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ORIGINAL RESEARCH

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The association of Dihydrofolate reductase (*DHFR*) expression and 10q25.3 with non-syndromic cleft lip with or without left palate in children residing in Northern China: a case-control study

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Abstract

Background: Non-syndromic cleft lip with or without cleft palate (NSCL/P) is one of the most common congenital deformities and notable for significant lifelong morbidity and complex etiology. Previous research demonstrated that genetic factors play a key role in the development of NSCL/P; however, no causative genes have been identified thus far. The aim of this study was to investigate the association between dihydrofolate reductase (*DHFR*) gene expression, the 10q25.3 chromosome, and NSLC with or without cleft palate. **Methods**: In total, we recruited 220 and 180 healthy controls to investigate the association between *DHFR* gene expression, the 10q25.3 chromosome, and NSLC with or without cleft palate in the northern Chinese population. In particular, we attempted to detect genetic variations by analyzing single nucleotide polymorphism (SNPs). Genomic DNA was extracted from peripheral blood samples and then analyzed by polymerase chain reaction (PCR) to determine target gene expression. PCR-restriction fragment length polymorphism (PCR-RFLP) was then used to identify the genotypes that were present in the experimental group. Finally, we performed case-controlled family analysis. **Results**: The gene frequency in the case and control groups all conformed to the Hardy-Weinberg (HW) equilibrium. Analysis revealed that there were no significant differences in the frequency distributions of genotypes or alleles in the experimental group and control group for two genes. The TT (GA) genotype of rs11742688 and rs7078160 was normal in the experimental groups (*DHFR* rs11742688, $p > 0.05$; 10q25.3 rs7078160, $p > 0.05$, respectively). Transmission disequilibrium testing further revealed that these were transmitted in equilibrium by rs11742688 and rs7078160. **Conclusions**: rs11742688 and rs7078160 polymorphism is not related to the development of NSCL/P in the population of north China. There is no significant difference in the C/T (G/A) genotype when compared between the experimental and control groups.

Keywords

Non-syndromic cleft lip with or without cleft palate (NSCL/P); *DHFR* gene polymorphism; Hardy-Weinberg equilibrium; Transmission disequilibrium test

1. Background

NSCL/P is one of the most common birth defects and is known to be associated with a complex etiological mechanism that involves both inherited and environmental factors [1]. NSCL/P is commonly divided into three different types: cleft lip (CL) only, cleft lip and palate (CLP), and cleft palate (CP) only. According to recent statistical analysis, NSCL/P is associated with a morbidity rate of 0.182% [2] across China; the morbidity rate in Shenyang city has been reported to be higher than the national average, at 0.192% [3]. Previous research has determined that NSCL/P represents a polygenic genetic disorder, although no single gene has been shown to play a specific role in pathological changes. It is possible that alterations in the expression of one or more genes may result in the development of NSCL/P. Once diagnosed with NSCL/P, patients need to receive a specific sequence of treatments. Therefore, there is an increasing need for maxillofacial surgeons to be able to treat patients affected by NSCL/P as such therapeutic strategies play a key role in the development of children.

Previous studies have reported that a number of genes play critical roles in the development of the lip and palate, and also that the effect of some individual genes may be related to geographical origin [4] along with racial and ethnic back-

grounds. A previous study reported that the dihydrofolate reductase (*DHFR*) gene and 10q25.3 contribute to the development of NSCL/P among with a large number of other candidate genes, and identified that the abnormal expression of *DHFR* and 10q25.3 is a risk factor for the development of NSCL/P [5]. The discovery of abnormal *DHFR* expression in NSCL/P provided significant inspiration for investigating the pathogenesis of NSCL/P. Furthermore, research has shown that good source of folate during the conception period can reduce the incidence of NSCL/P. *DHFR* and 10q25.3 expression has already been proven to represent a risk factor for NSCL/P in the populations of Europe and America [5–7], although this relationship is known to differ in the Asian population.

The purpose of the study was to investigate the relationship between *DHFR*, 10q25.3 and NSCL/P in a northern population of China.

2. Methods

2.1 Sample acquisition

In total, we recruited 220 patients and 187 healthy as controls. The patient group consisted of 116 males and 104 females (86 cases of NSCL, 65 cases of NSCP, 55 cases of NSCLO (nonsyndromic cleft lip only), and 14 cases of NSCLA). All patients were recruited from the Department of Oral-maxillofacial Surgery at the Affiliated Stomatology Hospital of China Medical University, located in the northeast of China.

Inclusion Criteria for the Case Group:

1. The patients are afflicted with congenital non-syndromic cleft lip and palate.
2. The patients' parents are their biological parents.
3. The patients' parents possess normal intellectual capacities and are capable of completing the questionnaire.
4. They do not present with any other congenital diseases or deformities.
5. They give their consent to participate in this experimental study.

Inclusion Criteria for the Control Group:

1. They have no congenital diseases or deformities.
2. They agree to take part in this experimental study.
3. The parents of the subjects have normal intelligence and are able to fill out the questionnaire.

Exclusion criteria

Patients with congenital craniofacial deformities or syndromes, and those with a history of facial trauma or previous orthognathic surgery excluded.

All subjects received chest radiography, electrocardiogram (ECG) and other preoperative examinations to screen for the presence of associated anomalies or syndromes, and only those who met strict criteria were included in the study. The subjects recruited into the control group exhibited a similar age and gender distribution (Table 1).

TABLE 1. The consist of the cases and controls.

Project	Cases (220)	Controls (180)
Han/Inner Mongolia ethnic	209/11	177/3
Males/Females	116/104	94/86
CLP/CL/CP	55/86/65	
Father	169	
Mother	122	

CLP: cleft lip and palate; CL: cleft lip; CP: cleft palate.

2.2 Selection of single nucleotide polymorphisms for analysis

DHFR (rs11742688) was first reported by Marcella *et al.* [8] in 2015; these authors reported that four SNPs showed strong association to NSCL/P and markers. We also selected 10q25.3 (rs7078160) based on previous NSCL/P-GWAS (Genome-wide association study) analysis [8, 9]. These SNPs have been confirmed to be associated with NSCL/P in both European and American subjects, but not Asian subjects. Table 2 shows some basic information relating to the SNPs selected for analysis.

2.3 Genotyping

DNA was isolated from peripheral blood samples with a Tianamp Genomic Blood DNA Kit. The reference sequences for rs11742688 and rs7078160 were obtained from the National Center for Biotechnology Information (NCBI) GenBank. Specific primer pairs were designed using Primer 5 software (San Francisco, CA, USA). The primers used to amplify rs11742688 were F-5′CATGGTGGTGGGTGACTG-3′ and R-5′GCTCTGTCTCCCAGGTGT-3′. The primers used to amplify rs7078160 were F-5′AGAGCCATAGGAAGTTG-3′ and R-5′TTCCCATTTCTCGTCTGC-3′. The genotyping of rs11742688 (in *DHFR*) and rs7078160 (on 10q25.3) were carried out on the Veriti 96 Well Thermal Cycler PCR platform (Applied Biosystems) using TaqMan exonuclease allelic discrimination assays. We used the *Dde I* restriction enzyme for rs11742688 and the *Hpy* enzyme for 188I (rs7078160). The expected fragment lengths were 122bp (rs11742688) and 314bp (rs7078160). For quality control purposes, 10% of all samples were randomly chosen for a second round of genotyping by either conventional genotyping methodology or direct sequencing (Fig. 1). The genotypes obtained from DNA sequencing were consistent with the pre-designed results.

2.4 Statistical treatment

Cases were compared to controls by SPSS software (version 13.0.1, IBM, Armonk, NY, USA). Allele and genotype frequencies were calculated by direct counting and were compared between the patient and control groups. The Chi-squared test was used to compare genotypic and allelic frequencies when the expected frequency in at least one cell was <5. The Hardy-Weinberg equilibrium was estimated from the genotypic distribution. A transmission disequilibrium test was also performed to investigate the wrong family typing which consisted of triad analyses featuring the child, mother and father.

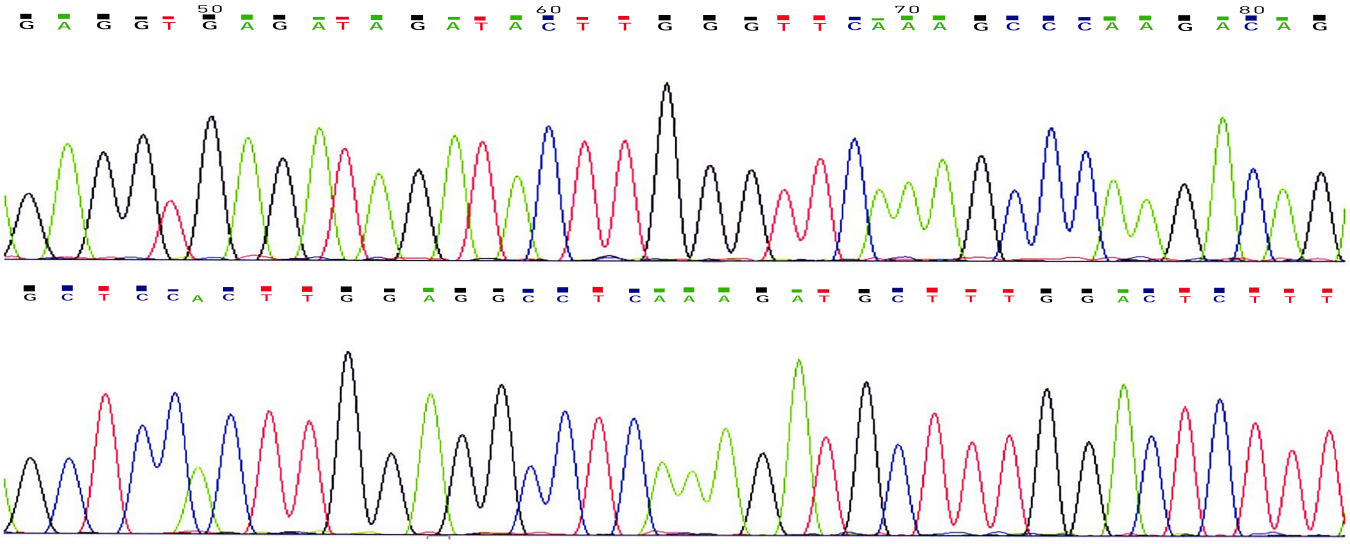

FIGURE 1. The results of direct sequencing.

3. Results

All genotypic and allelic distributions in the patient and control groups conformed to the Hardy-Weinberg equilibrium (data not shown). The allelic and genotypic frequencies of *DHFR* and 10q25.3 polymorphisms detected in the patient and control groups are given in Table 3 along with odds ratios (ORs), p values and 95% confidence intervals (95% CI). Chi-squared analysis showed that the wild homozygous (CC, AA), heterozygous (CT, AG), and mutant homozygous (TT, GG) genotypes did not differ significantly with respect to rs11742688 SNP ($p > 0.05$) or rs7078160 ($p > 0.05$) SNPs; the OR for rs11742688 and rs7078160 were 1.01 (95% CI: 0.304–4.827) and 1.03 (95% CI: 0.568–1.432), respectively. There was no significant association between the two SNPs and NSCL/P, according to conventional genotyping analysis (Tables 3 and 4).

For the case-parent trios with a heterozygous genotype, transmission disequnilibrium test (TDT) analysis revealed a non-significant over-transmission of the T (G) allele at rs11742688 and rs7078160 in a population in North China (Table 5). These data do not indicate a substantial contribution of the *DHFR* gene and 10q25.3 in the etiology of NSCL/P in a specific population in North China. These findings may be explained two key factors. First, the allele frequencies of Chinese subjects are different from those of European and North American populations. Second, the proportion of alleles may vary between different ethnic races.

4. Discussion

There is a high incidence of CL/P in North China. This represents a significant clinical issue because CL/P can exert impact on a patient's appearance and also their physical functionality. CL/P can be broadly divided into two major classes: syndromic oral clefts and non-syndromic oral clefts. The later can be further divided into three different categories: simple cleft lip, simple cleft palate, and cleft lip and palate [10]. Previous research has shown that alterations in the development process during different time periods can cause different types of malformation. Consequently, there is a critical need to gain a better understanding of the specific relationship between gene expression and CL/P.

Gene mapping projects involving NSCL/P have become a significant hotspot for research over recent years. This research has discovered that over 30 genes are associated with NSCL/P, including *MTHFR* [11], *TGFβ3* [12], *PVRL1* [13], *WNT* [14], *MSX1* [15], *BCL3* [16] and *PRR2* [17]. Oral-facial development is known to be a complex process that involves many genes and pathways. Previous studies have reached differing conclusions with respect to the relationship between gene expression and NSCL/P in different ethnic races and biogeographical regions, including *DHFR* (rs11742688) and 10q25.3 (rs7078160). The discovery of *DHFR*, as a neural tube defect (NTD), provided inspiration for investigating the etiology of NSCL/P; Subsequent research showed that a good source of folate during the conception period can reduce the incidence of NSCL/P [18]. DHFR plays an essential role in the folate pathway, participates in the synthesis of folic acid and is

TABLE 2. Candidate single nucleotide polymorphism (SNP) function and minor allele frequency (MAF) in northeast China.

Gene	Chromosome	SNPs	SNP function	Allele	MAF
DHFR	5q14.1	rs11742688	intronic	C/T	0.45
10q25.3	10q25.3	rs7078160	intronic	G/A	0.47

DHFR: Dihydrofolate reductase.

TABLE 3. Test of Hardy-Weinberg equilibrium, genotypic and allelic distribution and comparisons in rs11742688.

	CL	CP	Total	Controls
rs11742688 genotype				
CC	55 (78.6%)	35 (77.8%)	167 (75.9%)	145 (80.6%)
CT	14 (20.0%)	10 (22.2%)	48 (21.8%)	31 (17.2%)
TT	1 (1.4%)	0 (0%)	5 (3.3%)	4 (2.2%)
χ^2	0.400	1.529	1.335	
p	0.819	0.466	0.513	
Allele				
C	124 (88.6%)	80 (88.9%)	382 (86.8%)	321 (89.2%)
T	16 (11.4%)	10 (11.1%)	58 (13.2%)	39 (10.8%)
χ^2	0.036	0.006	1.025	
p	0.849	0.940	0.311	
TT and CT/CC in the family compared with controls				
CT + CC	15 (93.8%)	10 (100.0%)	53 (91.3%)	35 (89.7%)
TT	1 (6.2%)	0(0%)	5 (8.7%)	4 (10.3%)
χ^2	0.220	1.117	0.074	
p	0.439	0.291	0.785	
OR	1.714	1.114	1.211	
95% CI	(0.177–1.647)	(1.002–1.239)	(0.304–4.827)	

CL: cleft lip; CP: cleft palate; OR: odds ratios; CI: confidence intervals.

TABLE 4. Test of Hardy-Weinberg equilibrium, genotypic and allelic distribution and comparisons.

	CL	CP	Total	Controls
Genotype rs7078160				
AA	18 (25.7%)	11 (24.4%)	48 (21.8%)	40 (22.2%)
GA	34 (48.6%)	20 (44.4%)	112 (50.9%)	78 (43.3%)
GG	18 (25.7%)	14 (31.1%)	60 (27.3%)	62 (34.4%)
χ^2	1.774	0.207	2.873	
p	0.412	0.902	0.238	
Allete				
A	70 (50.0%)	42 (46.7%)	208 (47.3%)	158 (43.9%)
G	70 (50.0%)	48 (53.3%)	232 (52.7%)	202 (56.1%)
χ^2	1.518	0.225	0.913	
p	0.218	0.635	0.339	
GG and GA/AA in the family compared with controls				
AA + GA	52 (74.3%)	31 (68.9%)	160 (72.7%)	118 (65.6%)
GG	18 (25.7%)	14 (31.1%)	60 (27.3%)	62 (34.4%)
χ^2	1.765	0.179	2.402	
p	0.184	0.672	0.121	
OR	1.518	1.163	1.401	
95% CI	0.818–2.816	0.577–2.348	0.914–2.148	

CL: cleft lip; CP: cleft palate; OR: odds ratios; CI: confidence intervals.

TABLE 5. TDT analysis of rs11742688 and rs7078160.

	CL		CP		Total	
	Transmitted	Not transmitted	Transmitted	Not transmitted	Transmitted	Not transmitted
rs11742688						
C	14	12	16	12	48	56
T	16	18	10	18	58	50
χ^2	0.271		0.404		1.208	
p	0.402		0.525		0.272	
OR	1.313		0.677		0.739	
95% CI	(0.427–3.653)		(0.191–2.333)		(0.431–1.268)	
Rs7078160						
A	42	38	22	20	92	84
G	30	34	18	20	80	88
χ^2	0.450		0.201		0.748	
p	0.502		0.654		0.388	
OR	1.253		1.222		1.205	
95% CI	0.648–2.420		0.508–2.943		0.789–1.840	

CL: cleft lip; CP: cleft palate; OR: odds ratios; CI: confidence intervals.

known to play a key role in the metabolism of folate. Activity of the DHFR protein is essential for amino acid metabolism and nucleotide synthesis. Furthermore, DHFR can convert dihydrofolate into tetrahydrofolate and has been confirmed to be a risk factor associated with NSCL/P. 10q25.3 was identified by GWAS, a powerful and accurate method that was previously used to identify genes that might be associated with NSCL/P. A GWAS study also identified the *IRF6* gene as a key contributor to the etiology of NSCL/P [19]. NSCL/P is associated with significant genetic heterogeneity. Previous research demonstrated that 10q25.3 is associated with neural tube defects, which is also known to occur with the *DHFR* gene. Recent studies, involving European and American populations, demonstrated a significant association between NSCL/P and the rs7078160 polymorphism in 10q25.3, but did not consider the Asian population [20]. Thus, the main aim of our present study was to confirm the evidence of an association between SNPs in the *DHFR* gene and 10q25.3 and the risk of NSCL/P in a northern population of China.

In our study, we tested Hardy-Weinberg equilibrium in both patient and control groups. We detected Hardy-Weinberg equilibrium for three genotypes in the patient group, and a research of data association method was used in case and control groups. Statistical analysis showed that filial generation in the patient groups did not differ significantly for the three genotypes, or for the distribution of allele genes ($p > 0.05$). OR and 95% CI values were generated for carriers of the T allele (G) relative to the C allele C (A); this was not significant ($p > 0.05$), thus implying that the mutation of this gene did not increase the risk of morbidity in patients with NSCL/P.

In addition, we performed the TDT test based on core families; the case group represented allele transfer from parents while the control group did not include allele transfer. Analysis confirmed that the mutation of these genes did not increase the risk of morbidity in patients with NSCL/P. Statistical analysis revealed that filial generation in the case groups did not show any statistical significance across the three genotypes, or in terms of the distribution of allele genes ($p > 0.05$). We calculated OR and 95% CI values for carriers of the T allele (G) relative to the C allele (A) but did not identify statistical significance ($p > 0.05$); thus, the mutation of these genes cannot increase the risk of morbidity in patients with NSCL/P.

Replication analysis demonstrated that the polymorphic variant rs11742688 in *DHFR* and the rs7078160 variant in 10q25.3 were not significantly associated with NSCL/P in the northeast of China. The lack of association between these genes and NSCL/P may have been influenced by the small sample size and the limited study area. NSCL/P is a multifactorial disease, including race, SNPs and environmental factors. Our understanding of the etiology of NSCL/P remains limited and needs to be expanded to include many more genetic, ethnic, and environmental factors. Consequently, it is not possible to confirm a significant relationship between the genes analyzed herein and NSCL/P on a global basis. In addition, both environmental and ethnic factors should possible be considered as triggers because they can influence the manner in which genes compound, thus generating alterations in fetal cell differentiation during lip and palate formation. Consequently, there is a clear need for further studies to investigate the specific relationship between gene expression and NSCL/P.

This experiment also exhibited several limitations. First and foremost, the sample size was insufficiently large, rendering it impossible to eliminate the potential result bias stemming from a small sample. In the aspect of sample collection, the blood samples of the parents in the control group were not obtained, which precluded the implementation of a comparative study on the parental samples. Additionally, the disparities

in these research findings could potentially be attributed to diverse regional genetic backgrounds, ethnic variations, and the limitations inherent in the experimental design. Mutation frequencies demonstrate marked differences across different countries, distinct regions within the same country, and various ethnic groups within the same region.

5. Conclusions

This was the first study to demonstrate that the rs11742688 variant in DHFR and the rs7078160 variant in 10q25.3 do not represent candidate genes for NSCL/P in a northern population of Chinese individuals.

ABBREVIATIONS

NSCL/P, Non-syndromic Cleft lip with or without cleft palate; ECG, Electrocardiogram; PCR, polymerase chain reaction; DHFR, Dihydrofolate reductase; SNPs, single nucleotide polymorphism; RFLP, restriction fragment length polymorphism; HW, Hardy-Weinberg; CL, cleft lip; CLP, cleft lip and palate; CP, cleft palate; OR, odds ratios; CI, confidence intervals; MAF, minor allele frequency; NCBI, National Center for Biotechnology Information; TDT, transmission disequnilibrium test; NTD, neural tube defect.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

JTY—study design, wrote first draft; wrote final draft. YZ—analyzed data; interpreted results. All authors critically revised manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the ethics committee at the Hospital of Stomatology, Wenzhou medical university (#2019011). Informed consent was obtained from all participants.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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