Association of CRISPLD2 Single Nucleotide Gene Polymorphism and Non-Syndromic Cleft Lip With or Without Cleft Palate in an Iranian Population

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Introduction: Non-syndromic cleft lip with or without cleft palate is a malformation that may occur sporadically or with familial aggregation. Its inheritance is complex and many studies have focused on the role of genetics in the etiology of NSCL/P. rs1546124 is one of the most investigated polymorphims of the CRISPLD2 gene. This study aimed to investigate the association between CRISPLD2 rs1546124 gene polymorphism and the incidence of NSCL/P in Iranian population. **Materials and Methods:** In this case-control study, 81 case-parent trio cases have been included. All cases of congenital anomalies and major developmental problems were excluded to select only those with a NSCL/P. 5 ml of peripheral blood was taken from all subjects. PCR and electrophoresis were performed to demonstrate genotype status in each group. For evaluating the probability of transfer from parents to children transmission disequilibrium was performed. **Results:** The highest frequency of genotype in all three is related to CG and the highest allele frequency is related to C allele. The results of transmission disequilibrium showed that the probability of transfer from parents to children is not significant (P=0.38) and the risk of allelic transmission from each parent (with OR near 1) is not significant. **Conclusion:** This study did not find any association between CRISPLD2 and NSCL/P. Further studies with adequate sample size should be designed to investigate for possible polymorphisms in Iranian population.

Keywords: CRISPLD2; Cleft Lip; Cleft Palate; Single Nucleotide Polymorphism

Introduction

Oral and facial clefts are one of the most commonly encountered disorders, with an estimated 600 births (newborns) worldwide (1-3). The two most common types of these clefts are the cleft lip "with" or "without" cleft palate (CL/P) and cleft palate-only (CPO) (4). These can be part of the clinical manifestations of a complex syndrome or occur alone. It is estimated that non-syndromic cases include 70% of all CL/P cases and 50% of all CPO cases.(5) The inheritance is complex, showing incomplete penetrance, and different genetic and environmental factors may have effects on it.

Many studies have focused on the role of genetics in the etiology of cleft lips and palates, however, determining the specific genes and their role remains a conflicting topic (6). Candidate genes such as IRF6 and TGFA were identified by linkage and linkage disequilibrium investigations (7, 8); however, contradictory results from studies (9, 10) suggest the inter- and intra study populations genetic and phenotypic heterogeneity. Studies have demonstrated an association between NSCL/P and single nucleotide polymorphisms (SNPs) in the cysteine-rich secretory protein LCCL domain containing 2 (CRISPLD2) gene (11-17). One of the most studied polymorphisms of this gene is rs1546124 and previous studies showed conflicting results regarding the association of this polymorphism and NSCL/P. There were studies showing no association between rs1546124 polymorphism with NSCL/P among an Irish population in Dublin (18), nor in an Italian population (19); however association has been found between this polymorphism and NSCL/P in Caucasian and Hispanic populations (11), in an

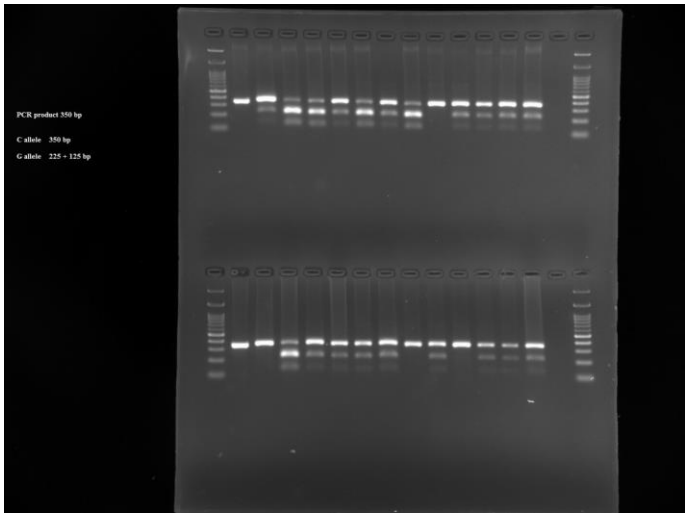

Figure 1. Electrophoresis results showing the genotype status in each group

American population (15), in northwestern Chinese population (14), and Xinjiang Uyghur population (20).

Few studies have been conducted on association between SNPs and NSCL/P in Iran. The results from these studies showed association of CDH1 and MSX1with NSCL/P (21). There were no association between TGFA TaqI polymorphism with NSCL/P (22). As rs1546124 polymorphism in CRISPLD2 gene has not been investigated in Iranian population and finding the exact etiology of the disease for preventive and treatment goals has of great importance, this study aimed to investigate the association between CRISPLD2 rs1546124 gene polymorphism and the incidence of NSCL/P in an Iranian population with trio study design.

Materials and Methods

Sample collection

This study was conducted in Cleft and Palate research center of Shiraz University of Medical Sciences. In this case-control study, 27 trio cases (27 children and 54 parents) have been included. All cases of congenital anomalies and major developmental problems were evaluated to select only those with a NSCL/P. The protocol of the study was approved by the Ethics Committee of the dental school of Shahid Beheshti University of Medical Sciences with the code IR.SBMU.RETECH.REC.1394.521 and carried out in agreement with the Declaration of Helsinki and its subsequent revisions. The participants were involved in the study after obtaining informed consent from their parents.

Designing questionnaire and collecting data

All of the patient and their parents were interviewed and data were recorded in a questionnaire. Environmental factors that appeared to contribute to the non-syndromic cleft palate and /or lips (23-27), such as: maternal smoking, pregnancy, body mass index, folic acid and zinc exposure, alcohol use, stress and fever were asked to exclude the participants who were severely affected by these factors as far as possible.

DNA extraction and genotyping

Ultimately, 5 ml of peripheral blood was collected from all participants in tubes containing 200 µl of 0.5 M of ethylenediaminetetraacetic acid. Samples were stored at -20° C. Genotyping of the CRISPLD2 polymorphism (rs1546124) in the CRISPLD2 gene was performed by polymerase chain reaction (PCR). The primers were designed by Tetra Arms-PCR primer software 1 and are represented in Table 1. The results were evaluated and analyzed on the 1.5% agarose gel.

Statistical analysis

For evaluating the association between CRISPLD2 rs1546124 polymorphism with NSCL/P transmission disequilibrium test (TDT) was performed using Beagle 5.0 (28).

Results

27 Children including 16 boys and 11 girls with mean age of 5.33±3.77 years with their parents were participated in this study. The mean age of fathers was 37.67±7.25 years. 7.4% of mothers aged 18-19 years during pregnancy, 18.5 % were in 20-24 years age group, 29.6% in 25-29, 37% in 30-34 and 7.4 in >35 years age group. Mother's BMI during pregnancy, drug use history, family history, food supplements, smoking and alcohol, mother's education, stress, and fever are presented in Table 2.

Allele and genotypes frequencies

Performing PCR and electrophoresis (Figure 1) demonstrated genotype status in each group. The abundance of CC, CG, and GG genotypes among children, mother and father are shown in Table 3. As it is evident, the highest frequency of genotype in all three is related to CG and the highest allele frequency is related to C allele.

Transmission disequilibrium test analysis

The results of transmission disequilibrium test showed that the probability of transfer from parents to children is not significant (P=0.38) and the risk of allelic transmission from each parent (with OR near 1) is not significant (Table 3).

Table 1. PCR primers for rs1546124 in the CRISPLD2 gene

Oligo Name	Oligo Sequence 5'--> 3'	Base No.
CRISPLD2-F	CCCTTGGCGTAAATCCCAGC	20
CRISPLD2-R	AGAGTGACGTTGGGCAGGAG	20

Table 2. Variables extracted from questionnaire

Variable		Frequency (%)
Maternal BMI during Pregnancy	Underweight (<18.5)	2 (7.4%)
	Normal (18.5-24.9)	21 (77.8%)
	Overweight (25-29)	2 (7.4%)
	Obese (>30)	2 (7.4%)
Use of drugs during pregnancy	Yes	7 (25.9)
	No	20 (74.1)
Maternal smoking during pregnancy	Yes	0 (0%)
	No	27 (100%)
Passive smoking during pregnancy	No	20 (74.1%)
	1-14	3 (11.1%)
	15-24	2 (7.4%)
	>25	2 (7.4%)
Folic acid supplements during pregnancy	Daily	20 (74.1%)
	Not Daily	1 (3.7%)
	Never	6 (22.2%)
Zinc supplements during pregnancy	Yes	21 (77.8%)
	No	6 (22.2%)
Familial history of Cleft Lip/ palate and Facial clefts	Yes	7 (25.9%)
	No	20 (74.1%)
Alcohol during pregnancy	Yes	0 (0%)
	No	27 (100%)
Stress during pregnancy	Yes	18 (66.7%)
	No	9 (33.3%)
Fever during pregnancy	Yes	1 (3.7&)
	No	26 (96.3%)
Mother education during pregnancy	0-8	11 (40.7%)
	9-11	15 (55.6%)
	12-15	1 (3.7%)
	>16	0 (0%)

Table 3. Genotype frequencies in each group and the risk of allelic transmission

	Genotypes frequency			OR(95% CI)	*P*-Value
	CG	CC	GG		
Children	70.4%	29.6%	0%	-	
Father	59.3%	37%	3.7%	1.1 (0.88-1.22)	0.38
Mother	70.4%	25.9%	3.7%	0.99 (0.77-1.05)	

Discussion

In this case-control study, there was no association between rs1546124 polymorphism in CRISPLD2 gene and the risk of nonsyndromic Cleft Lip and/or Cleft Palate in an Iranian Population.

Many studies have investigated the role of genetics in the etiology of NSCL/P. Chiquet *et al.* (11) studied the candidate gene for NSCL/P and genetic variation at the CRISPLD2 loci for the first time. Rs1546124 is one of the most investigated markers of the CRISPLD2 gene and located in exon2, 51 bp upstream of the ATG start codon. Sequence alteration in the putative promoter region disrupt the regulatory elements such as RNA polymerase binding or transcription factor activator/inhibitor binding of CRISPLD2 gene. This can lead to alterations in expression of proteins, and subsequently change the development processes (29, 30).

Although CRISPLD2 expression in the naso and oropharynx at E13.5, the mandible at E14.5, and the palate and cartilage primordia of the nasal septum at E17.5 have been

shown with in situ hybridization of mouse tissues, function of this gene has not been determined yet (11). CRISPLD2 containing an LCCL domain, plays role in structural or immunologic development or can influence the cell motility (31-33). When the LCCL domain misses, it may influence NSCL/P. Chiquet *et al.* (34) reported that the association of CRISPLD2 variations with NSCL/P was greater than CRISPLD1 alone. CRISPLD1 may have effects on NSCL/P through the interaction with CRISPLD2 and folate pathway genes. Moreover, CRISPLD2 can influence the neural crest cells derived tissues and therefor alter the normal craniofacial development (35).

The results of this study were similar to those studies suggested that the CRISPLD2 gene did not associate with NSCL/P among an Irish population in Dublin (18), nor in an Italian population (19); however there were previous studies with conflicting results showing that this locus has been associated with NSCL/P in Caucasian and Hispanic populations (11), in an American population (15), in northwestern Chinese population (14), and Xinjiang Uyghur population (20). These conflicting results may come from different ethnic origins, environmental differences, anthropologic variety, problems related to study designs, and complex genetic etiology of this disease.

Small sample size is one of the major limitations of present study. Therefore, our study was probably underpowered to detect a mild or moderate association between the SNPs and oral clefts; however, this study used a trio design for assessing the association of the polymorphism with NSCL/P in order to overcome the sample size problems.

Conclusion

In summary, polymorphism of the locus rs1546124 is not associated with NSCL/P in the assessed population. Therapy targeted for polymorphism of related genes need information regarding the associated SNPs in NSCL/P. Therefore, further studies with adequate sample size should be designed to investigate for possible polymorphisms in Iranian population.

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Conflict of Interest: 'None declared'.

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