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A case-control study of two polymorphisms of HIF1A in children with cleft lip/palate and in their mother

Yarık damak ve dudaklı çocuklar ve annlerinde HIF1A'nın iki polimorfizminin vaka kontrol çalışması

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Abstract

Aim: Palatogenesis is a metabolic event that occurs in the initial stages of fetal life. Reports indicate that hypoxia is a critical condition in the formation and elevation of the palate and fusion of the lips. It is known that both environmental and genetic factors play important roles in hypoxia. The ability of the cells to respond to changes in the oxygen pressure that lead to hypoxia depends on the activation of a transcription factor family known as hypoxia-inducible factors (HIFs). This study was conducted to determine the distribution of two polymorphisms of hypoxia-inducible factor 1, alpha subunit (HIF1A) in children with cleft lip/palate and their mothers.

Methods: Two polymorphic structures of HIF1A (Pro582Ser and Ala588Thr) were studied in children with cleft lip/palate and their mothers along with control group children and mothers using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. DNA fragments were monitored by agarose gel electrophoresis after cleavage.

Results: In Ala588Thr comparison, no difference was observed between mothers, children, and their controls. Regarding Pro582Ser, there were differences in the comparison of maternal and children genotypes with control groups (P=0.034 and P=0.023, respectively). In allelic comparisons, there was a difference between mothers of children with cleft lip/palates and the control group (P=0.001). Although this was different in children, it was not as high as in mothers (P=0.026).

Conclusion: HIF1A polymorphisms that change the proline residues may affect the activity or lifespan of HIF1A protein and play a role in the formation of cleft lip/palate.

Keywords: Hypoxia-inducible factor 1, Cleft lip, Cleft palate

Öz

Amaç: Palatogenez, fetal yaşamın erken evrelerinde ortaya çıkan metabolik bir olaydır. Raporlar, hipoksinin damak oluşumunda ve yükselmesinde ve ayrıca dudakların füzyonunda kritik bir durum olduğunu göstermektedir. Çevresel faktörlerin yanı sıra, genetik faktörlerin hipokside önemli rol oynadığı bilinmektedir. Hücrelerin hipoksiye yol açan oksijen basıncındaki değişikliklere cevap verebilme yeteneği, hipoksi ile indüklenebilir faktörler (HIF'ler) olarak bilinen bir transkripsiyon faktörü ailesinin aktivasyonuna bağlıdır. Bu çalışma, yarık dudak/damaklı çocuklar ve bu çocukların annelerinde hipoksi ile indüklenebilir faktör 1, alfa alt birimi (HIF1A)'nin iki polimorfizminin dağılımını belirlemek için yapılmıştır.

Yöntemler: HIF1A'nın (Pro582Ser ve Ala588Thr) iki polimorfik yapısı, çalışma grubu olan yarık dudak/damaklı çocuklar ve annelerinde ve kontrol grubu çocuklar ve annelerinde polimeraz zincir reaksiyonu fragment uzunluğu polimorfizmi (PCR-RFLP) yöntemi kullanılarak incelendi. DNA fragmentleri, kesim işleminden sonar agaroz jelde görüntülendi.

Bulgular: Polimorfik yapılardan Ala588Thr karşılaştırmasında, yarık dudak/damaklı çocuklar ve anneler ile kontrol grubu çocuklar ve anneleri arasında fark gözlenmemiştir. Pro582Ser karşılaştırmasında ise; anne ve çocuk genotiplerinin kontrol gruplarıyla karşılaştırılmasında farklılıklar görülmüştür (P=0.034 ve P=0.023, sırasıyla). Alell karşılaştırmalarında, yarık dudak/damaklı çocukların anneleri ile kontrol grubu anneleri arasında fark gözlenmiştir (P=0.001). Çocuklardaki allel karşılaştırmalarında da, annelerdeki kadar yüksek olmasa da fark görülmüştür (P=0.026).

Sonuç: HIF1A proteinindeki Prolin aminoasitlerinin değişimine neden polimorfizmler, HIF1A proteininin aktivitesini veya ömrünü etkiliyor ve yarık dudak/damak oluşunda rol alıyor olabilir.

Anahtar kelimeler: Hipoksi ile indüklenebilir faktör 1, Yarık dudak, Yarık damak

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Introduction

Cleft lip and palate, the most common craniofacial deformities seen in 700 to 1000 newborns, can be examined in two groups: Syndromic and non-syndromic [1]. Non-syndromic type is more frequent. Cleft lip is more common in males and mostly seen on the left side, while cleft palate is more commonly observed among females [2]. Environmental factors play a significant role in the genetic background of this deformity [3]. It has been determined that folate intake by pregnant women reduced the incidence of cleft lip and palate [1]. After this finding, studies focused on the genes involved in folate metabolism and some candidate genes were identified, which include transforming growth factor-alpha (TGFA), transforming growth factor-beta 2 (TGFB2), transforming growth factor-beta 3 (TGFB3), Msh homeobox 1 (MSX1), Methylenetetrahydrofolate Reductase (MTHFR) and protooncogene B-cell leukemia protein 3 (Bcl3) [1]. Results are controversial. While researchers found a relationship between cleft lip/palate and these genes in some studies, various other studies reported the contrary [1,4-6]. The major risk factors for cleft lip and palate formation have not been clearly elucidated, and it is unclear which of the mutant genes are causing this disease. The reason behind this is the complexity and diversity of the relevant mechanisms at the molecular level during embryogenesis [7].

When the development of the palate in the embryo is examined, the secondary palate elevation and tissue fusion of lips are seen to play important roles in this process. In the development of the second palate, glucosamine and hyaluronan-hydration are needed, and collagen fibers direct the elevator force [8,9]. Interestingly, the pathways of these three components intersect in the hypoxic event. There is a complex relationship between glucosamine, hyaluronan, collagen, and hypoxia. Hypoxia up-regulates hyaluronan, reduces collagen synthesis and enhances the effect of D-glucosamine to down-regulate HIF1A [10-12]. HIF1A, a component of HIF1 and a significant mediator of hypoxia, functions as a key regulator of cellular and systemic homeostatic response to hypoxia by coordinating the genes involved in energy metabolism, angiogenesis, apoptosis, and other genes whose protein products increase oxygen delivery or facilitate the metabolic adaptation to hypoxia [13,14]. HIF1A is rapidly degraded in cells under normoxic conditions by the von Hippel-Lindau tumor suppressor protein (pVHL), which targets the minimal N-terminal transactivation domain (N-TAD) (within the oxygen-dependent degradation domain (ODD)) of HIF1 [13,14]. Two substations of the HIF1A may change the lifespan of HIF1A: One encodes serine at codon 582 (Pro582Ser) and the other encodes threonine at 588 (Ala588Thr) position that is within or near the N-TAD domain.

In this study, we aimed to determine the distribution of these two polymorphisms in children with cleft lip and palate and their mothers to assess the role of these two polymorphisms in cleft lip/palate development and learn about the role of hypoxia in this pathology.

Materials and methods

A total of 76 children with non-syndromic cleft lip/palate and their mothers were included in the study group. This study was approved by the Ethics Committee of the Dicle University Medical Faculty, Diyarbakır, Turkey (No 69-18/03/2011). All parents provided informed consent before blood tests were performed. Control group consisted of 78 healthy children and mothers with no family history of clefting or other congenital disorders.

All subjects underwent peripheral blood sampling for genotype analyses. Genomic deoxyribonucleic acid (DNA) was isolated from peripheral blood by DNA isolation kit (Bio Basic Inc., EZ-10 Spin Column Genomic DNA Kit for Blood Samples; Ontario, Canada). Genotyping was performed by the PCR-RFLP method. The oligonucleotide primers for single-nucleotide polymorphisms (SNPs) were selected from a previous study and optimized for appropriate PCR conditions in our laboratory [15]. *Bsl1* and *Aci1* restriction enzymes were used for the digestion of amplicons according to protocols provided by the supplier of the enzymes. SNPs, oligonucleotide primers, and restriction enzymes are given in Table 1. Restriction enzyme-digested fragments were monitored by agarose gel electrophoresis.

Table 1: SNPs, primer pairs and restriction enzymes [15]

SNPs	Primers	Restriction Enzymes
rs11549465	12A F: 5'-TGTGGCCATTGTAAAAACTCA-3'	Bsl1
(Pro582Ser)	12 PR:5'-CTTGCGGAACTGCcTTCTAA-3'	
rs11549467	12A F: 5'-TGTGGCCATTGTAAAAACTCA-3'	Aci1
(Ala588Thr)	12A R: 5'-TTTAATTCATCAGTGGTGGCA-3'	

SNPs: single-nucleotide polymorphism

Statistical analysis

Descriptive statistics were expressed as count and percentage. Chi-square test was used to evaluate the significance of the distribution of polymorphisms between the study group and healthy controls. *P*-value <0.05 was considered statistically significant and SPSS (version-21) statistical program was used for all statistical computations.

Results

Associations of two known polymorphisms of HIF1A in cleft lip/palate were investigated. For this purpose, allele frequencies obtained from study group and mothers were compared with control individuals and mothers. Interestingly, we did not find any polymorphisms in Ala588Thr (rs11549467; G1790A) in any of the participants. All showed homozygous GG genotype. This result was confirmed by the DNA sequencing of randomly selected samples.

For Pro582Ser (rs11549465; C1772T), we found differences in the genotypes between children and between mothers of the study and control groups. Genotyping results and allele distribution of children (study and control groups) are given in Table 2. Results of the mothers are presented in Table 3.

CC and CT genotype frequencies of the control group children were higher than the study group children and TT genotype frequency of the study group was higher than that of the control group (*P*=0.034). Also, differences were seen in allele frequencies of the children: The C allele was seen more in the control group than the study group (*P*=0.026). Similar to the results of children, a difference was detected between the genotype frequencies of mothers (*P*=0.023). The C allele was seen higher in the control group than the study group (*P*=0.001).

Table 2: Genotype and allele frequencies of children for rs11549465 with cleft lip/palate and healthy controls

Genotypes	Case n=76	Controls n=78	Genotype frequencies of cases %	Genotype frequencies of control %	χ2	*P*-value
CC	48	64	63.2	82.1	8.75	0.034
CT	28	13	36.8	16.7		
TT	0	1	0	1.2		
Alleles	n=152	n=156	Odds ratio (95% CI)		χ2	*P*-value
C allele	124	141	2.1226 (1.084-4.1562)		4.97	0.026
T allele	28	15				

CI: Confidence Interval

Table 3: Genotype and allele frequencies of mothers of children with cleft lip/palate and mothers of healthy controls

Genotypes	Case (mother) n=76	Controls (mother) n=78	Genotype frequencies of cases %	Genotype frequencies of control %	χ2	*P*-value
CC	46	64	60.5	82.1	9.763	0.023
CT	24	13	31.6	16.7		
TT	6	1	7.9	1.2		
Alleles	n=152	n=156	Odds ratio (95% CI)		χ2	*P*-value
C allele	116	141	2.9172 (1.5221-5.591)		11.029	<0.001
T allele	36	15				

CI: Confidence Interval

Discussion

Cleft lip and palate are orofacial defects that occur during pregnancy. These individuals cannot breathe normally from the nose due to the inadequate mouth pressure, and pronunciation is also affected [16]. In addition, there is facial asymmetry. It is difficult to eat solid foods. There are deformities in the shape of the teeth and in their placement, and maintaining oral hygiene is a challenge [17]. Surgical treatment is the main solution.

The mechanism of the formation of cleft lip and palate is not entirely clear. After studies have shown that the use of folic acid reduces the incidence of cleft lip and palate, researchers focused on genes involved in folic acid metabolism and transport. Some of the candidate genes are folate receptor alpha (FR), reduced folate carrier (RFC), 5,10-methylenetetrahydrofolate reductase (MTHFR), cystathionine β-synthase (CBS), methionine synthase (MTR), and methionine synthase reductase dehydrogenase (MTHFD) [18-20]. HIF1, another one of the candidate genes, a heterodimer composed of an alpha and a beta subunit, plays an essential role in embryonic vascularization and pathophysiology of ischemic diseases [21]. HIF1 regulates the transcription of genes involved in apoptosis, angiogenesis, energy metabolism and those whose protein products increase oxygen delivery or facilitate metabolic adaptation to hypoxia [21]. Hypoxia is an important instrument in embryogenesis and HIF-1 is the master regulator of hypoxia. Homozygous inactivation of HIF-1 is lethal due to the lack of vascular formation in the embryo [22].

Under normoxic conditions, HIF1A protein degrades rapidly with the binding of von Hippel Lindau tumor suppressor protein (pVHL). pVHL targets the N-terminal transactivation domain (N-TAD) of HIF1A which is a part of the oxygen-dependent degradation domain (ODD) [13,14]. This blocks the subsequent activity of HIF1A, preventing HIF1A from translocating to the nucleus, and merging with HIF1 beta to form the transcription factor HIF1 [13]. N-terminal transactivation domain (N-TAD) is important in the degradation of HIF1A and affinity of HIF1A to pVHL is determined by proline residues within the N-TAD domain [13]. One of the polymorphisms we studied is the substitution of proline to serine within N-TAD domain, which may affect the lifespan of HIF1A protein. In this study, we found significant differences between children with cleft lip/palate, healthy control groups and their mothers.

HIF1, an important mediator of hypoxia, functions as a key regulator of cellular and systemic homeostatic response to hypoxia by regulating genes whose protein products increase oxygen delivery or facilitate metabolic adaptation [1,13]. During embryogenesis, hypoxia plays a key role in the development of palate in the embryo [23]. As mentioned above, hypoxia is an intersection point for the formation and elevation of the palate and the fusion of lips. HIF1 is the orchestra chef of this pathway and if its lifespan is longer than normal, it may downregulate collagen and upregulate hyaluronan and D-glucosamine, which may subsequently affect the formation of cleft lip/palate.

The strength of this study is that it involves mothers to investigate the intrauterine effects of these two polymorphisms of HIF1A on cleft lip/palate formation. One of the limitations is the relatively small number of patients and controls. It would be better to study in a larger population. Another limitation is the lack of research at protein and mRNA levels.

Conclusion

We studied two substitutions of HIF1A near the binding place of pVHL. We have not found any polymorphisms in Ala588Thr. The other substitution was the conversion of proline to serine amino acid at the 582nd position. pVHL binds to proline residues, and if proline residues change, this may affect the lifespan of HIF1A protein. This finding needs to be confirmed by messenger RNA (mRNA) and protein expression levels.

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