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Association between *PAX7* and *NTN1* gene polymorphisms and nonsyndromic orofacial clefts in a northern Chinese population

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Abstract

Background: Nonsyndromic orofacial clefts (NSOC) are the most common orofacial congenital defect with a complex etiology. Genome-wide association studies have identified paired box protein 7 (*PAX7*) and netrin-1 (*NTN1*) as candidate susceptibility genes for NSOC in both European and Asian populations. Here, possible associations between single-nucleotide polymorphisms (SNPs) in or near *PAX7* and *NTN1* were investigated in relation to risk of NSOC in a northern Chinese population.

Methods: A total of 602 individuals with NSOC and 510 controls were recruited from northern China. Polymerase chain reaction–ligation detection reactions were used to analyze 4 SNPs (rs742071, rs6659735, rs766325, and rs4920520) of *PAX7* and 2 SNPs (rs9904526 and rs9788972) of *NTN1*. Investigations of polymorphisms and risk of NSOC were conducted by using the PLINK software.

Results: *NTN1* rs9788972 AG was found to be associated with an increased risk of NSOC compared to the GG homozygous genotype (OR = 1.43, 95% CI = 1.11–1.86, $P = .006$). When the multifactor dimensionality reduction method was applied, *NTN1* rs9788972 still exhibited an increased risk for NSOC ($P = .008$). In contrast, SNPs in *PAX7* were not associated with any increased risk of NSOC.

Conclusion: *NTN1* rs9788972 is identified as a risk locus for NSOC susceptibility in a northern Chinese population.

Abbreviations: CIs = confidence intervals, GWAS = genome-wide association studies, HWE = Hardy–Weinberg equilibrium, LD = linkage disequilibrium, MDR = multifactor dimensionality reduction, NSOC = nonsyndromic orofacial clefts, NTN1 = netrin-1, ORs = odds ratios, PAX7 = paired box protein 7, PCR–LDR = polymerase chain reaction–ligation detection reaction, SNPs = single-nucleotide polymorphisms.

Keywords: nonsyndromic orofacial clefts, *NTN1* gene, *PAX7* gene, single-nucleotide polymorphisms

1. Introduction

Nonsyndromic orofacial clefts (NSOC) are the most common orofacial congenital defect that develop at the early in pregnancy when fusion between facial prominences or their derivatives does not occur.[1] The birth prevalence of NSOC has been found to vary among ethnic populations. For example, Asian populations have the highest prevalence rates with 1/500 births, Africans have the lowest birth prevalence with 1/2500 births, and European populations have an intermediate prevalence of 1/1000.[2] The average prevalence worldwide is about 1.2 per 1000 live births.[3] However, in China, live births exhibit a high prevalence rate of 1.42/1000 for NSOC.[4] Previous genetic and epidemiological studies of NSOC have revealed that the etiology of NSOC derives from both genetic and environmental risk factors.[3] In fact, several environmental risk factors have been investigated, including maternal smoking, maternal consumption of alcohol, malnutrition, viral infection, drug use, and exposure to environmental teratogens, in the workplace or home during early pregnancy.[2] Genetic linkage and genome-wide association studies (GWAS) have further provided evidence for the genetic etiology of NSOC in recent years, with roles for *IRF6*, *FOXE1*, *BMP4*, *TP63*, *JAG2*, and *PVRL1* investigated. However, the exact number of causal genes and associated biological mechanisms of NSOC remain unclear.[5–9]

Recently, a cohort of case–parent trios from both European and Asian populations were subjected to GWAS by Beaty et al.[10] Two new susceptibility loci associated with NSOC were identified as "second-tier" hits, *PAX7* at chromosome 1p36 and *NTN1* at chromosome 17p13. Further studies of animal models have identified biological functions for *PAX7* and *NTN1* showing that they affect development of the craniofacial region.[11,12] Moreover, in humans, *PAX7* is involved in the development of the dorsal neural tube and early migratory neural

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crest cells of early-stage human embryos.[13] Correspondingly, impaired neural crest development is related to human defects, including cleft palate.[14] Regarding *NTN1*, it has been shown to play a vital role in regulating cell migration during embryogenesis, and it is also expressed in the medial edges and oral sides of the palatal shelves.[12,15–17]

To our knowledge, no large studies have been conducted to investigate a possible association between *PAX7* and *NTN1* genes and risk of NSOC in a northern Chinese population. We examined the 4 SNPs (rs742071, rs6659735, rs766325, and rs4920520) of *PAX7* and 2 SNPs (rs9904526 and rs9788972) of *NTN1* previously suggested in the GWAS conducted by Beaty et al[10] and confirmed by Leslie et al[17] as risk markers for NSOC in a case–control study from northern Chinese population.

2. Materials and methods

2.1. Subjects

A total of 602 NSOC cases (326 males and 276 females), as well as 510 healthy individuals (307 males and 203 females), were recruited from the Affiliated Stomatology Hospital of Harbin Medical University, the Second Affiliated Hospital of Harbin Medical University, between October 2010 and November 2015. All of the NSOC patients received a diagnosis by well-trained oral surgeons and other congenital anomalies, syndromes involving oral clefts, and other major structural anomalies were excluded. Signed informed consent was obtained from each participant or their guardians and this study was approved by the Institutional Review Board of Harbin Medical University.

2.2. Marker selection and genotyping

The associations between *PAX7* rs742071 and *NTN1* rs4791774 and the risk of NSOC have been investigated in a southern Chinese population. However, northern and southern Chinese populations contain distinct ethnicities.[18] Therefore, based on GWAS that were conducted by Beaty et al,[10] as well as targeted sequencing of loci identified in GWAS performed by Leslie et al,[17] 4 SNPs (rs742071, rs6659735, rs766325, and rs4920520) of *PAX7* and 2 SNPs (rs9904526 and rs9788972) of *NTN1* were used to test for association with NSOC in a northern Chinese population.

Venous blood samples were collected from each participant and genomic DNA was isolated with a DNA purification kit (Axygen Biosciences, Union City, CA). Polymerase chain reaction–ligation detection reaction (PCR–LDR) was used for genotyping. Target DNA sequences were amplified using a multiplex PCR method, and the primer and probe sequences used are listed in Tables S1 and S2 (http://links.lww.com/MD/B666). Briefly, the PCR samples were amplified with an ABI 9600 system (Applied Biosystems, Foster City, CA) in a total volume of 20 μL which included 1 μL genomic DNA, 2 μL 1 × PCR buffer, 0.6 μL $MgCl_2$, 2 μL dNTP, 0.2 μL Hot-Start Taq DNA polymerase (Qiagen, Hilden, Germany), 12.2 μL ddH_2O, and 2 μL of each primer. The cycling parameters were: 95°C for 2 minutes; 40 cycles of 94°C for 30 seconds, 62°C for 90 seconds, and 72°C for 1 minute; and a final extension step at 72°C for 10 minutes. The subsequent ligation reaction for each PCR product included 1 μL 1 × buffer, 1 μL probe mix, 0.05 μL Taq DNA ligase (New England Biolabs, Ipswich, MA), 4 μL ddH_2O, and 4 μL PCR product in a total volume of 10 μL. The cycling parameters were: 95°C for 2 minutes, then 40 cycles at 94°C for 15 seconds and 50°C for 25 seconds. The resulting products were analyzed with an ABI Prism 3730XL DNA sequencer (Applied Biosystems) (Table S3, http://links.lww.com/MD/B666). GeneMapper software (Applied Biosystems) was used to analyze the sequencing data. For quality control, 10% of the samples were randomly selected to have their ligation reactions repeated, and 100% concordance between the duplicated results was observed.

2.3. Statistical analysis

Each SNP in both NSOC patients and healthy individuals were subjected to a Hardy–Weinberg equilibrium (HWE) analysis. SNPs were excluded from subsequent analyses if they deviated from HWE ($P < .05$). Genotype and allele frequencies of the measured markers between NSOC patients and control groups were compared using χ^2 and Fisher's exact tests. Statistical analyses were performed by using a free, open-source whole genome association analysis toolset, PLINK. Odds ratios (ORs) and 95% confidence intervals (CIs) were used in unconditional logistic regression analyses to estimate the association between genotypes and NSOC susceptibility. Combinations of multiple markers were subjected to haplotype analysis, and D' and r^2 values were used to estimate linkage disequilibrium (LD), which were calculated by SHEsis online software. Further investigations of the association between polymorphisms and risk of NSOC were performed using an R statistical language package of multifactor dimensionality reduction (MDR) software.[19] Statistical significance was evaluated with a 10-fold cross-validation and 1000-permutation testing process to avoid false positive findings, which could result from multiple comparisons. A *P*-value <.05 was used as the criterion for statistical significance.

3. Results

3.1. Markers analysis

All 6 of the SNPs examined conformed to HWE (Table 1) and their genotype and allele distributions are listed in Table 2. The distribution of allele frequencies of the *NTN1* polymorphisms

Table 1

Characteristics of the SNPs examined.

Gene	SNP	Position	HWE *P*	Call rate (%)	MAF	Alleles
PAX7	rs742071	18852461	1.000	100	0.055	T:G
	rs6659735	18856284	1.000	100	0.055	A:G
	rs766325	18829045	1.000	100	0.184	A:G
	rs4920520	18802429	.167	100	0.153	A:G
NTN1	rs9904526	8859701	.451	100	0.322	C:T
	rs9788972	8860355	.181	100	0.215	A:G

HWE = Hardy–Weinberg equilibrium, MAF = minor allele frequency, NTN1 = netrin-1, PAX7 = paired box protein 7, SNP = single-nucleotide polymorphism.

Table 2

Frequencies and ORs of *PAX7* and *NTN1* polymorphism genotypes and alleles in the NSOC cases and controls.

SNPs	Genotype/allele	Controls (%)	Cases (%)	OR (95% CI)	*P* value*
rs742071				1.05 (0.73, 1.52)	.792
	GG	457 (89.61)	535 (88.87)	1	–
	GT	51 (10.00)	66 (10.96)	1.11 (0.75, 1.63)	.611
	TT	2 (0.39)	1 (0.17)	0.43 (0.04, 4.72)	.488
	Dominant	GG vs (GT + TT)		1.08 (0.74, 1.58)	.693
	Recessive	(GG + GT) vs TT		0.42 (0.04, 4.67)	.482
	MAF	5.39	5.65		
rs6659735				1.03 (0.72, 1.49)	.858
	GG	457 (89.61)	536 (89.04)	1	–
	AG	51 (10.00)	65 (10.80)	1.09 (0.74, 1.60)	.674
	AA	2 (0.39)	1 (0.17)	0.43 (0.04, 4.71)	.487
	Dominant	GG vs (AG + AA)		1.06 (0.72, 1.56)	.759
	Recessive	(GG + AG) vs AA		0.42 (0.04, 4.67)	.482
	MAF	5.39	5.56		
rs766325				1.04 (0.84, 1.29)	.699
	GG	344 (67.45)	402 (66.78)	1	–
	AG	148 (29.02)	175 (29.07)	1.01 (0.78, 1.31)	.930
	AA	18 (3.53)	25 (4.15)	1.19 (0.64, 2.22)	.587
	Dominant	GG vs (AG + AA)		1.03 (0.80, 1.33)	.812
	Recessive	(GG + AG) vs AA		1.18 (0.64, 2.20)	.591
	MAF	18.04	18.69		
rs4920520				1.03 (0.82, 1.30)	.782
	GG	370 (72.55)	433 (71.93)	1	–
	AG	126 (24.71)	151 (25.08)	1.02 (0.78, 1.35)	.865
	AA	14 (2.75)	18 (2.99)	1.10 (0.54, 2.24)	.796
	Dominant	GG vs (AG + AA)		1.03 (0.79, 1.34)	.818
	Recessive	(GG + AG) vs AA		1.09 (0.54, 2.22)	.808
	MAF	15.10	15.53		
rs9904526				1.07 (0.90, 1.28)	.426
	TT	247 (48.43)	269 (44.68)	1	–
	CT	206 (40.39)	269 (44.68)	1.20 (0.93, 1.54)	.156
	CC	57 (11.18)	64 (10.63)		
	Dominant	TT vs (CT + CC)		1.16 (0.92, 1.47)	.212
	Recessive	(TT + CT) vs CC		0.95 (0.65, 1.38)	.771
	MAF	31.37	32.97		
rs9788972				1.20 (0.98, 1.47)	.07
	GG	338 (66.27)	356 (59.14)	1	–
	AG	143 (28.04)	216 (35.88)	1.43 (1.11, 1.86)	.006
	AA	29 (5.69)	30 (4.98)	0.98 (0.58, 1.67)	.947
	Dominant	GG vs. (AG + AA)		1.36 (1.06, 1.74)	.014
	Recessive	(GG + AG) vs AA		0.87 (0.51, 1.47)	.603
	MAF	19.71	22.92		

CI = confidence interval, MAF = minor allele frequency, NTN1 = netrin-1, NSOC = nonsyndromic orofacial clefts, OR = odds ratio, PAX7 = paired box protein 7, SNP = single-nucleotide polymorphism.
* Two-sided χ^2 test was used for the genotype and allele distributions between cases and controls.

examined, and the allele and genotype frequencies of the *PAX7* polymorphisms did not significantly differ between the NSOC cases and controls. However, the AG genotype at rs9788972 had an OR of 1.43 (95% CI = 1.11–1.86, *P* = .006) compared with the GG genotype. After correcting for multiple testing, the difference remained significant (corrected critical value *P* = .05/6 = .008). Further analysis of dominant and recessive genetic models showed the distribution of *NTN1* rs9788972 differed between the NSOC and control groups (*P* = .014). After combining the AG and AA genotypes of *NTN1* rs9788972 compared with the GG homozygous genotype in a dominant genetic model, the AG + AA genotype was identified as a possible risk factor for NSOC (OR = 1.36, 95% CI = 1.06–1.74). However, the AG + AA genotype for rs9788972 was no longer significant as a risk factor when the Bonferroni correction for multiple comparisons was applied. For *PAX7*, we found no significant associations between the *PAX7* SNPs examined and risk of NSOC by logistic regression analysis.

3.2. Haplotype analysis

The LD pattern of the 6 SNPs examined is displayed in Fig. 1. A haplotype block was constructed in this region by the D' and r^2 values (Table 3). There were no haplotypes associated with NSOC based on the observed haplotype distributions between NSOC patients and control groups ($P > .05$; Table 4).

3.3. MDR analysis

A MDR analysis was performed using the most significant SNP rs9788972 model achieved the best cross validation consistency (10/10) with a testing accuracy (TA) of 0.54 (Table 5). After this model was adjusted using a 1000-fold permutation test, it reached statistical significance for predicting susceptibility to NSOC (*P* = .008). However, MDR analysis did not find a significant gene–gene interaction among the 6 SNPs.

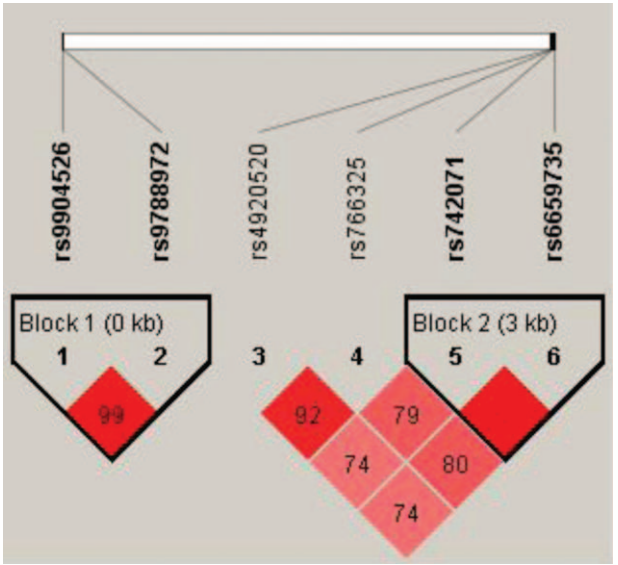

Figure 1. Linkage disequilibrium blocks for the *NTN1* and *PAX7* haplotype analysis.

4. Discussion

NSOC is a common development deformity that involves a complex and heterogeneous etiology including environmental and genetic risk factors. Substantial advances in gene identification studies for NSOC have identified a large number of new potential candidate genes associated with this condition.[20–22] In particular, associations between polymorphic markers in *PAX7* and *NTN1* genes and risk of NSOC have been identified in different populations.[17,23–28] To our knowledge, the present study is the first to identify an association between *NTN1* rs9788972 and an increased risk of NSOC in a northern Chinese population. Our research further demonstrated that the distribution of the AG genotype at rs9788972 significantly differed between the case and control groups ($P = .006$), while the AG + AA genotype of rs9788972 may increase NSOC susceptibility in northern Chinese populations (OR = 1.36, 95% CI = 1.06–1.74, $P = .014$, under a dominant model). However, the former result, and not the latter result, remained significant after Bonferroni correction for multiple comparisons. It is possible that this discrepancy may be due to the small sample size of our study, which did not have sufficient power to detect the magnitude of NSOC risk that may be associated with this genotype.

The *NTN1* gene, which is located on chromosome17p3, encodes a protein that belongs to a family of laminin-related secreted proteins. NTN1 was originally identified as an important guidance molecule in the nervous system.[15] and the *NTN1* gene has since been proven to be expressed during organogenesis, angiogenesis, tumorigenesis, and inflammation-mediated tissue injury.[15,29,30] Expression of *NTN1* has also been shown to play a role in regulating the processes of cell

Table 3

Pairwise linkage disequilibrium measures for *PAX7* and *NTN1*.

SNPs	rs742071	rs6659735	rs766325	rs4920520	rs9904526	rs9788972
rs742071		1	0.796	0.734	0.145	0.116
rs6659735	0.991		0.794	0.731	0.140	0.110
rs766325	0.165	0.163		0.922	0.071	0.054
rs4920520	0.174	0.171	0.682		0.054	0.037
rs9904526	0.003	0.002	0.002	0.001		0.997
rs9788972	0.003	0.003	0.002	0.001	0.570	

D' value are located above the diagonal; r^2 value are below the diagonal.
NTN1 = netrin-1, PAX7 = paired box protein 7, SNP = single-nucleotide polymorphism.

Table 4

Haplotype analysis of the *PAX7* and *NTN1*.

Haplotypes	Total frequency	Case frequency (%)	Control frequency (%)	*P* value
Block1 TG	0.672	843.0 (66.80)	787.0 (67.61)	.6701
Block1 CA	0.216	288.3 (22.84)	234.6 (20.15)	.1076
Block1 CG	0.113	130.7 (10.36)	142.4 (12.23)	.1442
Block2 GG	0.946	1189.0 (94.36)	1102.0 (94.84)	.6084
Block2 TA	0.054	71.0 (5.63)	60.0 (5.16)	.6084

NTN1 = netrin-1, PAX7 = paired box protein 7.

Table 5

Interactions identified with the MDR method.

Model	Testing balanced accuracy (%)	Cross-validation consistency	Odds ratio (95% CI)	*P* value*
rs9788972	54.00	10/10	1.20 (0.98, 1.47)	.008
rs9788972-rs6659735	53.71	8/10	1.04 (0.59, 1.82)	.750
rs9788972-rs6659735-rs766325	51.03	6/10	0.50 (0.20, 1.26)	.508
rs9788972-rs766325-rs4920520-rs742071	54.28	8/10	0.45 (0.09, 2.37)	.342

* Significance of accuracy, empirical *P*-value based on 1000 permutations.
Odds ratio: describes the disorder risk with exposure to binary classification in the selected model of MDR.
MDR = multifactor dimensionality reduction.

adhesion, migration, survival, differentiation, and branching morphogenesis.[31] Recently, Leslie et al[17] detected expression of NTN1 in the medial edges, the basement membrane, and the oral sides of the palatal shelves. In the same study, NTN1 was found to mediate a critical step in palatal fusion in mouse embryos (E13.5). In another study, *NTN1* was found to be upregulated in dental pulp stem cell cultures originating from NSOC cases, thereby revealing the significance of *NTN1* in the development of NSOC.[28]

Despite these insights, however, there is very little information regarding *NTN1* gene polymorphisms as risk factors for NSOC. *NTN1* was first identified as a risk factor of NSOC in European and Asian population based on GWAS conducted by Beaty et al,[10] and also in subsequent follow-up studies.[24] In accordance with the results of Beaty et al,[10]*NTN1* rs9788972 was found to be associated with risk of NSOC in the present study in a northern Chinese population. Correspondingly, the minor allele frequencies of *NTN1* rs9788972 in our control group were similar to those observed in the HapMap CHB populations (National Center for Biotechnology Information) (0.20 vs 0.17, respectively). Additionally, Sun et al[28] found that rs4791774 in the intron of *NTN1* showed a strong association with NSOC in a case–control study of a population from southern China (which included 858 cases and 1248 controls). Thus, despite differences in ethnicities and geographic locations in these southern and northern populations, it appears that the *NTN1* gene is a potential risk factor for NSOC in Chinese populations.

In accordance with research by Pan et al,[23] we found no association between rs742071 of the *PAX7* gene and risk of NSOC in the northern Chinese population examined here. Moreover, the genotype frequencies of GG, GT, and TT for rs742071 in the study by Pan et al[23] versus allele frequencies in the present study were very similar (89.26%, 10.74%, and 0% vs 88.87%, 10.96%, and 0.17%, in each study, respectively), as were the minor allele frequencies for rs742071 (0.054 vs 0.057, respectively). Taken together, these data suggest that these *PAX7* gene variants may not be relevant to NSOC susceptibility in the Chinese population. It should also be noted that we failed to replicate an association between *PAX7* polymorphisms and risk of NSOC previously demonstrated by Beaty et al.[10] For example, the minor allele frequencies of rs742071, rs6659735, rs766325, and rs4920520 (corresponding to rs742071 T, rs6659735 A, rs766325 A, and rs4920520 A, respectively) in our controls were 0.05, 0.05, 0.18, and 0.15, respectively, compared with frequencies of 0.45, 0.54, 0.56, and 0.52, respectively, reported for these minor alleles in HapMap CEU populations (National Center for Biotechnology Information). These inconsistencies between these results may be due to differences in the origin of the different NSOC patient and control datasets.

When the MDR method was used to investigate potential interactions among the tested SNPs, rs9788972 was the best single marker model with the highest test accuracy and good cross-validation consistency. Hence, rs9788972 was identified as a risk factor for NSOC. However, no associations between any combinations of markers *PAX7* and *NTN1* SNPs and NSOC were observed in this study. These results may be limited by the sample size which may not have been large enough to detect a modest effect of other tested gene variants.

However, to our knowledge, the present findings provide the first evidence that *NTN1* rs9788972 is associated with increased risk of NSOC in individuals living in northern China, and this insight may enrich our understanding of the etiology of human orofacial defects. However, there were limitations associated with the present study. First, our research was a hospital-based, case–control investigation, and thus, selection bias could not be completely avoided. Second, the sample size was too small to detect subtle genetic abnormalities within a population. Therefore, it will be important for future studies to include various ethnic populations and environmental factors to better identify the etiology of NSOC.

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