Investigation of the SNPs of IRF6 Gene in Non-syndromic Cleft lip and/or Palate (NSCLP) in a Turkish Population

Türk Popülasyonunda Non-sendromik Dudak ve/veya Damak Yarıklarında (NSDDY) IRF6 Genindeki SNP'lerin Araştırılması

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Abstract

Introduction Non-syndromic cleft lip and/or palate (NSCLP) is a complex malformation with both genetic and environmental effects and interferon regulatory factor 6 (IRF6) is the most focused gene to unravel the genetic etiology of the disease since its important role in the formation and maintenance of the oral periderm to ensure appropriate palatal adhesion has been demonstrated. The aim of this study was to investigate the association between two SNPs (rs2235371 and rs2013162) of IRF6 gene and NSCLP in Turkish population.

Materials and Methods Genotyping of both SNPs were performed with Polymerase Chain Reaction-Restriction Fragment Length Polymorphism method in 38 NSCLP patients, their mothers and the same number of healthy control individuals.

Results Our results did not indicate significant differences in neither genotypic nor allelic distributions between children and controls. The same situation was observed when the mothers of children were also compared with the controls.

Conclusion Considering both contradictive results in different populations worldwide and our relatively small sample size that may be a limitation for this study, we strongly encourage the replication studies with larger sample sizes to draw a precise conclusion about the role of IRF6 gene variants for susceptibility to NSCLP.

Keywords Cleft lip, Cleft palate, Interferon Regulatory Factor 6 Gene

Özet

Amaç Non-sendromik dudak ve/veya damak yarığı (NSDDY) hem genetik hem de çevresel etkilere sahip kompleks bir malformasyondur ve uygun palatal adezyonu sağlamak için oral periderm oluşumu ve devamlılığındaki önemli rolünden dolayı IRF6 hastalığın genetik etiyolojisini çözmek için üzerinde en çok yoğunlaşılmış gendir. Bu çalışmanın amacı, Türk popülasyonunda IRF6 genindeki iki SNP (rs2235371 ve rs2013162) ile NSDDY arasındaki ilişkiyi araştırmaktır.

Gereç ve Yöntemle Her iki SNP'nin genotiplemesi, Polimeraz Zincir Reaksiyonu-Restriksiyon Fragment Uzunluk Polimorfizmi yöntemi ile 38 NSDDY hastasında, annelerinde ve aynı sayıda kontrol grubu bireylerde yapıldı. Bulgular: Sonuçlarımız, çocuklar ve kontroller arasında ne genotipik ne de allelik dağılımlarda önemli farklılıklar belirtmedi. Aynı durum çocukların anneleri ile kontroller karşılaştırıldığında da gözlendi.

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Sonuç Hem dünya çapındaki farklı popülasyonlardaki çelişkili sonuçlar hem de bu çalışma için bir sınırlama olabilecek nispeten küçük örneklem büyüklüğümüz göz önüne alındığında, NSDDY'ye yatkınlık oluşturmada IRF6 gen varyantlarının rolü hakkında kesin bir sonuç çıkarabilmek için daha büyük örneklem boyutlarına sahip replikasyon çalışmalarını güçlü bir şekilde desteklemekteyiz.

Anahtar Kelimeler Yarık dudak, Yarık damak, Interferon Regülatör Faktör 6 Geni

INTRODUCTION

Cleft lifts with or without cleft palate (CLP) are common birth defects of complex genetic/environmental etiology in more than 300 recognizable syndromes. Non-syndromic CLP (NSCLP) often observed as an isolated birth defect affects approximately 1/700 individuals worldwide and does not manifest any other cognitive or craniofacial structural abnormalities (1, 2). Both epidemiological and animal studies offer environmental risk factors during early pregnancy such as smoking, alcohol consumption, viral infections such as Rubella (RUBV), Influenza, and Varicella-zoster virus (VZV), and deficiency of vitamins/micronutrients such as folic acid and zinc as a risk factor for NSCLP (3).

Interferon regulatory factor 6 (IRF6) gene is expressed in the medial edge epithelia of the palatal shelves immediately before and during fusion, as well as it plays a key role in formation and maintenance of the oral periderm essential to ensure appropriate palatal adhesion (3). Besides with their contributions also in cancer, intricate interactions between TGF-β signaling, IRF6, and tumor protein 63 (p63) during palatogenesis were identified and thus disruption of the regulatory loop in which p63 and IRF6 co-operate with the mutations in p63 or IRF6 may cause congenital clefting. IRF6 is also the mediator of TGFβ3 since ablation of IRF6 resulted in a delay in TGFβ3-regulated palatal fusion (4-7).

Genetic studies have identified several candidate genes associated with CLP up to now and IRF6 is one of the strongest of these genes. Though IRF6 gene mutations are accepted as the general reason of Van der Woude syndrome which is a syndromic CLP condition, some associations have also been shown for non-syndromic form too (2). Up to 12% of NSCLP cases result from variants in IRF6 and show a recurrence risk in families with one affected child tripled in case of an IRF6 variant was segregated into the family (8).

The aim of this study was to investigate the role of two IRF6 variations (rs2235371 and rs2013162) in Turkish patients with NSCLP and to analyze the possible effects of these variants in NSCLP susceptibility.

MATERIAL and METHODS

Patients

All samples belonged to the Turkish population. Thirty-eight NSCLP patients, their mothers (38 mothers) and thirty-eight healthy control individuals (with no congenital malformation, no family history of genetic disease) were included in the study. Written informed consent was obtained from all subjects and the Clinical Research Ethics Committee of Dicle University (Approval number: 69/2011) approved the study.

Genotyping

Peripheral blood samples (5 ml) were collected from each participant and frozen to be used in further genotyping experiments. The DNA extraction was performed according to the standard salting-out method of Miller et al. (9). All genotyping experiments were performed with PCR-RFLP method. The used primers in PCR experiments were taken from previous studies (10, 11) and they were shown in Table 1. Randomly selected 30% of the samples were repeated and the results were coincident with the first genotyping. SNP information and the results of the expected genotyping results are depicted in Table 1.

Table 1. SNP information, primers, restriction enzymes, and the expected fragments

SNP ID	Position	Allelex	Primer pairs	Amino acid	Restriction enzyme	Fragments (bp)
rs2235371	exon 7	T/C	F: 5'-ATATGCTCAGGACCTGGGAATTTGA-3' R: 5'-CTCAGGGCTGCCGACTCTTCTA-3'	V/I	Bsp143I	CC: 109 bp TT: 86 bp, 23 bp CT: 109, 86, 23 bp
rs2013162	exon 5	A/C	F: 5'-CCCTGGGATGAGAAGGATAA-3' R: 5'-ACCTCTGACTCCCACTTGCT-3'	S/S	Dde1	CC: 264 bp AA: 199 bp, 65 bp CA: 264 bp, 199 bp, 65 bp

X minor allele listed first, in bold .

Statistical analysis

Hardy-Weinberg equilibrium (HWE) was assessed for the selected SNPs in cases (children and their mothers) and the control group. Case-control statistical analysis was performed with statistical package IBM SPSS Statistics V22.0 software. The data were analyzed by x2 test. Associations between SNPs and NSCLP were calculated by computing the odds ratio (OR) and 95% confidence intervals (95% CI) from logistic regression analyses. The statistical level of significance was defined as $p<0.05$.

RESULTS

All SNPs were in HWE. Allele and genotype frequencies of rs2235371 and rs2013162 in IRF6 gene are listed in Table 2 and Table 3, respectively. There were no significant differences in the allele frequencies of rs2235371 (p=0.6026) between NSCLP children and the control group. No significant association was observed in the allele frequencies between mothers of NSCLP children and the controls, too (p=0.3802). Allele frequencies of rs2013162 did not differ significantly between NSCLP children and the control group (p=0.1336) and the same situation was valid between mothers of NSCLP children and the control group (p=0.6801).

DISCUSSION

In the past decade, a strong effort has been achieved to enlighten genetic background leading to NSCLP pathogenesis and transcription factor IRF6 located on chromosome 1q32.3-q41 has been one of the mostly focused genes. Since Zucchero et al. (8) proved that IRF6 gene variants were associated with the risk of non-syndromic orofacial clefts (NSOC) in a very large set of ~2000 families from multiple populations in the world, replication studies in different populations and ethnic groups have continued to be performed. There are several studies in literature representing the associations/disassociations of the two selected SNPs in this study in terms of liability to NSCLP. A brief recapitulation of these studies was shown in Table 4 to offer an insight and also to provoke the other studies both in our population and different populations with larger cohorts.

That different studies have yielded significant results for

Table 2. Genotypic and allelic distributions for rs2235371

	Genotypes	**Cases**	**Controls**	**Genotype frequencies**	**Genotype frequencies**	**χ^2**	**p**
	Children	**n=38**	**n=38**	**of cases %**	**of controls %**		
IRF-6	CC	19	17	50.0	44.7		
	CT	15	16	39.5	42.1	0.254	0.8805
rs2235371	TT	4	5	10.5	13.2		
C/T	**Alleles**						
	C	53	50	OR (95% CI)		0.271	0.6026
Children	T	23	26	0.8345 (0.4223-1.6493)			
	Genotypes	**Cases**	**Controls**	**Genotype frequencies**	**Genotype frequencies**	**χ^2**	**p**
	Mothers	**n=38**	**n=38**	**of cases %**	**of controls %**		
IRF-6	CC	18	19	47.4	50.0		
	CT	14	17	36.8	44.7	2.317	0.3139
rs2235371	TT	6	2	15.8	5.3		
C/T	**Alleles**						
	C	50	55	OR (95% CI)		0.770	0.3802
Mothers	T	26	21	1.3619 (0.6825-2.7177)			

Table 3. Genotypic and allelic distributions for rs2013162

	Genotypes	Cases	Controls	Genotype frequencies	Genotype frequencies	χ^2	p
	Children	n=38	n=38	of cases %	of controls %		
IRF-6	CC	18	24	47.4	63.2		
	CA	17	13	44.7	34.2	2.390	0.3026
rs2013162	AA	3	1	7.9	2.6		
C/A	Alleles						
	C	53	61	OR (95% CI)		2.25	0.1336
Children	A	23	15	1.7648 (0.8358-3.7263)			
	Genotypes	Cases	Controls	Genotype frequencies	Genotype frequencies of	χ^2	p
	Mothers	n=38	n=38	of cases %	controls %		
IRF-6	CC	25	23	65.8	60.5		
	CA	12	14	31.6	36.9	0.237	0.8882
rs2013162	AA	1	1	2.6	2.6		
C/A	Alleles						
	C	62	60	OR (95% CI)		0.17	0.6801
Mothers	A	14	16	1.3487 (0.3804-1.8851)			

an association between IRF6 and NSCLP (10-13; 16-23) suggests that this is an important gene and clearly warrants further study. Though this study does not indicate that IRF6 may play a role in the etiology of NSCLP in Turkish population in terms of genotype/allele frequencies, there are some limitations stemming from the relatively small numbers included. Thus, we strongly recommend the replication studies with larger cases in our population as well as in different populations worldwide to have a deeper insight about the exact role of the selected IRF6 variations in NSCLP.

Significantly different polymorphism distributions can be observed even in the same population inhabiting in different geographical locations as this is also the case for the Chinese population represented in Table 4. This is also a clearly discussed issue in the article of Song et al. (20) who found an association with NSCLP and IRF6 SNPs rs2073485, rs2235371, rs2236909, and rs861020. Though SNPs rs2013162, rs2235375, and rs2236909 are located in the same linkage disequilibrium (LD) block in the HapMap CHB database. The data showing no significant association between NSCLP susceptibility and rs2013162 in the study of Huang et al. (11) (Table 4) and it may be mostly and reasonably linked to the different locations even in the same population.

On the other hand, some replication studies even in the same population may also show a difference in terms of sub-phenotypic groups as in the case of Letra et al. (16). For IRF6, the authors found a positive association between rs2235371 and complete left cleft lip/palate in contrast to the study of Paranaíba et al. (15) (Table 4). Some other studies evaluated IRF6 gene variants comparing only the three major cleft categories (cleft lip, cleft lip/palate and cleft palate), implying the fact that though IRF6's role as a risk factor may be contradictive, this does not rule out the possibility of IRF6's specific contributions such as controlling the side of unilateral cleft. Also, the complex ethnic admixture of the Brazilian population may be one of the reasons to contribute to such discrepancies. The observation of stronger results for IRF6 among Eastern Asia populations may imply that genetic backgrounds are prone to the haploinsufficiency of IRF6 variants to cause NSCLP. All the same, the studies with supporting evidence with larger sample sizes are necessary to draw a more precise conclusion. One of the future suggestions that sounds reasonable is studying IRF6 gene with some other genes in

Table 4. Summary studies that have investigated the role of IRF6 variants in the risk of NSCLP in different populations

Population	rs2235371 association	rs2013162 association	Reference
Polish	NE	-	(2)
Italian	NE	+	(12)
Norwegian	-	+	(13)
German	-	NE	(14)
Northern Chinese	-	NE	(1)
Brazilian	-	NE	(15)
Brazilian	+	-	(16)
Honduran	+	NE	(17)
West China	+	-	(11)
Central European	+	+	(18)
Malay	+	NE	(19)
Chinese Han	+	NE	(10)
Northeast Chinese	+	NE	(20)
Northeast Chinese	+	+	(21)
Northeast Chinese	+	+	(22)
Northeast Chinese	+	NE	(23)
Turkish	-	-	This study
NE: Not evaluated			

which it may be in interaction as Song et al. (20) has already provided such gene-gene interaction evidence between IRF6 and PAX9 genes for susceptibility to NSCLP.

CONCLUSION

As a result, the inconsistent outcomes may arise from different ethnic origins, environmental differences, anthropological diversity, and the complex genetic etiology of the disease. One of the main limitations in our study is the relatively small sample sizes and therefore we strongly recommend additional studies conducted in our population as well as in other populations using larger samples. A more comprehensive analysis of these SNPs and identification of new risk SNPs in IRF6 and/or other genes for NSCLP development could be quite beneficial for recognizing high-risk populations and genetic counselling.

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