFBS, transcription factor binding site.

Table 3 Putative regulatory SNPs indicated by bioinformation analysis.

SNP	RegulomeDB	3DSNP		HaploReg				
	Rank	Score	TFBS	Promoter Histone marks	Enhancer Histone marks	DNase	Protein bound	Motifs changed
rs198198	4	11.61	12	BLD	9 tissues	7 tissues	JUND, CJUN, JUNB, TAL1	Arid3a_2, CDP_6, CEBPG, Cdc5, Dbx1, Foxa_known4, Foxl1_1, Ncx_2, TATA_known1, Zfp105
rs174828	2b	11.04	11	BLD	9 tissues	5 tissues	JUND	CDP_7, Mef2_disc3, Pax-6_1

TFBS, transcription factor binding site.

normal craniofacial development.[25,26,33] Previous research by Balasubramanian et al[28] demonstrated that PKC isoenzymes exhibit dynamic spatial and temporal expression patterns during palatal development.

PRKCB, a highly expressed member of the conventional PKC family, is activated by Ca^{2+} and the second messenger diacylglycerol, mediating various cellular events essential for normal development.[34] In addition to secalonic acid D,[35] PKC signalling is also implicated in cell proliferation inhibition induced by glucocorticoids, retinoic acid and other teratogenic compounds, suggesting that *PRKCB* might be a target for these teratogens.[25,36-38] The present study represents the first comprehensive investigation of *PRKCB* as a candidate gene for NSOC susceptibility and conducted an association study between *PRKCB* polymorphisms and NSOC subtypes, aiming to identify genetic variants associated with NSOC and provide evidence for the role of PKC signalling genes in orofacial cleft pathogenesis.

NSOC is a heterogeneous disorder influenced by both genetic and environmental factors.[39] Although NSCLO and NSCLP are often grouped as NSCL/P, substantial evidence indicates their genetic origins are distinct.[11] Moreover, marked phenotypic diversity exists even within the same NSOC subtype, which can reduce the statistical power of genetic studies when diverse phenotypes of potentially different aetiologies are pooled.[40] To overcome this limitation, the present authors performed detailed clinical classification and conducted stratified association analyses among the 2,724 NSOC patients, aiming to improve the detection of subtype-specific susceptibility SNPs and better elucidate potential genetic heterogeneity obscured in aggregate analyses. It should be noted that observed associations do not imply causality but may suggest risk modifying effects of these SNPs on specific clinical subtypes.

The present results revealed distinct and sometimes opposing effects of SNPs within *PRKCB* across NSOC subtypes. Although rs174828 exhibited only a borderline association with NSCL/P (P = 0.03) in the allelic model, it showed variable associations across different NSOC subtypes in the genotypic model. Notably, significant relationships were observed in the genotypic model for overall NSOC (P = 0.01), NSCL/P (P = 0.02) and NSCPO (P < 0.01) in the genotypic model. Neither SNP showed significant associations with NSCLO, further supporting the concept of genetic heterogeneity among NSOC subtypes. These findings align with growing evidence suggesting that susceptibility variants may be masked by heterogeneous signals when association

analyses combine cases with diverse phenotypes. For instance, Huang et al[7] reported that *IRF6* rs72741048 functions as a risk allele for NSCPO but as a protective allele for NSCLO, highlighting differential genetic contributions across NSOC subtypes. The present study provides additional support for this phenomenon, underscoring the importance of detailed phenotypic classification in genetic studies of complex disorders like NSOC.

Although both rs198198 and rs174828 are located within intronic regions, the present authors' bioinformatics analyses using the RegulomeDB, 3DSNP and HaploReg databases suggest that these SNPs may influence transcriptional regulation. Motif analysis predicted that rs198198 could alter binding sites for multiple transcription factors, including Dbx1 and Foxa, both of which play established roles in craniofacial development.[41] Similarly, rs174828 was predicted to affect binding sites for transcription factors such as Mef2 and Pax-6, which have been involved in craniofacial disorders and development. *MEF2C*, for example, is known as the causative gene for 5q14.3 deletion syndrome (MIM#613443), characterised by cleft lip/palate and facial dysmorphism,[42] whereas PAX6 has also been associated with orofacial clefts and craniofacial development in both animal and human studies.[43] These functional predictions provide plausible mechanisms through which these intronic variants might influence NSOC susceptibility.

Taken together, the present findings not only provide the first genetic evidence supporting the involvement of *PRKCB* in NSOC susceptibility within a non-canonical Wnt/Ca^{2+} signalling framework but also highlight that subtype-specific analyses are critical to uncovering such associations. Nonetheless, the precise biological mechanisms underlying these effects require further investigation through targeted functional studies.

Conclusion

In conclusion, the present study identified significant associations between two PRKCB SNPs (rs198198 and rs174828) and specific NSOC subtypes within Chinese Han population. The A allele of rs198198 exhibited a risk effect against NSCL/P, whereas rs174828 showed significant associations with multiple NSOC phenotypes, particularly in genotypic models. These findings establish *PRKCB* as a susceptible gene for NSOC and highlight the genetic heterogeneity among NSOC subtypes. The results emphasise the importance of detailed phenotypic classification in genetic studies of orofacial clefts and provide new insights for future investigations into the molecular mechanisms underlying this common congenital disorder.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

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Author contribution

Drs Si Di ZHANG, Yi Xin YANG and Li GAN contributed to the data collection, analysis and manuscript draft and revision; Dr Hao Lang ZHAO contributed to the manuscript rivision; Dr Bing SHI contributed to study supervision; Dr Zhong Lin JIA contributed to the conceptualisation, methodology and study supervision.

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