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Letter to the Editor

TYMS gene 5′- and 3′-untranslated region polymorphisms and risk of non–syndromic cleft lip and palate in an Indian population

Dear Editor:

Increased homocysteine levels due to vitamin B6 or B12 deficiency or genetic defects in folate pathway genes are associated with an increased incidence of non-syndromic cleft lip with or without cleft palate (NSCLP)[1]. Thymidylate synthase (TS) is a folate-dependent enzyme that catalyzes methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to 2′-deoxythymidine-5′-monophosphate (dTMP), a rate-limiting step in DNA synthesis, for which 5,10-methylene-tetrahydrofolate (CH_2-THF) is the methyl donor. TS competes with 5,10-methylenetetrahydrofolate reductase (MTHFR) for the availability of CH_2-THF. The *TYMS* gene is located on chromosome 18p11.32 and is about 30 kb in length with 7 exons[2]. Two most extensively studied *TYMS* variants are located in the promoter enhancer region of the 5′-untranslated region (UTR) and 3′-UTR. The VNTR polymorphism (rs45445694) is located in 5′-UTR, consisting of 2 or 3 tandem repeats of 28 bp (2R or 3R). A 6-bp insertion and deletion polymorphism (indel) has been identified in the 3′-UTR (rs16430) of the *TYMS*[3]. These 2 polymorphisms have been extensively studied for association with cleft lip and palate[4]. Although the *TYMS* plays a critical role in fetal development, so far it has not yet been reported to be associated with NSCLP in the Indian population. In this study, we investigated the effects of *TYMS* functional variants (rs45445694 and rs16430) on the risks of NSCLP in a southern Indian population.

The study was carried out in 283 ethnically matched unrelated subjects, including 142 unrelated NSCLP patients (123 with cleft lip and palate and 19 with cleft palate only) and 141 healthy controls without family history of cleft. Subjects with malformation syndromes and major developmental disorders were excluded. The study was approved by the local institutional ethics committee and written informed consent was obtained from all the participants. Peripheral blood (3 mL) was collected from each subject and DNA was obtained using a standard procedure. *TYMS* 6-bp indel genotyping was performed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method[5]. *TYMS* 5′-UTR VNTR was genotyped according to the PCR method[6]. Allele frequencies were estimated by the gene counting method. Hardy-Weinberg equilibrium (HWE) was performed to assess the cases and control groups using chi-square test. Association between two *TS* SNPs and different cleft phenotypes (NSCLP, CLP and cleft palate only (CPO)) was analyzed by χ^2-test. Odds ratio and 95% confidence inter-

Table 1 **Association of TYMS VNTR polymorphism with NSCLP**

	Control	NSCLP	OR (95% CI)	*P*	CLP	OR (95% CI)	*P*	CPO	OR (95% CI)	*P* value
3R3R	65	59	Reference		54	Reference		5	Reference	
2R3R	60	79	1.45 (0.89-2.36)	0.006*	67	1.34 (0.81-2.21)	0.003*	12	2.60 (0.86-7.81)	0.213*
2R2R	16	4	0.27 (0.08-0.81)		2	0.15 (0.03-0.69)		2	1.62 (0.28-9.15)	
2R3R+2R2R	76	83	1.20 (0.75-1.92)	0.440	69	1.09 (0.67-1.77)	0.720	14	2.39 (0.81-7.10)	0.102
3R allele	190	197	Reference		173	Reference		20	Reference	
2R allele	92	87	0.91 (0.64-1.30)	0.610	71	0.85 (0.58-1.23)	0.383	16	1.65 (0.82-3.34)	0.158

NSCLP: non-syndromic cleft lip; OR: odd risk; CI: confidence interval; CLP: cleft lip; CPO: cleft palate only. *P value by χ^2 test (df=2).

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Table 2 **Association of *TYMS* gene 6 bp indel with NSCLP**

	Control	NSCLP	OR (95%CI)	*P*	CLP	OR (95%CI)	*P*	CPO	OR (95%CI)	*P*
-6 bp/-6 bp	32	19	Reference		19	Reference		0	Reference	
+6 bp/-6 bp	69	77	1.89(0.98-3.61)	0.124*	62	1.51(0.78-2.94)	0.284*	15	-	0.023*
+6 bp/+6 bp	40	46	1.94(0.95-3.93)		42	1.77(0.87-3.61)		4	-	
(+6 bp/-6 bp)+(-6 bp/-6 bp)	109	123	1.90(1.02-1.833)	0.041	104	1.61(0.86-3.01)	0.136	19	-	
-6 bp allele	133	115	Reference		100	Reference		15	Reference	
+6 bp allele	149	169	1.31(0.94-1.83)	0.109	146	1.31(0.92-1.85)	0.132	23	1.37(0.69-2.73)	0.372

NSCLP: non-syndromic cleft lip; OR: odd risk; CI: confidence interval; CLP: cleft lip; CPO: cleft palate only. *P value by χ^2 test (df=2).

vals (CI) were calculated using low risk genotypes or alleles as the reference group.

The genotype frequencies of *TYMS* VNTR and 6 bp indel are shown in ***Table 1*** and ***Table 2***, respectively. The genotype frequencies were in HWE in the control group of *TYMS* VNTR (P=0.704) and 6 bp indel (P=0.830). TYMS VNTR polymorphism showed significant difference in genotypic frequencies between NSCLP (P= 0.006) and CLP (0.003) compared to the controls (***Table 1***). The *TYMS* 6 bp indel genotype and allele frequencies were not significantly different between the NSCLP and control group (***Table 2***). In subgroup analysis, 6 bp indel showed significant association with the CPO group (***Table 2***). To estimate relative risk of NSCLP, we calculated OR and 95%CI in co-dominant, dominant and allelic models. None of the models revealed significant association between the TYMS VNTR and NSCLP group (***Table 1***). For TS 6 bp indel, risk analysis showed increased risk in all 3 models and increased risk reached significant level in the dominant model for the NSCLP group (OR=1.90; 95%CI=1.02-1.83 and P=0.041) (***Table 2***).

Although TS is a folate-dependent enzyme, there are very few studies on the association of the *TYMS* gene with human orofacial clefts. TS is an autoregulatory protein composed of 313-amino acids and binds to its messenger RNA (mRNA) directly and inhibits mRNA translation. A Norway family based association study did not find evidence of an association between several *TYMS* variants and folate intake on risk of orofacial clefts[6]. A family-based association study of NSCLP with *TYMS* gene variants showed altered familial transmission of haplotypes in the non-Hispanic group for cleft risk[7]. However, these two studies did not investigate the TYMS variants in
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The 5′-UTR VNTR was found to influence the efficiency of *TYMS* expression. *TS* genes with the 2R2R repeat sequence showed that the expression activity of the gene was lower than that of the gene with the 3R3R repeat sequence[8]. A recent case-control study showed that the homozygous 2R2R repeat sequence influenced CP risk[4]. Another 6 bp indel suggest that the homozygous insertion (+6 bp/+6 bp) had significantly higher *TS* mRNA levels compared to individuals with homozygous deletion (-6 bp/-6 bp), which is associated with decreased *TYMS* mRNA stability[9]. Individuals with the homozygous (-6 bp/-6 bp) genotype have higher red blood cell folate levels and lower plasma homocysteine levels compare to (+6 bp/+6 bp) or (+6 bp/-6 bp) genotypes[10]. A recent case-control study does not indicate that SNP rs16430 genotype contributes to CLP or CP risks either alone or in combination with folate intake[4].

In conclusion, we report that the *TYMSS* 5'UTR VNTR and 3'UTR 6-bp indel are significantly associated with increased risk of NSCLP in a southern Indian population. To obtain more evidence on the association between *TYMS* polymorphisms and NSCLP, population studies conducted among other ethnicities are required. Furthermore, the analysis of biochemical mechanisms of the *TYMS* polymorphisms in the pathogenesis of NSCLP requires investigation.

Yours sincerely,

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