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Transforming Growth Factor-β3 Gene *Sfa*N1 Polymorphism in Korean Nonsyndromic Cleft Lip and Palate Patients

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The nonsyndromic cleft lip and palate (NSCL/P) is a congenital deformity of multifactorial origin with a relatively high incidence in the oriental population. Various etiologic candidate genes have been reported with conflicting results, according to race and analysis methods. Recently, the ablation of the TGF-β3 gene function induced cleft palates in experimental animals. Also, polymorphisms in the TGF-β3 gene have been studied in different races; however, they have not been studied in Koreans. A novel A → G single nucleotide polymorphism (defined by the endonuclease *Sfa*N1) was identified in intron 5 of *TGF-β3* (IVS5+104A > G). It resulted in different genotypes, AA, AG, and GG. The objective of this study was to investigate the relationship between the *Sfa*N1 polymorphism in *TGF-β3* and the risk of NSCL/P in the Korean population. The population of this study consisted of 28 NSCL/P patients and 41 healthy controls. The distribution of the *Sfa*N1 genotypes was different between the cases and controls. The frequency of the G allele was significantly associated with the increased risk of NSCL/P [odds ratio (OR) = 15.92, 95% confidence interval (CI) = 6.3-41.0]. The risk for the disease increased as the G allele numbers increased (GA genotype: OR = 2.11, 95% CI = 0.38-11.68; GG genotype: OR = 110.2, 95% CI = 10.67 2783.29) in NSCL/P. A stratified study in patients revealed that the *Sfa*N1 site IVS5+104A > G substitution was strongly associated with an increased risk of NSCL/P in males (p<0.001), but not in females. In conclusion, the polymorphism of the *Sfa*N1 site in *TGF-β3* was significantly different between the NSCL/P patients and the control. This may be a good screening marker for NSCL/P patients among Koreans.

Keywords: Cleft lip and palate, Polymorphism, TGF-β3

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Introduction

Clefts of the lip and palate affect about 1/1,000 births and have diverse causes. These can be classified as environmental factors of either geographical or socioeconomic origin and genetic factors (Murray, 2002). Clefts can be divided into nonsyndromic and syndromic forms. In nonsyndromic clefts, the affected individuals have no other physical or developmental abnormalities. The syndromic clefts can be subdivided into three different types; chromosomal syndrome, teratogenic syndrome, and uncategorized syndrome (Mitchell *et al.*, 2000). Clefts also can be classified as either those that involve the lip, with (CL/P) or without the palate (CL), and those that involve the palate only (CPO).

Nonsyndromic cleft lip, with or without palate (NSCL/P), is a common complex multigenic birth defect that has been extensively studied (Maestri *et al.*, 1997; Romitti *et al.*, 1999). However, the nature of the genetic or environmental contribution to the etiology of this malformation remains to be elucidated. The probable environmental factor causes are nutritional deficiency, intrauterine trauma, smoking, and drug abuse (Murray, 2002). Some genetic defects can also induce cleft palate. Thus, it would be difficult to find any single gene that has a definite relation to the disease. However, statistical approaches that are aimed at discovering evidence of an unequal distribution of genetic variants for candidate genes in the case and control populations could provide the identification of the role for a candidate gene in a particular disorder (Lander, 1994). Efforts to investigate the genes that are involved in nonsyndromic cleft also include the evaluation of allelic variants at candidate loci. Associations have been identified between the allelic variants of the genes for the transforming growth factor α (TGF-α) (Machida *et al.*, 1999), retinoid receptor α, B-cell CLL/Lymphoma 3 (BCL3) (Gaspar *et al.*, 2002), MSX1, and the transforming growth factor β3 (TGF-β3) (Lidral *et al.*, 1997).

Candidate genes that potentially play a role in the pathogenesis of congenital deformities can be identified from

animal models or studies of tissue and embryonic-stage-specific expression (Murray, 1995). Recently, a TGF-β3 knock-out mouse showed a cleft palate and delayed pulmonary development (Kaartinen *et al.*, 1995; Proetzel *et al.*, 1995). Also, the polymorphism in exons 2 and 5 of *TGF-β3* had a high correlation with the cleft palate (Maestri *et al.*, 1997). The polymorphism of *TGF-β3*, or an adjacent DNA sequence on the Japanese population, revealed that it might contribute to the development of cleft lip and palate (Sato *et al.*, 2001). However, no relationship was found between the TGF-β3 gene and the development of cleft lip and palate in the Philippines (Lidral *et al.*, 1997). It is, therefore, possible that there may be some difference in the cleft-related gene in various countries.

Although there is high incidence of cleft palate in oriental countries, there has been little genetic epidemiological study on the Korean population. In the present study, we evaluated the association of a novel *Sfa*N1 polymorphism, which resides in intron 5 of *TGF-β3*, with the increased risk of NSCL/P in the Korean population.

Materials and Method

Subjects The samples were collected from NSCL/P patients who were examined at the Department of Orthodontics, as well as from students as the control in the College of Dentistry, Seoul National University (Seoul, Korea) from 2000 to 2002. All of the patients were examined for the presence of cleft lip, with or without secondary clefting of the palate, but not for any other associated abnormalities that would be suggestive of syndromic variants. The distribution of cleft types was 8 bilateral CLP, 18 unilateral CLP, and 2 CPO. The total samples consisted of 28 NSCL/P and 41 controls.

Collection of blood samples The DNA was collected from blood samples. Blood was absorbed in filter paper and dried. A small disc was punched with a 1.5 mm diameter and then put intoa PCR tube. After adding 200 μl of the lysis buffer (1% SDS and 0.2 N NaOH), it was vortexed and placed at room temperature for 5 min. The tube was centrifuged at 12,000 rpm for 15 s. The aqueous solution was removed. This procedure was repeated twice. The disc was then washed twice with 70% ethanol. Finally, the disc was dried in the PCR tube at room temperature.

TGF-β3 genotying When the disc was completely dried, PCR was performed as follows: To determine the TGF-β3 at the *Sfa*N1 site genotype, genomic DNA was amplified by a PCR using primer sets. The sense primer was 5'-TGTCACTTTCCTTCCCTTCTTC-3' and the antisense primer was 5'-TTCTTCCTGGAGATGTTTG TGA-3'. The PCR products were directly sequenced. The other genotyping was performed using different PCR primers (upper primer: 5'-GAGCAGGAGATTGTCACTTTC-3', lower primer: 5'-TGACCAAGCATCTCCAATC-3') and digestion of the PCR products with *Sfa*N1 (New England Biolab Co., Beverly, USA). The PCR reaction was comprised of 32 cycles of amplification with initiation at 94°C for 4 min, denaturation at 94°C for 20 s, annealing at 51°C for 20 s, and extension at 72°C for 30 s. After the *Sfa*N1 digestion, there were three fragments (506, 331, and 194 bp) in AG heterozygote, two (331 and 194 bp) in GG, and one (506 bp) in AA genotype.

Statistical Method The χ^2 test calculated the statistical significance of the frequency differences of the variant at the*Sfa*N1 polymorphism in the *TGF-β3* genotypes in groups. The risk of NSCL/P that was associated with the genotype was estimated using the odds-ratios (OR) and 95% confidence intervals (CI) for the marker allele. The two means were compared by the Welch unpaired t-test. A *P* value of less than 0.05 was considered to be statistically significant. All of the analyses were performed using Statistical Analysis Software for Windows, version 6.12 (SAS institute Inc., Cary, USA).

Results

The NSCL/P was clinically determined. Table 1 presents the cleft type of the cases. Among 28 patients, 18 were unilateral CLP, 8 were bilateral CLP, and 2 were CPO. We first sequenced all of the exons of the TGF-β3 gene to see whether the mutation was observed in the cases. There was no mutation in the coding region in both the cases and controls; however, a single nucleotide polymorphism was observed in the intron 5 of *TGF-β3* in our PCR products. It was A → G mutation at position +104, relative to exon 5/intron 5 junction (IVS5+104A > G, *Sfa*N1 site) (Fig. 1). The *Sfa*N1 digestion was related in the A and G genotypes (Fig. 2A). The distribution of the allele frequency at *Sfa*N1 among the controls and cases was different in the Korean population (Table 2). The respective frequency of the AA, AG, and GG genotypes in the NSCL/P individuals (17.9, 14.3, and 67.8%, respectively, P<0.01) was significantly different from that of the controls (70.7, 26.8, and 2.4%, respectively). When the AA genotype was used as the reference group, the risk of the disease increased as the G allele number increased (AG genotype: OR = 2.11, 95% CI = 0.38 – 11.68 and GG genotype: OR = 110.2, 95% CI = 10.67 – 2783.29). The frequency of the G allele among the NSCPL patients was significantly higher than in the control population (OR = 15.92, 95% CI = 6.3 – 41). Male patients with the G allele type were particularly strongly associated with NSCL/P (OR

Table 1. Summary of patients

Sex	Male	17
	Female	11
	CLP	26
	CPO	2
Cleft lip	Uni-Rt	1
	Uni-Lt	17
	Bilateral	8

CLP, cleft lip and palate; CPO, cleft palate only; Uni-Rt, unilateral right; Uni-Lt, unilateral left.

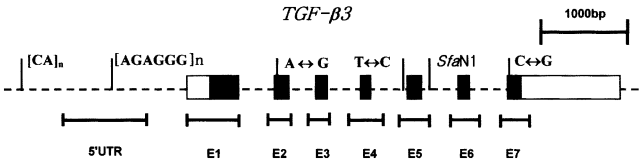

Fig. 1. The locus of a single nucleotide pleomorphism of *TGF-β3*. The exons are indicated as a box, and the *Sfa*N1 site is shown between exon 5 and intron 5. The cartoon was modified from http://genetics.uiowa.edu/data/candidateGenes/TGFB3.shtml. Several SNP are indicated through the exon or intron sites.

= 38.9, 95% CI = 9.91 – 168.3); however, the female patients were not as strongly associated with NSCL/P (OR = 1.23, 95% CI = 0.4 – 3.84). There was also no significant difference between the unilateral and bilateral cases with NSCL/P.

Discussion

This study reveals that the G allele at the *Sfa*N1 polymorphism of *TGF-β3* is associated with an increased risk of NSCL/P in the Korean population. It is also specifically associated with the male NSCL/P patients.

The genotypes of the variant at the *Sfa*N1 site consist of the AA, AG, and GG sequences. Individuals in the control had similar genotype; whereas, those with the GG genotype were significantly associated with the NSCL/P cases. In this study, the relative risk of having the G allele was 15.92 (95% CI = 6.3 – 41) for NSCL/P in the Korean population. The risk was significantly higher for males (OR = 38.9, 95% CI = 9.91 – 168.3), but not for females (OR = 1.23, 95% CI = 0.4 – 3.84). A characteristic finding of this study was that there was a gene-dosage effect with the G allele. The result showed that the polymorphism at the *Sfa*N1 site in *TGF-β3* was strongly associated with an increased risk of NSCL/P in Koreans.

Several polymorphic sites of *TGF-β3* were analyzed in the case and control studies with regard to association with clefts (Lidral *et al.*, 1998; Romitti *et al.*, 1999). Based on the published data, nearly equal proportions of the case and control infants were typed for the TGF-β3 markers. However, the transmission/disequilibrium test (TDT) analysis of other sites in *TGF-β3* was documented as a significant association with NSCL/P in the Caucasian (Lidral *et al.*, 1998; Romitti *et al.*, 1999) and Japanese populations (Sato *et al.*, 2001). Lidral *et al*. (1998) showed that a linkage disequilibrium between the TGF-β3 marker and either CL/P or CPO was not detected in the case-control design, while a linkage disequilibrium was

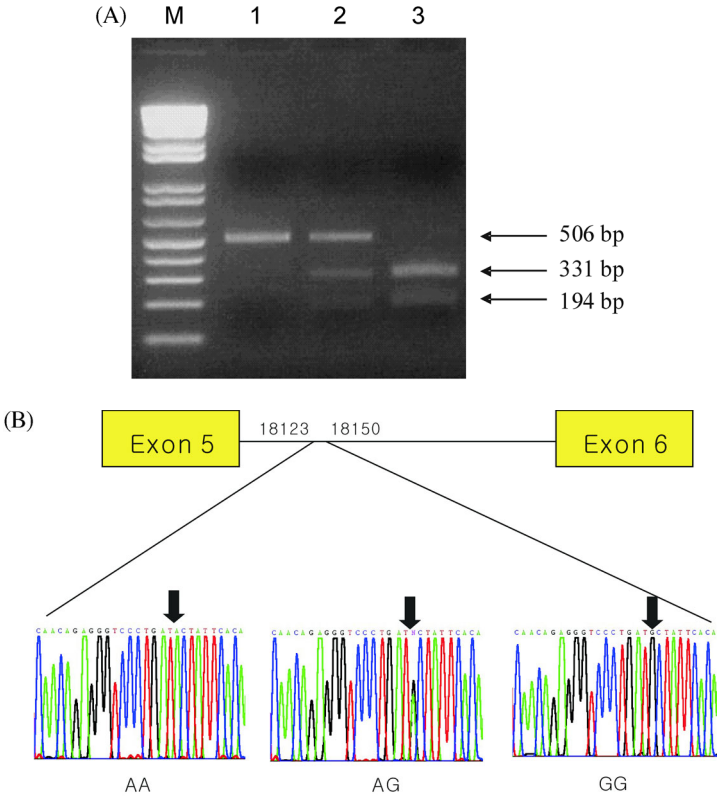

Fig. 2. The *Sfa*N1 polymorphism in the TGF-β3 gene. (A) The PCR products were digested with *Sfa*N1 and electrophoresised in a 2% agarose gel. Lane M, size marker; Lane 1, homozygous AA *Sfa*N1 genotype; Lane 2, heterozygous AG *Sfa*N1 genotype; Lane 3, homozygous GG *Sfa*N1 genotype. (B) Sequencing results of each genotype. The numbers are from GenBank (access number AY140241).

Table 2. Genotypic and allelic distributions of the *Sfa*N1 SNP in CLP patients and the control[a]

Subjects(n)	Genotype			Allele type (%)			P Value	Odd ratios (95% CI)
	G/G	A/G	A/A	G	A	χ^2		
CP/L								
All (28)	19	4	5	42 (75)	14 (25)	46.126	<.001	15.92(6.3-41.0)
M (17)	14	1	2	29 (85)	5 (15)	42.212	<.001	38.9(9.91-168.3)
F (11)	5	3	3	13 (59)	9 (41)	5.905	0.015	1.23(0.4-3.84)
Control								
All (41)	1	11	29	13 (15.8)	69 (84.2)			
M (27)	0	7	20	7 (13)	47 (87)			
F (14)	1	4	9	6 (21.4)	22 (78.6)			

[a]Differences in allelic distribution between subpopulations of CPL patients and the controls were analyzed using the χ^2 test.

observed between CL/P and the TGF-β3 X5.1 variant, which is the T → C mutation at position −24 relative to the intron 4/exon 5 junction by the TDT analysis. The same group showed that maternal smoking is associated with a risk for CP at the TGF-β3 X5.1 variant and CP/CLP at the 5 UTR.1 variant (Romitti *et al.*, 1999). Also, drinking alcohol was a risk factor for CLP and CP in relation to the variants TGF-β3 CA2, X5.1, and 5 UTR (Romitti *et al.*, 1999).

This study adds a new *Sfa*N1 polymorphic site that shows a significant association of the G allele at the IVS5+104A > G site of the TGF-β3 gene with NSCL/P using the case-control design. We expect that a TDT analysis of the family cases at the *Sfa*N1 site may show a significant association with the increased risk of NSCL/P in the Korean population. In addition, the analysis of the *Sfa*N1 site of *TGF-β3* in other populations will help in detecting how much is contributed by population heterogeneity or other environmental influences.

The TGF-β3 gene has been known as one of the strong candidates for NSCL/P, based on the knock-out mouse phenotype. The homozygous TGF-β3 null mouse shows a cleft palate and it has no other concomitant craniofacial abnormalities (Kaartinen *et al.*, 1995; Taya *et al.*, 1999). TGF-β3 is specifically expressed at a high level in the epithelial tissue of the medial edge of the palatal shelves. Also, inhibition of the TGF-β3 function by an antibody or antisense oligonucleotide prevents normal palate fusion (Brunet *et al.*, 1995). In a recent study, the exogenous addition of TGF-β3 could induce palatal fusion in the cleft palate model of the chicken embryo, and TGF-β3 decreased the epidermal growth factor receptor immunoreactivity in mesenchymal cells beneath the medial edge epithelium of the palatal process (Yang *et al.*, 2001). The comparative loss-of-function and gain-of-function studies indicate that the down-regulated TGF-β3 level might be one of causal factors for CL/P. It will be very interesting to explore the contribution of the TGF-β3 pathway genes to palatogenesis. Further study is required to discover whether the *Sfa*N1 site in *TGF-β3* can affect the TGF-β3 expression level, and also why the polymorphism has a strong association with NSCL/P in males, etc.

Collectively, the *Sfa*N1 polymorphism in *TGF-β3* proved to be a predictor of NSCL/P, especially in Koreans males.

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