

## BHMT Gene Polymorphisms as Risk Factors for Cleft Lip and Cleft Palate in a Chinese Population\*

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### Abstract

**Objective** Convincing evidence suggests a link between increased risk of nonsyndromic cleft lip with or without cleft palate (NSCL/P) and low intake of folic acid by the mother during pregnancy. The present study was designed to explore if genetic variation in the betaine-homocysteine methyltransferase (BHMT) gene contributes to NSCL/P.

**Methods** DNA was obtained from 166 individuals with NSCL/P and 285 healthy subjects. Three known single nucleotide polymorphisms (SNPs) present in the BHMT gene (rs651852, rs3797546, and rs3733890) were investigated by real-time PCR-based TaqMan genotyping.

**Results** Neither allelic nor genotypic association was found between NSCL/P and SNPs rs651852 and rs3733890. SNP rs3797546 did not show allelic association with NSCL/P; however, a higher proportion of NSCL/P patients carry the CC genotype compared with the TT+CT genotype ( $P=0.020$ ,  $OR=2.10$ ,  $95\% CI=1.11-3.95$ ).

**Conclusion** Our study suggests that polymorphism rs3797546 in the BHMT gene may confer genetic risk of NSCL/P in a recessive manner.

**Key words:** Nonsyndromic cleft lip with or without palate; Homocysteine; Folate; Polymorphisms

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### INTRODUCTION

**N**onsyndromic cleft lip with or without cleft palate (NSCL/P) (OMIM 119530) is one of the most common congenital malformations. The global prevalence of NSCL/P ranges from 1 in 300 to 1 in 2000, and is determined by a number of factors, including geographical origin, ethnicity, and socioeconomic status<sup>[1-2]</sup>. The Asian population (especially Chinese and Japanese races) has a higher incidence than the African-American population<sup>[3-4]</sup>. Despite considerable improvements in our understanding, the etiology and pathogenesis of NSCL/P remain poorly understood. Genome-wide linkage studies have identified several regions in the

human genome that are likely to contain susceptibility genes for orofacial clefting<sup>[5-6]</sup>. This reiterates not only the complexity and diversity of the anomaly, but also the wide range of likely molecular mechanisms involved during embryogenesis within NSCL/P. A combination of genetic and environmental factors is inextricably linked to the development of the anomaly<sup>[7]</sup>.

Periconceptional vitamin supplementation with folic acid has been indicated as a preventative measure that reduces the risks of this oral facial cleft<sup>[8-12]</sup>. The precise mechanisms that are modified to reduce these risks have not been determined, but genetic variation in genes that alter cellular absorption, transport, and metabolism of

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folate/homocysteine may either confer or reduce susceptibility to the disease<sup>[13]</sup>. Betaine-homocysteine methyltransferase (BHMT) is an essential enzyme that drives the remethylation reaction from homocysteine to methionine, and is involved in conserving methionine and detoxifying homocysteine. Given that *BHMT* is responsible for up to 50% of the homocysteine methylation capacity of the liver<sup>[14]</sup>, dysfunction of this enzyme might result in the accumulation of homocysteine. Several studies have identified hyperhomocysteinemia (HyperHcy) in children and mothers<sup>[15-16]</sup> with NSCL/P. This suggests that a disturbed folate-dependent homocysteine (Hcy) metabolism may be involved in NSCL/P. The human *BHMT* gene is located on chromosome 5q13.1-5q15 and encodes a protein of 406 amino acids. The gene spans approximately 20 kb of DNA and consists of eight exons. Significantly, and of relevance amongst SNPs present in *BHMT*, SNP rs3733890 has been found to confer a risk of neural tube defects (NTD) rather than conotruncal heart defects<sup>[13,17-18]</sup> by interacting with 5,10-methylenetetrahydro folate reductase (MTHFR). However, this SNP did not show an association with NSCL/P in American children<sup>[19]</sup>. Homozygotes of rs3733890 (in a Norwegian population) have a low risk of NSCL/P where there has been maternal supplementation with >400 µg folate before or during conception<sup>[8]</sup>.

Given that *BHMT* plays a pivotal role in folic acid metabolism and Hcy methylation, disease-underlying variants of the *BHMT* gene or abnormal expression of the gene may contribute to the etiology of NSCL/P. There has been no previous report on the *BHMT* gene and NSCL/P in a Chinese population. Therefore, the present study was undertaken to investigate the *BHMT* gene as a risk factor for NSCL/P with a Chinese sample.

## MATERIALS AND METHODS

### Subjects

A cohort of 166 NSCL/P patients, which included isolated cleft lip with or without palate, was identified from individuals who attended the Beijing Stomatological Hospital, Capital Medical University, Beijing, between 2007 and 2009. These patients were clinically evaluated and a detailed family history was taken. Syndromic patients, and those patients whose mothers declared use of periconceptional drugs were, excluded from the study. A total of 285 sex-matched control samples were provided by the Cardiovascular Institute and Fu

Wai Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing. These healthy samples were obtained from workers on the Qingdao pier, Shandong province, and were selected for an absence of both other severe forms of physical diseases and a family history of any birth defects. All the subjects were of Han Chinese origin. Patients (104 men and 58 women) were aged between six months and 43 years, and control subjects (189 men and 97 women), were between 18 and 49 years. The study protocol was approved by the Ethics Committee of Beijing Stomatological Hospital and Fu Wai Hospital. Written informed consent for genetic analysis was obtained from all the subjects or their guardians. Genomic DNA was extracted from peripheral blood leukocytes using a genomic DNA extraction kit (Relaxgene Blood DNA kit, Tiangen, Beijing, China).

### SNPs Selection and Genotyping Assay

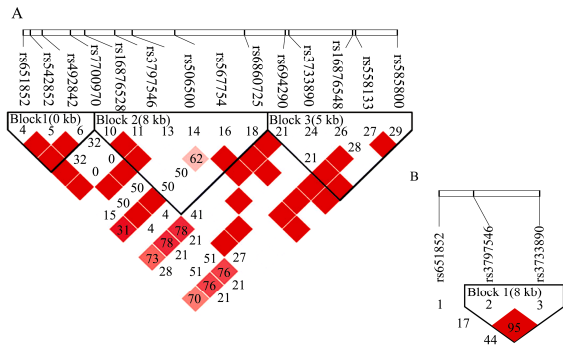
Three single nucleotide polymorphisms (SNPs) present in the *BHMT* gene (rs651852, rs3797546, and rs3733890) were selected based on their presence in the HapMap database for the Han Chinese in Beijing (CHB) population ([www.hapmap.org](http://www.hapmap.org)), their haplotype-tagging properties, and their minor allele frequency (MAF). These three SNPs were prioritized based on prior evidence of an MAF of >0.1 and location in the three linkage disequilibrium (LD) blocks that were determined by the Haploview™ program (version 4.0), respectively (Figure 1A). The SNPs were detected using a real-time PCR-based TaqMan genotyping assay (Assay-On-Demand ID: C\_978718, C\_11646606\_20 and C\_27517894) on an ABI PRISM 7500HT sequence Detection System (Applied Biosystems, USA). Genotype calling was manually performed, based on the distribution of three clusters resulting from plotting the amplification of allele 1 versus allele 2.

### Statistical Analysis

The Haploview program (version 4.0) was applied to estimate the LD measures ( $D'$  and  $r^2$ ) between paired SNPs and also to assess the Hardy-Weinberg Equilibrium for genotypic distribution of all three SNPs, tested separately within both case and control classes. The UNPHASED program (version 3.1.3) performed a likelihood ratio test for allelic and haplotypic association<sup>[20]</sup>. Odds ratios (OR) and 95% confidence intervals (CI) were used to estimate risks.

**RESULTS**

Analysis with the Haploview program (version 4.0) showed that rs3797546 and rs3733890 were in the same haplotype block, spanning about 8kb of DNA. The LD measures between them were 0.95 ( $D'$ ) and 0.17 ( $r^2$ ) respectively, while rs651852 was not strongly tagged to the two others (Figure 1B). The genotypic distributions of the three SNPs showed no deviation from the Hardy-Weinberg equilibrium, in either the patient group or in the control group. Furthermore, gender distribution did not result in deviation between CL/P and controls.



**Figure 1.** Haploview LD plots (A) and the LD measures for paired SNPs in the *BHMT* gene (B).  $D'$  values are indicated in the pair wise squares. Three SNPs were in the three blocks respectively, according to the Solid spine of LD criteria (A). The Haploview program showed that rs3797546 and rs3733890 were in the same block, spanning about 8 kb of DNA, while the rs651852 was not strongly tagged to the other two (B).

The distributions of allele frequencies between controls and patients are presented in Table1. None of these three SNPs in the *BHMT* gene showed allelic association with NSCL/P. The genotype frequencies in cases and controls are displayed in Table 2. There was neither allelic nor genotypic association between rs3733890 and NSCL/P. Interestingly, a significant association was detected for the rs3797546 CC genotype, with an OR of 2.10 (95% CI 1.11-3.95,  $\chi^2 = 5.42$ ,  $P=0.020$ ). With regard to haplotypic association for multiple loci, we applied a permutation test (10000 times) using UNPHASED to correct the global  $P$  value ( $\chi^2 = 0.066$ ,  $P=0.797$ ). There was also no significant association among individual locus combinations (Table 3).

**DISCUSSION**

Prenatal folic acid supplementation provides a protective effect against NSCL/P<sup>[21]</sup>; therefore, intense interest has focused on a role of the genes encoding folate receptors and transporters, and enzymes in the folate metabolic pathways<sup>[22-26]</sup>. *BHMT*, which lies at a critical juncture of the folate and homocysteine cycles, is thought to account for up to half of the homocysteine remethylation capacity<sup>[14]</sup>.

The present study genotyped three SNPs at the *BHMT* locus, including rs651852 in intron 1, rs3797546 in intron 2, and rs3733890 in exon 6. Although none of these three SNPs showed allelic association with NSCL/P, the rs3797546-CC genotype carriers might have an increased risk of the disease compared with those carrying the CT or TT genotypes

**Table 1.** *BHMT* SNPs Allele Frequencies in Patients and Controls

SNPs	Allele	Controls (Frequencies)	CL/P Cases (Frequencies)	$\chi^2$	$P$	Odds Ratio	95% CI <sup>a</sup>
rs651852	A	337(59.1)	199(60.0)	0.06	0.810	0.97	0.73-1.27
	G	233(40.9)	133(40.0)				
rs3797546	T	383(69.1)	213(64.9)	1.65	0.199	1.21	0.91-1.62
	C	171(30.9)	115(35.1)				
rs3733890	G	378(70.5)	236(73.3)	0.76	0.383	0.87	0.64-1.19
	A	158(29.5)	86(26.7)				

**Note.** <sup>a</sup>CI, confidence interval.

**Table 2.** Genotypic Association between *BHMT* SNPs and Risks for CL/P

SNPs	Genotype	Controls (Frequencies)	CL/P Cases (Frequencies)	$\chi^2$	$P$	Odds Ratio	95% CI <sup>a</sup>
rs651852	AA+ GA	100+137(83.2)	62+75(82.5)	0.029	0.864	1.05	0.63-1.73
	GG	48(16.8)	29(17.5)				
rs3797546	TT+ CT	126+131(92.8)	72+69(86.0)	5.420	0.020*	2.10	1.11-3.95
	CC	20(7.2)	23(14.0)				
rs3733890	GG+ GA	130+118(92.5)	90+56(90.7)	0.461	0.479	1.27	0.63-2.57
	AA	20(7.5)	15(9.3)				

**Note.** <sup>a</sup>CI, confidence interval.

**Table 3.** Haplotypic Analysis between *BHMT* SNPs and Risks for CL/P

Haplotype	CL/P Cases (Frequencies)	Controls (Frequencies)	$\chi^2$	P
G-C-A	0.003	0.004	0.003	0.954
G-T-A	0.162	0.162	0.000	0.999
G-T-G	0.165	0.136	2.025	0.155
A-C-A	0.072	0.060	0.802	0.370
A-C-G	0.223	0.232	0.156	0.692
A-T-A	0.166	0.183	0.682	0.408
A-T-G	0.222	0.209	0.295	0.587
Total	-	-	0.066	P=0.797

(Table 2). Several studies showed a significant association of several putative susceptibility loci with NSCL/P, indicating that this condition is either polygenic or heterogeneous, or both. While the mode of inheritance of NSCL/P has been investigated in many studies, lack of consistency across all the studies has not established which model might best describe the transmission of NSCL/P. The results from this study raise the possibility that the *BHMT* gene may play a role in predisposition to NSCL/P, in a recessive manner, among the Chinese population. Our finding seems to be consistent with that reported by Marazita and co-workers, who used a complex segregation analysis in 2 000 NSCL/P families in Shanghai, China, and found that the best-fitting model was an autosomal recessive major locus<sup>[27]</sup>. Within the same population, they also found a significant association for the DNA markers D5S807, D5S2501, D5S5816 and D5S1456 on chromosome 5<sup>[28]</sup>. Interestingly, D5S5816 is located in the *BHMT* gene, further supporting the view that *BHMT* is very likely to confer a genetic risk of NSCL/P in a recessive manner. Our findings do not agree with other studies that found no association between *BHMT* and NSCL/P in a South American population<sup>[19]</sup> and showed no evidence to support the major locus<sup>[29]</sup>. To the best of our knowledge, this is the first report of a genetic analysis of the *BHMT* gene for NSCL/P in the Chinese population.

In the present study, rs3733890 did not show either allelic or genotypic association with NSCL/P, as previously reported in studies of children with NSCL/P<sup>[9,19]</sup>. There is evidence that suggests that this SNP is related to an increased risk of neural tube defects (NTD) and placental abruption, but it not related to risk of conotruncal heart disease<sup>[13,30]</sup>. *BHMT* was significantly associated with NTD, particularly when the *MTHFR* rs1801133 T allele is preferentially transmitted by the parents. However, there is no obvious role for *BHMT* in developing vascular disease<sup>[31]</sup>. SNP rs3733890 results in a substitution of glutamine to arginine at position 239 (Q239R). The presence of arginine may not have a

major impact on folding of the protein, subsequently affecting protein function<sup>[32]</sup>, as the secondary structure predicted by Chou & Fasman analysis does not lead to divergent structures. This SNP also does not appear to influence circulating homocysteine levels, which is compatible with functional genomic results. Thus, there is no evidence confirming that rs3733890 can influence homocysteine metabolism, and this SNP can be considered as a benign polymorphism contributing to the etiology of NSCL/P. By contrast, rs3797546 showed genotypic association with NSCL/P in a recessive manner, suggesting that this intronic SNP might play a role in transcriptional regulation; this hypothesis can be supported by a previous study of human nonsyndromic CL/P, which showed an increase in protein levels<sup>[32]</sup>. Although both rs3797546 and rs3733890 are located in the same LD block, only the CC genotype of rs3797546 showed significant association with NSCL/P. This finding raises the possibility that rs3797546's association may result from a disease-underlying variant present in a nearby LD block. In such a case, rs3797546 instead of rs3733890 tags the variant.

The present study has some potential limitations. Detailed information on maternal folate status was absent. Furthermore, transient elevation of folate in maternal serum because of supplementation or dietary intake could prevent orofacial clefts by overcoming genetically determined metabolic deficiencies or folate transport problems. However, we were unable to derive this information by testing samples from mothers to verify whether maternal nutritional status could have affected the infant condition. In addition, a small proportion of the patients came from regions of China other than North China; therefore, the impact of the differences in the origin of the patients and healthy individuals on our study should be taken into account.

In summary, NSCL/P is a complex disorder involving many genetic and environmental factors. Folate-related NSCL/P could be attributable to

epigenetic factors or interactions with disease-underlying genes, and this will require research into those genes that are related to NSCL/P.

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