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Original Research

Studying the C1772T polymorphism of *Hif-1α* and *TGF-β3* IVS5+104 A/G polymorphism in children with congenital non-syndromic neural tube defects and their mothers

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Abstract: Prevalence of neural tube defect (NTD) has reduced after folic acid intake. However; which mechanisms are effective in NTD are not known exactly. In this study; due to the possible effects on hypoxic pathway and embryonic development, particularly on extracellular matrix components, *Hif-1α* Pro582Ser and *TGF-β3* IVS5+104 A/G SfaN1 polymorphisms were studied by PCR-RFLP method both on children with NTDs and mothers. Statistical differences were seen for *Hif-1α* and *TGF-β3* IVS5+104 A/G SfaN1 polymorphisms in children with NTDs but no difference was seen in mothers. Both genes are effective on many pathways and our results suggest that regulation of extracellular matrix components of children during fetal life is important in neural tube defects formation. The results of this study show that *Hif-1α* Pro582Ser and *TGF-β3* IVS5+104 A/G SfaN1 polymorphisms may play a role in NTDs.

Key words: NTD; Hypoxia, *Hif-1α*; *TGF-β3; SfaN*1.

Introduction

NTDs seen in 3 of 1000 live births (1). After determining the effect of folic acid in the prevention of NTD, folic acid use became widespread and therefore the incidence of NTD decreased (2). As folic acid use significantly reduced NTD cases, genes involved in folic acid metabolism and transport were targeted. These candidate genes include *Folate Receptor* (*FR*), *Reduced Folate Carrier* (*RFC*), *Cystathionine B-Synthase* (*CBS*), *Methionine Synhtase* (*MTR*), *Methionine Synthase Reductase* (*MTRR*), and *Methylenetethrahydrofolate Dehidrogenase* (*MTHFR*) (3,4). It has been identified that there was a relationship between NTD and a few of these genes and later these genes were presented as important risk factors (4,5).

While in some studies, a relationship between oxygen pressure and neural tube closure (6-8) has been identified, in some other studies, the role of apoptosis in the neural tube closure has been identified (9,10). In these studies, it has been shown that hypoxia is essential in inducing the apoptotic processes for the neural tube closure. During the critical period of development, degradation in the oxygen level regulation was observed to produce similar results as using caspases that block the neural tube closure (9). In mice, around forty genes have been identified to cause NTDs (11) and in studies, *proto-oncogene ski* (12), *homeobox gene Cart1* (13) and tumor suppressor gene *p53* (14) which were estimated to have a role in apoptosis and neural tube were found to be regulated by hypoxia. Studies have shown that hypoxia is effective in normal embryonic development by causing changes also in gene expression. Mouse embryos with homozygous *Hif-1α* mutations could not survive and were found to have NTDs and cardiovascular anomalies (6,8). Studies have indicated that HIF activity should be induced by hypoxia for embryo and placental development (15,16). Moreover, antisense inhibition of *Hif-1* in villus explants has been shown to downregulate *TGF-β3* mRNA expression (17). A study carried out in the *TGF-β3* knock out mice revealed that these mice had cleft lip/palate, NTD, and delayed lung development (18). *TGF-β3* has also been shown to have effects on collagen, glycosaminoglycan and hyaluronan expression, which play an important role in neural tube closure (19).

Undesirable conditions that lead to hypoxia on placenta and embryo may be caused by the maternal side of the placenta or the fetal side of the placenta. There are very limited publications about the hypoxic complications that can be considered as negative, if they caused by the fetus along with maternal factors. Theoretically, both maternal and fetal hypoxia and thrombophilia may be thought to increase complications. In this study, we aimed to understand the role of polymorphic structures of *TGF-β3* and *Hif-1α* in the development of NTD by studying the polymorphism of *TGF-β3* IVS5+104 A/G SfaN1 polymorphism and *Hif-1α* Pro582Ser polymorphism. *TGF-β3* IVS5+104 A/G SfaN1 polymorphism plays an important role in the degradation of *Hif-1α* which has importance in hypoxia.

Materials and Methods

A total of 68 patients with NTDs and their mothers were included in the study. This study was approved by

Table 1. Studied SNPs, primer pairs and restriction enzymes.

	SNPs	Primer Pairs	Restriction endonuclease
Hif-1α	rs11549465 1772 C/T	F 5'-TGTGGCCATTGTAAAAACTCA R 5'-CTTGCGGAACTGCcTTCTAA	*Bsl*1
TGF-β3	IVS5+104A/G	F 5'TGTCACTTTCCTTCCCTTCTTC R 5'TTCTTCCTGGAGATGTTTG	*SfaN*1

the Ethics Committee of the Dicle University Medical Faculty, Diyarbakır, Turkey. All parents provided informed consent before the blood test was performed. Control group consisted of 78 healthy individuals and mothers with no family history of NTD or other congenital disorders.

All subjects underwent peripheral blood sampling for genotype analyses. Genomic DNA was extracted from peripheral blood by DNA isolation kit (Bio Baisc Inc., EZ-10 Spin Column Genomic DNA Kit for Blood Samples; Ontario, Canada). The oligonucleotide primers for SNPs were selected from a previous study (20,21) and optimized for appropriate PCR conditions in our laboratory. Genotyping was performed by the restriction enzyme digestion of amplicons according to protocols provided by the supplier of the enzymes. SNPs, oligonucleotide primers and restriction enzymes are given in Table 1. Fragments digested by restriction enzymes were monitored in agarose gels in concentrations between of 2% to 3%.

Statistical Analysis

Descriptive statistics were expressed as count and percent. C*hi*-square test was used to test the significance of the distribution of mutations between patients and control groups. Statistical significance levels were considered as 0,05 and SPSS (ver. 13) statistical program was used for all statistical computations.

Results

In this study, we investigated the polymorphism of the *Hif-1α* which substitutes the Proline amino acid to Serine amino acid and SfaN site polymorphism of *TGF-β3* in children with NTDs. We also decided to assess the effects of these polymorphisms in the intrauterine stage and we included mothers in the study. For this purpose, allele frequencies obtained from cases and mothers compared with healthy control individuals and their mothers. According to the results obtained from *Hif-1α*, there was a statistical difference between the comparison of allele frequencies of children with NTD and healthy control. In genotype comparisons, the p-value was found to be slightly higher than 0.05. We have not found any difference in the comparison between mothers of children with NTD and mothers of healthy controls both in genotype and allele frequencies. The results are shown in Table 2.

In this study, we also evaluate the SfaN site (IVS5+104 A/G) polymorphism of TGF-β3 which is thought to be related to the HIF1 pathway in NTDs. We found statistical differences in genotype and allele comparisons of children with NTDs and healthy controls. We have not found any difference between comparisons of mothers. Results are shown in Table 3.

Discussion

The response to changes in oxygen concentration may either be to adapt to the reduced oxygen concentration or to prefer less reliable alternative pathways. In hypoxia adaptation; changes in gene expression, metabolic function and ion channel activation are realized. All these are necessary for the survival of the cell. The ability of the cell to respond to changes in oxygen pressure depends on the activation of a transcription family known as Hypoxia-Inducible Factors (HIFs) (22).

HIF protein is first identified as the hypoxically responsive element (HRE) on the enhancer 3 of the erythropoietin gene (23). Subsequent studies have shown that the HIF protein is regulated by cell oxygen concentration and these proteins bind to DNA under hypoxic conditions (24). HIF proteins are continuously synthesized in the cell but rapidly degraded by von Hippel Lindau

Table 2. Results of *Hif-1α* C1772T (Pro582Ser) polymorphism in children and mothers.

	Genotypes	Case n=68	Controls n=78	Genotype frequencies of case %	Genotype frequencies of control %	χ2	*p*
	CC	44	64	64.7	82.0		
Hif-1α	CT	24	13	35.3	16.7	7.323	0.063
	TT	0	1	0.0	1.2		
C1772T	**Alleles**						
	C	112	141	OR (95% CI)		4.05	0.044
Children	T	24	15	2,0143 (1,0091-4,0206)			
	Genotypes						
	CC	48	64	70.6	82.0		
Hif-1α	CT	20	13	29.4	16.7	1.105	0.128
	TT	0	1	0.0	1.2		
C1772T	**Alleles**						
	C	116	141	OR (95% CI)		1.78	0.182
Mothers	T	20	15	1,6207 (0,7943-3,3068)			

Table 3. Results of *TGF-β3* IVS5+104 A/G SfaN site polymorphism in children and mothers.

	Genotypes	Case n=65	Controls n=68	Genotype frequencies of case %	Genotype frequencies of control %	χ^2	*p*
***TGF-β3* IVS5+104 A/G Children**	AA	33	48	50.7	70.6	7.367	0.025
	AG	29	20	44.6	29.4		
	GG	3	0	4.7	0.0		
	Alleles						
	A	95	116	OR (95% CI)		6.049	0.0139
	G	35	20	2.1368 (1.1579-3.9433)			
	Genotypes						
***TGF-β3* IVS5+104 A/G Mothers**	AA	42	42	64.6	61.8	2.466	0.291
	AG	21	26	32.3	38.2		
	GG	2	0	3.1	0.0		
	Alleles						
	A	105	110	OR (95% CI)		0.001	0.975
	G	25	26	1.0073 (0.547-1.8552)			

protein (pVHL) via the ubiquitin-proteasome system in the presence of high oxygen concentration (25,26). The pVHL protein carries out this function by binding to the amino acids in the N-terminal Transactivation Domain (NTAD) region of the *Hif-1α* (27). In this study, we tried to reveal the frequency of Pro582Ser polymorphism, which causes the change of Serine amino acid to Proline in the NTAD region of Hif-1α protein. A statistical difference in the allele comparison of children with NTDs and healthy children was observed. However, in genotype comparisons, the *p* value was found to be slightly higher than 0.05. When the results from the allele comparison and the genotypes are evaluated together, it may be thought that if the number of subjects increased, statistical differences can be seen in the comparison of genotypes. In this study, Pro582Ser polymorphism of *Hif-1α* was also studied in mothers of children with NTDs. Unlike the results obtained from children, we observed that there was no statistical difference in allelic comparison and genotype comparisons of mothers.

In the case of Hif-1α protein cannot be degraded by pVHL protein due to the change of Proline amino acids in the NTAD domain of Hif-1α, collagen synthesis is downregulated. In a study conducted by Hosper et al., it was shown that collagen synthesis decreased in amniotic cells obtained from the NTD embryos (28). Hif-1α protein may cause to the reduce of collagen synthesis, when undegraded by binding pVHL. As mentioned above, the pVHL protein binds to the amino acids Proline in the NTAD domain of the Hif-1α, delivering the Hif-1α to the ubiquitin pathway, thus may be affecting collagen synthesis. Collagen is one of the most important components of the extracellular matrix, and the decrease in its expression leads to a lack of healthy development in many tissues, including the neural tube closure.

In this study, *TGF-β3* IVS5+104 A/G polymorphism was also studied in children with NTDs and mothers. It has been shown that TGF-β3 plays a vital role in trophoblastic differentiation due to the effect of hypoxia and it was overexpressed in preeclamptic placenta (29). Besides, antisense inhibition of HIF-1 has been shown to downregulate *TGF-β3* mRNA expression in villus explants (17). Although *TGF-β3* is important in trophoblastic differentiation, the molecular mechanism of activating TGF-β3 via hypoxia/HIF-1 is not indicated clearly. In this study, we found statistical differences in the substitution of *TGF-β3* IVS5+104 A/G in both genotype and allele distribution in comparisons of children with NTDs to healthy individuals. This situation which we have identified in children has not been determined in mothers' comparisons.

Various studies have suggested that TGF-β3 is important in controlling the expressions of glycosaminoglycan, hyaluronan, and collagen, which have been shown to play an important role in the development of the extracellular matrix. It is not clear how the *TGF-β3* IVS5+104 A/G polymorphism affects the expression of *TGF-β3*, the life-span of the protein, or other functions of TGF-β3. The result obtained from children with NTDs suggests that this polymorphism may play an important role in the function of TGF-β3.

In conclusion, in this study we conducted in children with NTDs and mothers; we observed that *Hif-1α* Pro582Ser and *TGF-β3* IVS5+104 A/G polymorphisms are important in children with NTD. We have not seen any statistical difference in mothers for both polymorphisms. Studying with a larger group of subjects and identifying how *TGF-β3* IVS5+104 A/G affects TGF-β3 and its functions will contribute to the role of these polymorphic structures in neural tube development.

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Conflicts of Interest
There is no any conflict of interest.

Author's contribution
İbrahim Halil Yıldırım and Nadir Koçak conceived of the presented idea together and carried out all the stage of this study together. Both authors discussed the results and contributed to the final manuscript.

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