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Original Article

Thyroid cancer causal gene *FOXE1* involved in orofacial clefts in Western Han Chinese

Shi-Jun Duan[1*], Bi-He Zhang[1*], Bing Shi[1,2*], Jia-Yu Shi[3], Sha He[1], Shu-Yuan Jiang[1], Ni Zeng[1,2], Zhong-Lin Jia[1]

[1]State Key Laboratory of Oral Diseases, [2]Department of Cleft Lip and Palate Surgery, West China Hospital of Stomatology, Sichuan University, Chengdu, 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: *FOXE1* is causal gene for thyroid cancer and has been identified to play an essential role in non-syndromic orofacial clefts (NSOCs) among European population, but its effects remain controversial in Chinese Han population. To investigate its roles among Western Han Chinese population, we selected three SNPs (single nucleotide polymorphism): rs894673, rs3758249 and rs4460498 at *FOXE1* gene based on the published literatures and genotyped them in 440 case-parent trios by using SNPscan technology. We performed transmission disequilibrium test (TDT), pair-wise LD, sliding-window haplotype analysis and parent-of-origin effect analysis to evaluate the association. Allelic TDT results showed strong significance among NSOCs and NSCLP (Lowest P=0.0050) for all the three SNPs. Genotypic TDT results showed that A/A homozygotes at rs894673 and T/T homozygotes at rs3758249 were significantly under-transmitted from parents to affected individuals among NSOCs (lowest P=0.016), which indicated that they were protective for NSOCs. Rs894673 A allele (P=0.028), rs3758249 T allele (P=0.028), rs4460498 T allele (P=0.016) was paternally over-transmitted for NSOCs. LD results showed that they tightly linked with each other (D'>0.8 and r^2>0.8), and several haplotypes display association with NSOCs and NSCLP. These SNPs at *FOXE1* were associated with NSOCs among Western Han Chinese population, which could give more evidence of *FOXE1* gene in NSOCs and new insights for future research.

Keywords: *FOXE1*, single nucleotide polymorphisms, non-syndromic orofacial clefts

Introduction

As the most common birth defects, non-syndromic orofacial clefts (NSOCs) have a prevalence rate range from 1/700 to 1/1400 live birth worldwide [1], and it is the highest in Asian descendant than that in other populations. Based on the epidemiological data and inheritance patterns, NSOCs usually divided into two sub-classes: syndromic clefts and non-syndromic clefts. The former is accompanied with other physical and developmental abnormalities; the latter is isolated clefts occupied about 70% of all clefts. It is becoming clearer that multiple genes and environmental modifiers determine the risk to non-syndromic clefts [2, 3], but the major effect causal gene was still unclear. Cleft palate is another kind of orofacial clefts with lower occurrence rate than cleft lip with or without palate (CL/P), and it has different etiologic mechanism from CL/P, about 50% of newborns with cleft palate were considered as non-syndromic (NSCPO) and few genes or loci were associated with NSCPO [4].

To identify the causal genes associated with NSCPO and NSCL/P, the researchers worldwide performed numerous different experiments. Of all, GWAS and linkage study contributed a lot to the new findings of NSCL/P and NSCPO. GWASs reported more than 15 loci were associated with NSCL/P [5-10] and only rs41268753 at GRHL3 was risk for NSCPO [4, 11, 12]. The genome-wide linkage scan was performed on 388 multiplex families from seven different populations, and located the risk region to the 9q22-q33 for NSCL/P [12]. Then Moreno et al. 2009, using parallel positional cloning and candidate gene strategies, confirmed that the 9q22-q33 region was risk for NSOCs, and found common variants rs3758249 and rs4460498 at *FOXE1* gene had significant roles in the etiol-

Table 1. Types of non-syndromic orofacial clefts

	NSCLO	NSCLP	NSCL/P	NSCPO	NSOCs
Male	74	113	186	50	237
Female	56	55	110	82	193
Unknown sex	4	6	10	0	10
Total	134	174	308	132	440

Note: NSCLO, non-syndromic cleft lip only; NSCLP, non-syndromic cleft lip with cleft palate; NSCL/P, non-syndromic cleft lip with or without cleft palate, including NSCLO and NSCLP; NSCPO, non-syndromic cleft palate only; NSOCs, the total cases including NSCL/P and NSCPO.

ogy of both of NSCL/P and NSCPO in Caucasian of USA, Colombian and Filipino [13].

Forxhead box 1 *(FOXE1)* (NM_004473), located at 9q22, as a key regulator of the function of thyroid cells [14], plays an crucial part in the pathogenesis of thyroid cancer [15, 16]. Except *FOXE1*, several genes have been evidenced that they are not only associated with NSOCs but with cancer as well [14-16], individuals with CL/P could decrease overall lifespan, with higher rates of certain cancers postulated to account for observed mortality risk [17, 18]. Men born with NSCLP significantly increased incidence of primary lung cancer in Denmark, women born with NSCPO primary increased brain cancer, and women born with NSCL/P increased breast cancer [18].

As a member of forkhead/winged-helix domain transcription factor family, *FOXE1* was involved in the embryogenesis and the pathogenesis of NSOCs. Based on the previous GWAS findings, *FOXE1* rs3758249 (2.0 kb 5' of *FOXE1*) and rs4460498 (4.2 kb 3' of *FOXE1*) were strongly associated with both NSCL/P and NSCPO in a recent research from Caucasian of USA, Colombian and Filipino [13]. Rs4460498 was also associated with both NSCL/P and NSCPO from Germany and Mayan population [19]. And region between rs3758249 and rs4460498 has a high risk of NSCL/P among Northeastern European population even the sample size was limited [20]. In contrary, the positive associations between these SNPs and NSCL/P was failed to be confirmed in another three independent replication studies [21-23].

Except for the candidate genes, environmental factors and gene-environmental factor interactions contributed a lot to NSOCs, exposure to environmental risk factors (maternal smoking or passive smoking, illness, medication use, et al.) during early pregnancy have been demonstrated that they could increase the risk of NSOCs by many epidemiological investigations and animal experiments [24, 25].

Preceding studies suggested that FOXE1 gene was a good candidate gene for NSOCs. Therefore, we took three SNPs (rs894673, rs3758249 and rs4460498) and selected numerous associated maternal environmental exposure factors during the first trimester (e.g. illness, medication use, smoking, passive smoking and multi-vitamin supplementation [26] to validate the role of FOXE1 gene and G×E interactions among 440 case-parent trios from Western Han Chinese.

Materials and methods

Subjects

The family-based samples comprised 440 trios from Western Han Chinese population with non-syndromic orofacial clefts (134 NSCLO, 174 NSCLP and 132 NSCPO), they were recruited between 2008 and 2013 from the Cleft Lip and Palate Surgery Department of West China College of Stomatology, Sichuan University. Parents were asked about family history of orofacial cleft in first and second relatives. All mothers were required to finish the environmental factors questionnaire which included the nausea and vomiting, infection, pain and other relative environmental factors (maternal smoking, passive smoking, drinking history, et al) during first trimester of pregnancy. All the participants or their guardians were provided written informed consent before enrollment in the study and study protocol was approved by Hospital Ethics Committee of West China Hospital of Stomatology, Sichuan University. The types of clefts and gender were shown in **Table 1**.

Genotyping

Venous blood was collected and extracted DNA by the protein precipitation method. Genotyping of these three SNPs were performed by using SNPscan method (Shanghai BioWing Applied Biotechnology Company http://www.biowing.com.cn/).

Statistical analysis

We undertook the quality control analysis on the raw genotyping data by performing Hardy-

Table 2. Allelic TDT results for SNPs in *FOXE1* from FBAT

Phenotype	SNP	Miner Allele	MAF	Z	P
NSCLO	rs894673	A	11.07%	-0.29	0.77
	rs3758249	T	11.03%	-0.29	0.77
	rs4460498	T	11.07%	-0.44	0.66
NSCLP	rs894673	A	12.61%	**-2.32**	**0.020**
	rs3758249	T	13.15%	**-2.05**	**0.041**
	rs4460498	T	12.76%	**-2.22**	**0.027**
NSCL/P	rs894673	A	11.94%	-1.94	0.052
	rs3758249	T	12.23%	-1.74	0.081
	rs4460498	T	12.02%	-1.96	0.050
NSCPO	rs894673	A	9.40%	-1.12	0.26
	rs3758249	T	9.21%	-1.09	0.27
	rs4460498	T	9.28%	-1.15	0.25
NSOCs	rs894673	A	9.40%	**-2.81**	**0.0050**
	rs3758249	T	9.21%	**-2.81**	**0.0050**
	rs4460498	T	9.28%	**-2.75**	**0.0060**

Note: NSCLO, Non-syndromic cleft lip only; NSCLP, Non-syndromic cleft lip with cleft palate; NSCL/P, Non-syndromic cleft lip with or without cleft palate, including NSCLO and NSCLP; NSCPO, non-syndromic cleft palate only; NSOCs, the total cases including NSCL/P and NSCPO; SNP, Single Nucleotide Polymorphism; MAF, Minor Allele Frequency; Z, vector of the large sample Z statistic; Bold characters indicate the items with *P*-value less than 0.05.

Weinberg equilibrium (HWE) test and minor allele frequency (MAF) determination at each SNP among the normal parents. The SNPs passed the quality control (HWE *P*-value >0.01 and the MAF was consistent with the CHB data from 1000 Genome) was included into the following analysis. Pairwise linkage disequilibrium (LD) (D' and r^2) was calculated among the family-based samples by Haploview program. Transmission-disequilibrium test (TDT) and haplotype analysis were performed for with family-based association test (FBAT) program. Parent-of-origin effect was also conducted to distinguish the parental preference of transmission on a disease variant. The SNP genotype and the environmental exposure factors were taken account into G×E interaction analysis.

Results

HWE and LD

All of the SNPs were passed the Hardy-Weinberg equilibrium threshold (P>0.01) and the minor allele frequencies were consistent with the CHB data from 1000 Genome. So we included them in the latter analysis. LD analysis showed that they were tightly linked (D'>0.8 and r^2>0.8).

TDT analysis of alleles and genotypes

TDT was implemented with heterozygous informative parents. In allelic TDT analysis, rs894673 T allele (Z=-2.81, P=0.0050), rs3758249 T allele (Z=-2.81 P=0.0050) and rs4460498 A allele (Z=-2.75, P=0.0060) showed a statistically significance for under-transmission among NSOCs trios (**Table 2**), which indicate that they could decrease the risk for NSOCs. The similar result is also observed among NSCLP trios, rs894673 A allele (Z=-2.32, P=0.020), rs3758249 T allele (Z=-2.05 P=0.041) and rs4460498 T allele (Z=-2.22, P=0.027). In genotypic TDT analysis, rs895673 T/T homozygote (Z=2.15, P=0.032) and rs3758249 C/C homozygote (Z=2.15, P=0.032) show a statistically significance for over-transmission among NSOCs while rs895673 A/A homozygote (Z=-2.40, P=0.016) and rs3758249 T/T homozygote (Z=-2.40, P=0.016) under-transmission among NSOCs. No significant associations were identified between rs4460498 and sub-phenotype by genotypic TDT analysis (**Table 3**).

Parent-of-origin effects

Rs894673 A allele (P=0.028), rs3758249 T allele (P=0.028), rs4460498 T allele (P=0.016) was paternally over-transmitted for NSOCs, while no significant difference between paternal and maternal was founded in NSOCs, NSCL/P and NSCPO (**Table 4**).

Haplotype analysis

Because these three SNPs were highly linked with each other, so we used the sliding widow haplotype analysis to test if the adjacent SNPs were transmitted together from parents to affected kids. The analysis of these three SNPs showed statistical significance (lowest P is 0.011). The haplotypes T-C of rs894673-rs3758249 C-C of rs3758249-rs4460498 T-C of rs894673-rs4460498 and T-C-C of rs894673-rs3758249-rs4460498 were significant over-transmitted in NSOCs trios. Meanwhile, the haplotypes A-T of rs894673-rs3758249 T-T of rs3758249-rs4460498 A-T

Table 3. Genotypic TDT results for SNPs in *FOXE1* from FBAT

SNP	Genotype	NSCLO		NSCLP		NSCL/P		NSCPO		NSOCs	
		Z	P	Z	P	Z	P	Z	P	Z	P
rs894673	T/T	0.078	0.94	1.68	0.093	1.27	0.204	0.84	0.40	**2.15**	**0.032**
	T/A	0.15	0.88	-0.78	0.44	-0.39	0.70	-0.49	0.62	-1.15	0.25
	A/A	--	--	**-2.26**	**0.024**	**-2.29**	**0.022**	--	--	**-2.40**	**0.016**
rs3758249	T/T	--	--	**-2.26**	**0.024**	**-2.29**	**0.022**	--	--	**-2.40**	**0.016**
	T/C	0.15	0.88	-0.51	0.61	-0.19	0.85	-0.48	0.63	-1.15	0.25
	C/C	0.078	0.94	1.39	0.70	1.06	0.29	0.82	0.41	**2.15**	**0.032**
rs4460498	C/C	1.721	0.085	1.78	0.076	2.43	0.015	-0.81	0.42	0.85	0.40
	C/T	-1.02	0.31	-1.55	0.12	-1.90	0.058	0	1	-1.01	0.31
	T/T	-0.86	0.39	-0.074	0.94	-0.45	0.65	1.12	0.26	0.42	0.68

Note: NSCLO, Non-syndromic cleft lip only; NSCLP, Non-syndromic cleft lip with cleft palate; NSCL/P, Non-syndromic cleft lip with or without cleft palate, including NSCLO and NSCLP; NSCPO, Non-syndromic cleft palate only; NSOCs, the total cases including NSCL/P and NSCPO; SNP, Single Nucleotide Polymorphism; Z, vector of the large sample Z statistic; Bold characters indicate the items with *P*-value less than 0.05.

Table 4. Parent-of-Origin Effects for SNPs in *FOXE1*

Phenotype	SNP	Minor Allele	Paternal		Maternal		Z	P
			T/U	P	T/U	P		
NSCLO	rs894673	A	13/14	0.85	10/10	1	-0.13	0.90
	rs3758249	T	13/14	0.85	10/10	1	-0.13	0.90
	rs4460498	T	13/14	0.85	9/10	0.82	0.052	0.96
NSCLP	rs894673	A	11.5/22.5	0.059	10.5/20.5	0.072	-0.004	1
	rs3758249	T	12.5/22.5	0.091	11.5/20.5	0.11	-0.019	0.98
	rs4460498	T	11.5/22.5	0.059	10.5/19.5	0.10	-0.099	0.92
NSCL/P	rs894673	A	24.5/36.5	0.12	20.5/30.5	0.16	-0.0035	1
	rs3758249	T	25.5/36.5	0.16	21.5/30.5	0.21	-0.023	0.98
	rs4460498	T	24.5/36.5	0.12	19.5/29.5	0.13	0.039	0.97
NSCPO	rs894673	A	5.5/9.5	0.30	10.5/13.5	0.54	-0.437	0.66
	rs3758249	T	6.5/9.5	0.45	10.5/14.5	0.42	-0.087	0.93
	rs4460498	T	4.5/9.5	0.18	10.5/12.5	0.68	-0.81	0.42
NSOCs	rs894673	A	9/12	**0.028**	13/24	0.071	-0.44	0.66
	rs3758249	T	9/12	**0.028**	13/24	0.071	-0.44	0.66
	rs4460498	T	8/12	**0.016**	13/22	0.13	-0.81	0.42

Note: NSCLO, Non-syndromic cleft lip only; NSCLP, Non-syndromic cleft lip with cleft palate; NSCL/P, Non-syndromic cleft lip with or without cleft palate, including NSCLO and NSCLP; NSCPO, Non-syndromic cleft palate only; NSOCs, the total cases including NSC/LP and NSCPO; SNP, Single Nucleotide Polymorphism; Z, vector of the large sample Z statistic; T/U, transmitted/untransmitted; Bold characters indicate the items with *P*-value less than 0.05.

of rs894673-rs4460498 and A-C-C of rs894673-rs3758249-rs4460498 were statistically under-transmitted (**Table 5**). To sub-phenotypes, the haplotypes show equally result among NSCLP trios (**Table 6**). But no significant association was founded in other trios of sub-phenotypes.

G×E interaction

Based on our previous findings [26], we took the associated maternal environmental exposure factors during the first trimester in this analysis to evaluate if these exposure factors interacts with the *FOXE1* SNPs. however, there was no interactions identified (data not shown).

Discussion

FOXE1 gene located in the region 9q22, and there are several possible candidate genes of NSOCs in this region, such as *PTCH*, *ROR2*, *ZNF189*, while only SNPs in or near *FOXE1* showed genome-wide significant associations

Table 5. Markers and sliding window haplotypes in 440 Chinese case-parent trios in NSOCs

Marker/Haplotype			Allele freq	Trios (N)	P
rs894673	rs3758249	rs4460498			
Over-transmitted					
T	C	-	0.89	132	**0.029**
-	C	C	0.89	129	**0.022**
T	-	C	0.89	129	**0.022**
T	C	C	0.89	129	**0.022**
Under-transmitted					
A	T	-	0.11	133	**0.036**
-	T	T	0.11	129	**0.034**
A	-	T	0.11	128	**0.027**
A	T	T	0.11	129	**0.034**

Note: N, Number of informative case-parent trios; P, The *p*-value for a statistical model with whole haplotypes consisting of two or three SNPs; Bold characters indicate the items with *P*-value less than 0.05.

Table 6. Markers and sliding window haplotypes in 440 Chinese case-parent trios in NSCLP

Marker/Haplotype			Allele freq	Trios (N)	P
rs894673	rs3758249	rs4460498			
Over-transmitted					
T	C	-	0.88	56	**0.011**
-	C	C	0.88	56	**0.011**
T	-	C	0.88	56	**0.011**
T	C	C	0.88	56	**0.011**
Under-transmitted					
A	T	-	0.12	56	**0.011**
-	T	T	0.12	55	**0.015**
A	-	T	0.12	55	**0.011**
A	T	T	0.12	55	**0.015**

Note: N, Number of informative case-parent trios; P, The *p*-value for a statistical model with whole haplotypes consisting of two or three SNPs; Bold characters indicate the items with *P*-value less than 0.05.

with NSOCs through multipoint linkage results (HLOD and GSMA) [27]. *FOXE1* gene associated with both of the syndromic clefts and non-syndromic clefts, so far, six mutations of it have been reported to participate in Bamforth-Lazarus Syndrome with the features of congenital hypothyroidism and cleft palate [28, 29]. Developmental data showed that *FOXE1*-null mice has an extensive cleft of the secondary palate with athyreosis or ectopic thyroid gland [30]; *FOXE1* is highly expressed in media edge epithelium from E12.5-E14.5 and if it was over expressed at the high point during the palatogenesis could result in the palate shelf failing to fuse [31].

The target genes of *FOXE1*, *MSX1* and *TGF-b3* were the critical genes during the craniofacial development [32]. Mutated *FOXE1* could regulate and decrease the expression of *MSX1* and *TGF-b3*, and *MSX1* and *TGF-b3* failed to express in palate shelf in *FOXE1-/-* compared with *FOXE1+/-* embryos. Furthermore, *FOXE1* also interacted with *GLI2* in *Shh/Gli* pathway [33, 34] which is involved in primary palatogenesis.

From the information above, it is easy to know that *FOXE1* would be a good candidate gene to study its role in orofacial clefts. Since it was identified to be associated with NSOCs, there were numerous replication studies across different populations, however, the results were inconsistent and none of relevant study was performed in Western Han Chinese population. So we selected three SNPs with good evidence to test them.

TDT results showed three SNPs was associated in both of allelic and genotypic model. Specially, in allelic analysis, A allele at rs894673 T allele at both rs3758249 and rs4460498 were strongly under-transmitted which indicated that they were protective for NSOCs and could decrease the risk of having a NSOCs baby. It is supposed by the result of genotypic analysis that A/A at rs894673 and T/T at rs3758249 were under-transmitted but T/T at rs894673 and C/C at rs3758249 were over-transmitted, pointing the effect of increasing the risk of NSOCs.

And it is interesting to find that the haplotype consisted of the under-transmitted allele was also showed under-transmitted: A-T of rs8946-73-rs3758249 T-T of rs3758249-rs4460498 A-T of rs894673-rs4460498 and A-C-C of rs894673-rs3758249-rs4460498 were under-transmitted among NSOCs and NSCLP, suggesting they were protective factor to against NSOCs and NSCLP (**Tables 5**, **6**).

Effects of parent-of-origin were important in NSCL/P [35], maternal over-transmission of *FOXE1* alleles in the Colombian and Filipino population, and paternal over-transmission of *FOXE1* alleles in the USA population were reported [18]. In this study, we want to know if the under-transmitted allele was originated

from one of the parent, we performed the parent-of-origin effect analysis, and we observed a paternally over-transmitted allele A at rs894673 T at rs3758249 and T at rs4460498 for NSOCs, but no significance between maternal and paternal transmission. G×E interaction played important roles in the etiology of orofacial clefts [36, 37], however, we didn't find any significant factors based on the current exposure risks [26].

There is a replication study performed in Northeastern Han Chinese population with rs3758249 and r4460498 [38] and the results showed that they were associated with NSCLP, which is consist with our result, indicating the etiologic mechanism between NSCLO and NSCLP probably distinct; and another case-control study [39] based on the population of Southeastern Han Chinese population found rs7043516 was associated with NSCLO. China is a huge country and different region has various genetic backgrounds, so it is necessary to perform replication studies. Rs4460493 was once delightedly discovered the contribution to both NSCL/P and NSCPO in central Europe population [19], but none of these studies performed in Chinese population identified associations between with NSCPO and *FOXE1.*

In conclusion, our study showed that rs894673, rs3758249 and rs4460498 was associated with NSOCs from Western Han Chinese population. It could give more evidence for future research in search for the causal genes of NSOCs.

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Disclosure of conflict of interest

None.

Address correspondence to: Zhong-Lin Jia, State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, No. 14, 3rd Section, Renmin Nan Road, Chengdu 610041, China. Tel: +86-02885503462; Fax: +86-02885502848; E-mail: zhonglinjia@sina.com

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