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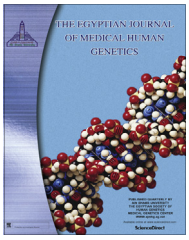

ORIGINAL ARTICLE

Association of MTHFR polymorphisms with nsCL/P in Chinese Uyghur population

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Abstract *Background:* nsCL/P (nonsyndromic cleft lip with or without cleft palate) is a complex disorder with a multifactorial etiology that involves both genetic and environmental factors. A number of studies identified that MTHFR (methylenetetrahydrofolate reductase) was associated with nsCL/P in different populations.

Aim: In this study, we aim to investigate the association between the polymorphism in MTHFR gene (rs1801131, rs1801133) and nsCL/P in Chinese Uyghur population.

Subjects and methods: We conducted a case–control study comparing 120 nsCL/P patients to 100 controls. The distribution of MTHFR genotypes and frequency of alleles were compared between patients and controls by chi-square test. The odds ratios (OR) and corresponding 95% confidence intervals (95% CIs) were calculated to estimate the strength of association of MTHFR gene (C677T and A1298C).

Results: For rs1801131, allele C frequency was higher in cases than in controls (30.4% > 19.0%), and the difference was statistically significant ($\chi^2 = 7.538$, $P = 0.006$). The genotype distribution at rs1801131 was different between cases (AC > AA > CC, 49.2% > 45.0% > 5.8%) and controls (AA > AC > CC, 65.0% > 32.0% > 3.0%), and the difference was statistically significant ($\chi^2 = 8.883$, $P = 0.012$). AC genotype was found to increase the susceptibility of nsCL/P (OR = 2.219, 95% CI = 1.266–3.892, $P = 0.005$). No association was found between rs1801133 and nsCL/P.

Conclusion: This study indicated that rs1801131 polymorphism in MTHFR was associated with nsCL/P in Chinese Uyghur population. Given the unique genetic and environmental characters of the Uyghur population, these findings may be helpful for exploring the pathogenesis of this complex disease.

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1. Introduction

Non-syndromic cleft lip with or without cleft palate (nsCL/P)[2] is one of the most frequently occurring birth defects worldwide [1]. It has a significant adverse effect on children's feeding, hearing, speaking and social integration, and imposes a substantial financial risk for families with a concomitant societal burden [2]. In China, the prevalence of nsCL/P is estimated to be as high as 1.42/1000, making it one of the five leading causes of perinatal deaths [3]. Therefore, the prevention and treatment of nsCL/P remains to be a great challenge in China.

The etiology of nsCL/P is complex, encompassing both genetic and environmental factors. Epidemiologic studies found that mothers who used multivitamins containing folic acid showed a significant reduction in risk for offspring with nsCL/P compared with mothers not using multivitamins [4]. Evidence from experimental studies also supports the hypothesis that folic acid supplement is associated with a reduced risk of a nsCL/P [5]. However, in a large meta-analysis including 2586 cases and 59,684 controls, there is no strong association between oral clefts and folic acid intake alone [6]. The inconsistent results may in part be due to the genetic variations in folate metabolism gene in different populations.

From a genetic standpoint, a polymorphic mutation in the gene encoding 5,10 methylenetetrahydrofolate reductase (MTHFR) has been shown to correlate with an increased risk of nsCL/P [7]. Two common single nucleotide polymorphism (SNP) variants of the MTHFR gene, C677T (rs1801133) and A1298C (rs1801131), have been studied as candidate genetic factors for nsCL/P risk. Semiç-Jusufagiç et al. found that the mothers of the study group had a higher frequency of 677TT genotype, with a threefold increased risk of having nsCL/P offspring [8]. A large meta-analysis based on 17 case-control studies found that CT heterozygote, TT homozygote, and CT/TT of infants' MTHFR C677T variant could contribute to elevated risks of nsCL/P in the Asian population [9], including Turkey, India and China. However, the studies about the association between MTHFR gene and risk of nsCL/P in the Chinese population were mainly conducted in the eastern China and Han population. It is unclear to what extent these associations apply to other ethnic populations and regions in China. More study is needed to confirm the association between MTHFR gene and risk of nsCL/P in the Chinese population.

Uyghur is the second largest minority group Xinjiang Uyghur Autonomous region (western China) [10]. The living environment, religion, racial background, custom, and socioeconomic status of Uyghur is distinct from the Han Population. It has a population of more than 10 million with the incidence (1.96/1000) of nsCL/P, which is greater than the average national level [11]. However, to our knowledge, very few studies have been conducted on the candidate genes associated with nsCL/P in the Uyghur population and no study has been published in the English literature. In the present study, we performed a case-control study to determine if MTHFR gene polymorphism has an association with nsCL/P in Xinjiang Uyghur population. We focused on the SNP variants in the MTHFR gene (C677T and A1298C) and the risk of nsCL/P in Uyghur population, trying to provide some beneficial information about the etiology of nsCL/P.

[2] *Abbreviations:* nsCL/P, non-syndromic cleft lip with or without cleft palate; MTHFR, 5,10 methylenetetrahydrofolate reductase; HW, Hardy–Weinberg (HW) equilibrium.

2. Subjects and methods

2.1. Subjects

The study had been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving human subjects. The inclusion criteria were (a) Uyghur nationality; (b) congenital nsCL/P (c) non-syndromic cleft lip with cleft palate. The exclusion criteria were (a) Cleft and palate associated with any of the following: neural tube defects, congenital heart disease, Kabuki syndrome, Meckel syndrome and velocardiofacial syndrome; (b) with serious systemic diseases, such as hypertension and coronary heart disease. The study sample is composed of 220 biologically unrelated patients of Xinjiang Uyghur origin, 120 cases with nsCL/P and 100 individuals without clefts or a family history of clefting were considered as controls. All cases and controls were consecutively recruited from January 2010 to December 2012 at 5 hospitals in the Xinjiang province (the first Affiliated hospital of Xinxiang Medical University, People's Hospital of Xinxiang Uyghur autonomous region, the Yili People's Hospital, the Hotan People's Hospital, the Kashgar People's Hospital).To verify the nsCL/P status and to exclude known teratogenic influences, all the subjects were examined clinically by two oral and maxillofacial surgeons for their individual phenotypic features, and their medical history was asked on the specific questions about the familial presence of other somatic and neurological disorders and the use of drugs known to cause oral clefts (phenytoin, warfarin, and ethanol). Parental informed consent was given for each study participant for both the blood collection and subsequent genotyping. The study was approved by the Ethics committee of Xinjiang Medical University.

2.2. DNA extraction, library preparation, and sequencing

Genomic DNA was isolated from the 2 ml peripheral blood taken from individuals by following the manufacturer's standard procedure using QIA quick Gel Extraction kit (Qiagen, Hilden, Germany). Then, DNA Purity was tested by a ratio of OD values at 230:260:280 using Invitrogen Qubit Spectrophotometer. The amplification reactions were carried out on a AB 2720 Thermal Cycler (Life Technologies Corporation, USA) with the following conditions: the cycling program was: predenaturation at 94 °C for 5 min; 30 cycles at 94 °C for 30 s, annealing at 55 °C for 40 s, and elongation at 72 °C for 45 s, and extension at 72 °C for 10 min. Then, the polymerase chain reaction (PCR) productions of 220 samples were visualized using 1.7% agarose gel electrophoresis and purified with the help of exonucleases ExoI and FastAP. PCR products were genotyped using an ABI PRISM 3730XL DNA Sequencer (Applied Biosystems) according to the manufacturer's instructions.

2.3. Statistical analysis

Hardy–Weinberg (HW) equilibrium was evaluated in both affected individuals and controls by using a Pearson chi-

square test. Comparison of genotype and allele frequencies between cases and controls was analyzed by the χ^2 test and Fisher exact tests. The odds ratios (OR) and corresponding 95% confidence intervals (95% CIs) were calculated to estimate the strength of association of MTHFR gene (C677T and A1298C) SNPs between cases and controls using SPSS version 17 (SPSS Inc, Chicago, Illinois, USA), and a P value less than 0.05 was considered statistically significant. Pairwise linkage disequilibrium (LD) between polymorphisms was estimated and expressed in terms of the D' and r^2 statistics, as implemented in SHEsis software. The SHEsis software was also used for haplotype analysis of MTHFR polymorphism at the rs1801131 and rs1801133 loci.

3. Results

The average age of patients for cases and control groups was 1.98-year and 1.81-year, respectively .The age and gender distributions were similar between case and control group ($P > 0.05$) (Table 1). Three genotypes of rs1801131 (AA,AC, CC) and rs1801133 (CC,CT,TT) were detected by SNaPshot analysis (Fig.1). The genotype distribution of rs1801131 polymorphism and rs1801133 polymorphism did not deviate from expectation based on Hardy–Weinberg equilibrium in both control ($P = 0.692$ for rs1801131 and $P = 0.971$ for rs1801133, respectively) and nsCL/P groups ($P = 0.077$ for rs1801131 and $P = 0.609$ for rs1801133, respectively) (Table 2).

The rs1801131 C allele was significantly more frequent in the nsCL/P (30.4%) group than in controls (19.0%) ($\chi^2 = 7.538$, $P = 0.006$), yielding an odds ratio of 1.864 (95% CI:1.191–2.916) (Table 3). Distribution of genotypes was significantly different between cleft patients and controls. For rs1801131 the presence of risk alleles (AC + CC) was more frequent in all cleft subjects (55%) in comparison to controls (35%) and the difference was statistically significant (OR = 2.27, 95% CI: 1.32–3.92, $P = 0.002$) (Table 4). For rs1801133, the presence of risk alleles (CT + TT) was almost similar in all cleft subjects (70.8%) in comparison to controls (78.0%) and the difference was not statistically significant (OR = 0.68, 95% CI: 0.37–1.27, $P = 0.227$) (Table 3).

In this study, the pairwise LD analyses showed that the D' and r^2 value between rs1801131 and rs1801133 was very low (0.206 and 0.014, respectively), indicating no significant LD between these two SNPs. Four different haplotypes were prepared (AC, AT, CC, CT). The haplotype AC and CT showed no evidence of association with nsCL/P ($\chi^2 = 0.040$, $P = 0.841$ and $\chi^2 = 2.535$, $P = 0.111$ respectively). In contrast,

Table 1 General characteristics between nsCL/P and control groups.

Variable	nsCL/P $N = 120$	Control $N = 100$	T value or χ^2	P value
Age (years, mean ± SD)	1.98 ± 0.91	1.81 ± 0.71	1.396	0.165
Gender (male/female)	62/58	55/45	0.243	0.622
Body mass index (normal/abnormal)	106/14	78/12	0.132	0.833

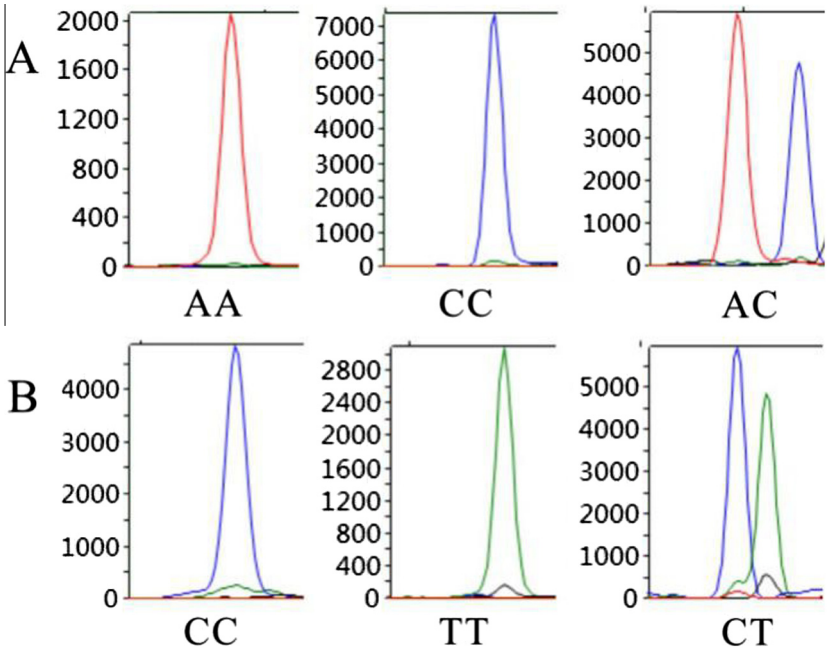

Figure 1 Genotyping of rs1801131 and rs1801133. (A) AA, CC and AC genotypes of rs1801131, AA genotype appeared as red peak in the chromatogram, CC genotype appeared as blue peak, AC genotype appeared as double peaks; (B) CC, TT and CT genotypes of rs1801131, CC genotype appeared as blue peak in the chromatogram, TT genotype appeared as green peak, CT genotype appeared as double peaks.

Table 2 Hardy–Weinberg equilibrium analysis for rs1801131 and rs1801133.

Group		rs1801131			χ^2	*P*	rs1801133			χ^2	*P*
		AA	AC	CC			CC	CT	TT		
nsCL/P	Observed	54	59	7	3.130	0.077	35	57	28	0.262	0.609
	Expected	58.1	50.8	11.1			33.6	59.8	26.6		
Control	Observed	65	32	3	0.157	0.692	22	50	28	0.001	0.971
	Expected	65.6	30.8	3.6			22.1	49.8	28.1		

Table 3 MTHFR rs1801131 and rs1801133 polymorphism in nsCL/P and controls (*n*, %).

SNP	nsCL/P (n = 120)	Control (n = 100)	χ^2	*P*
rs1801131				
Genotype frequency, *N* (%)			8.883	0.012
AA	54(45.0)	65(65.0)		
AC	59(49.2)	32(32.0)		
CC	7(5.8)	3(3.0)		
Allele frequency, *N* (%)			7.538	0.006
A	167(69.6)	162(81.0)		
C	73(30.4)	38(19.0)		
rs1801133				
Genotype frequency, *N* (%)			1.618	0.445
CC	35(29.2)	22(22.0)		
CT	57(47.5)	50(50.0)		
TT	28(23.3)	28(28.0)		
Allele frequency, *N* (%)			1.528	0.216
C	127(52.9)	94(47.0)		
T	113(47.1)	106(53.0)		

Table 4 Logistic regression analysis of rs1801131 AC genotype and C allele.

Variable	Control	*P*	OR	95% CI
C allele	A allele	**0.006**	1.864	1.191–2.916
AC genotype	AA genotype	**0.005**	2.219	1.266–3.892

Table 5 The distribution of the main haplotype frequencies of two MTHFR genetic polymorphisms in nsCL/P case and control in Uygur.

Haplotype	Uygur frequency		χ^2	*P* value	Odds ratio (95% CI)
	Case	Control			
AC	0.346	0.356	0.040	0.841	0.961(0.648–1.423)
AT	0.349	0.454	5.024	**0.025**	**0.645(0.439–0.947)**
CC	0.183	0.114	3.957	**0.047**	**1.731(1.004–2.984)**
CT	0.121	0.076	2.535	0.111	1.691(0.881–3.245)

the haplotype AT was associated with a significantly decreased risk of nsCL/P (χ^2 = 5.024, P = 0.025; OR = 0.645, 95% CI: 0.439–0.947). The haplotype CC frequency was significantly higher in cases than in controls (χ^2 = 3.957, P = 0.047; OR = 1.731, 95% CI: 1.004–2.984) and it was associated with an increased risk of nsCL/P (Table 5).

4. Discussions

As a complex disease, many genes and loci were demonstrated to be risk factors to nsCL/P, but few of them were replicated in different populations, particularly those from central and eastern Asia [8,12]. Therefore, studies with samples from different populations are essentially required, as part of the multistep approach in the identification of polymorphism-disease associations. It is not only vital for the identification of the common or population-specific risk alleles, but also the construction of the best model to explain this complex disease. We conducted a case–control study with independent samples to verify the contribution of MTHFR rs1801131 (A1298C) and rs1801133 (C677T) polymorphisms to nsCL/P in the Uyghur population. This approach is based on the premise that if the same effect of a disease-marker association can be obtained from a case–control study, the magnitude of information is strong and true.

MTHFR is a key enzyme in folate metabolism [13]. It catalyzes the conversion of 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate, the predominant circulating form of folate [14]. MTHFR gene polymorphisms can affect MTHFR activity and reduce the concentration of folate in serum, plasma and red blood cells, and mildly increase plasma total homocysteine, which was supposed to be associated with increased risk of nsCL/P [15]. However, though a number of studies had explored the role of MTHFR gene polymorphisms in nsCL/P, the results were still contradictory [16]. In the present study, we found that rs1801131 (A1298C), but not C677T (rs1801133), was associated with increased risk of nsCL/P in

the Uyghur population. These results were in accordance with the findings from Iran [17], Middle China [18], but different from those in eastern China [19] and western countries. These differences may be due to the ethnic variations of MTHFR gene polymorphisms. In a meta-analysis based on studies that were mostly performed on Caucasian populations, the MTHFR C677T (rs1801133) polymorphism and nsCL/P showed pooled ORs (95% CI) of 1.2 (0.9–1.5) in mothers and 1.0 (0.9–1.2) in children, whereas those estimates for the A1298C (rs1801131) polymorphism were 1.0 (0.7–1.2) in mothers and 0.9 (0.6–1.2) in children [20]. In contrast, in another meta-analysis based on Asian population, the MTHFR C677T polymorphism and nsCL/P showed pooled ORs (95% CI) of 1.41(1.23–1.61) in children, and 1.70(1.19–2.42) in mothers [21]. Xinjiang province located at the crossroads of Eurasian to Europe, was strategically placed on the ancient trading routes from Asia to Europe, known as the Silk Road [10]. It's probable that the genetic Eurasian admixture between the Caucasoid and Mongoloid populations, namely genetic excursion induced by emigration along the ancient Silk Road, may influence the association between MTHFR polymorphism and nsCL/P in Uyghur populations.

In addition, the geographical distribution of MTHFR gene may also influence the association between MTHFR polymorphism and nsCL/P in Uyghur populations [22]. Yang et al. found that there are marked geographical variations in the prevalence of C677T (rs1801133) and A1298C (rs1801131) among Chinese Han populations [22]. For MTHFR C677T, the frequencies of the 677T allele and the 677TT genotype were significantly higher among northern populations. In contrast, for MTHFR A1298C, the 1298C allele and the 1298CC genotype frequencies were significantly higher among southern populations [22]. Previous studies had indicated that nutritional and environmental factors, such as folate intake [23] and ultraviolet (UV) radiation [24] could influence the distribution pattern of MTHFR C677T polymorphism. The relative low intake of folate [25]and high UV radiation exposure [26] in Uyghur may influence the association between MTHFR polymorphism and nsCL/P in these populations.

There were several drawbacks associated with our study. First, the sample size of our study was relatively small, raising the possibility of false-positive and false-negative errors. Second, the information about family history was not obtained from the patients, which may limit the further exploration of the role of MTHFR polymorphism on nsCL/P in Uyghur. Nevertheless, we enrolled the subjects according to strict inclusion and exclusion criteria, which may increase the power of the study.

In conclusion, the results from our study suggested that rs1801131 (A1298C) was associated with increased risk of nsCL/P in the Uyghur population. To our knowledge, this is the first study that revealed the association between MTHFR polymorphism and nsCL/P occurrence in Uyghur. Our findings supplement worldwide reports on MTHFR polymorphisms and may be helpful for exploring the pathogenesis of this complex disease.

Conflict of interest

There is no conflict of interest.

Acknowledgements

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