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The ethnic difference of the prevalence of SfaN polymorphism in the nonsyndromic cleft palate

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Nonsyndromic cleft lip and/or palate (NSCLP) is one of the most common congenital deformities and its prevalence in Far East Asia, such as within Korean and Japanese populations, is relatively high. However, in the eastern part of Europe, clefts are relatively rare situations. These ethnic differences infer a genetic background of the disease. The objective of this study was to compare the frequency of single nucleotide polymorphism (SNP) in TGF-β3 between Korean and Romanian cleft families.

Korean cleft families samples were collected from twenty-six families (n=78) and Romanian cleft families samples were collected from eighteen families (n=41). For sequencing, the blood or saliva of the subjects was sampled. A single nucleotide plolymorphism was observed in the intron 5 of TGF-β3 (A18141G).

The frequency of each allele was significantly different between the Korean and Romanian samples. The AA allele was present in 18 out of 78 Korean samples (23.1%) and in 27 out of 41 Romanian samples (65.9%). The AG was present in 27 (34.6%) out of 78 Koreans and in 13 (31.7%) out of 41 Romanians. The GG was found in 33 (42.3%) Koreans and in 1 (2.4%) Romanian. The difference between the groups was significant ($p<0.001$).

In conclusion, the frequency of observed SNP was significantly different between the two countries. SNP in TGF-β3 in the Korean population seemed to have a higher possibility of occurrence for nonsyndromic cleft palate than the Romanian population.

Key words : Nonsyndromic cleft palate, SNP, TGF-β3, SfaN1

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Cleft palate is one of the most common congenital deformities and many authors have studied the causes of this disease.[1,2] Probable causes can be enlisted as nutrient deficiency, intrauterine trauma, drug abuse, and so on.[3] Certain genetic defects can also induce cleft palate. Candidate genes that potentially play a role in the pathogenesis of congenital deformities can be identified from animal

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models or studies of tissue and embryonic-stage-specific expression.[4] Recently, transforming growth factor-β3 (TGF-β3) knock-out mouse showed a cleft palate and delayed pulmonary development.[5,6] Lidral et al[7] studied TGF-α, Bcl3, Dlx2, Msx1 and TGF-β3 as candidate genes and found some variants in Msx1 and TGF-β3 .

The prevalence of a cleft lip with or without palate differed according to race. The highest prevalence was among Native Americans and Orientals.[8,9] In the study on Japanese populations, the TGF-β3 gene or an adjacent DNA sequence might contribute to the development of a cleft lip and palate.[10] However, there was no relationship between the TGF-β3 gene and cleft lip and palate development in the Philippines.[11] Thus, it is possible that there may be some differences in the cleft related gene among countries. However, there have been few genetic epidemiological studies conducted in Korea.

Several models were suggested as modes of inheritance for nonsyndromic cleft palate: a single locus, several interacting loci, and a multifactorial threshold model.[12] Environmental factors are also important.[1] There is a lesser probability that a single gene polymorphism will directly induce nonsyndromic cleft, except for a complete loss of function mutation. And the loss of function mutation usually results in a fatal outcome or syndrome. We previously studied the genetic polymorphism in exon 2 and exon 5 with their surrounding intron sequences in a normal population (n=41). There was no polymorphism in exon 2 and exon 5, but there was a polymorphism in intron 5 of TGF-β3 and its locus was previously reported.[13] In the previous study, the IVS5-104A/G (SfaN1) polymorphism in the TGF-β3 gene was significantly associated with an increased risk of NSCL/P in the Korean population.[14] However, the genetic background is different among races. Therefore, the SfaN1 polymorphism must be studied in other races as well. As Romania is geographically far from Korea, the Romanian cleft can be a good sample for comparative study.

The objective of this study was as follows: (1) to study the frequency of SfaN1 polymorphism in Korean and Romanian nonsyndromic cleft lip and/or palate, (2) to study the statistical difference in the frequency of SfaN1 between Korean and Romanian populations.

Patients and Methods

Population Description

Samples were collected from patients and their families who were seen and examined from 2000 to 2002. Samples in Korea were taken at the Department of Orthodontics, Seoul National University, and the Department of Oral and Maxillofacial Surgery, Hallym University Hospital, and in Romania at the Department of Craniomaxillofacial Surgery, Iuliu Hatieganu University of Medicine and Pharmacy (Table 1). Blood or saliva samples for DNA analysis were obtained at the time of initial examination for orthodontic or surgical treatment.

All patients were evaluated for the presence of associated abnormalities that would be suggestive of syndromic variants. The syndromic cases were excluded.

The Korean group consisted of twenty-six families. Each family sample was composed of three people (trio): a patient and his/her parents. In two families, one of the parents was the cleft case. A total of 78 samples were collected from Korean patients and family groups. Among them were 28 affected individuals (26 probands and 2 additional nonproband affected individuals).

Eighteen families were selected in the Romanian patients group. Romanian family samples consisted of an affected individual and his/her parents (trio), or an affected individual and his/her father or mother (two people). In two families, one of the parents was a cleft case, but one parent that exhibited a cleft was not sampled. A total of 41 samples were collected from the Romanian clefts and their family groups.

Extraction of Genomic DNA and PCR

The samples were collected from blood or saliva, and then absorbed in filter paper and dried. A small disc (diameter: 1.5mm) was punched and it was put in the PCR tube. Buffer A contained 10% SDS 200ml and 10

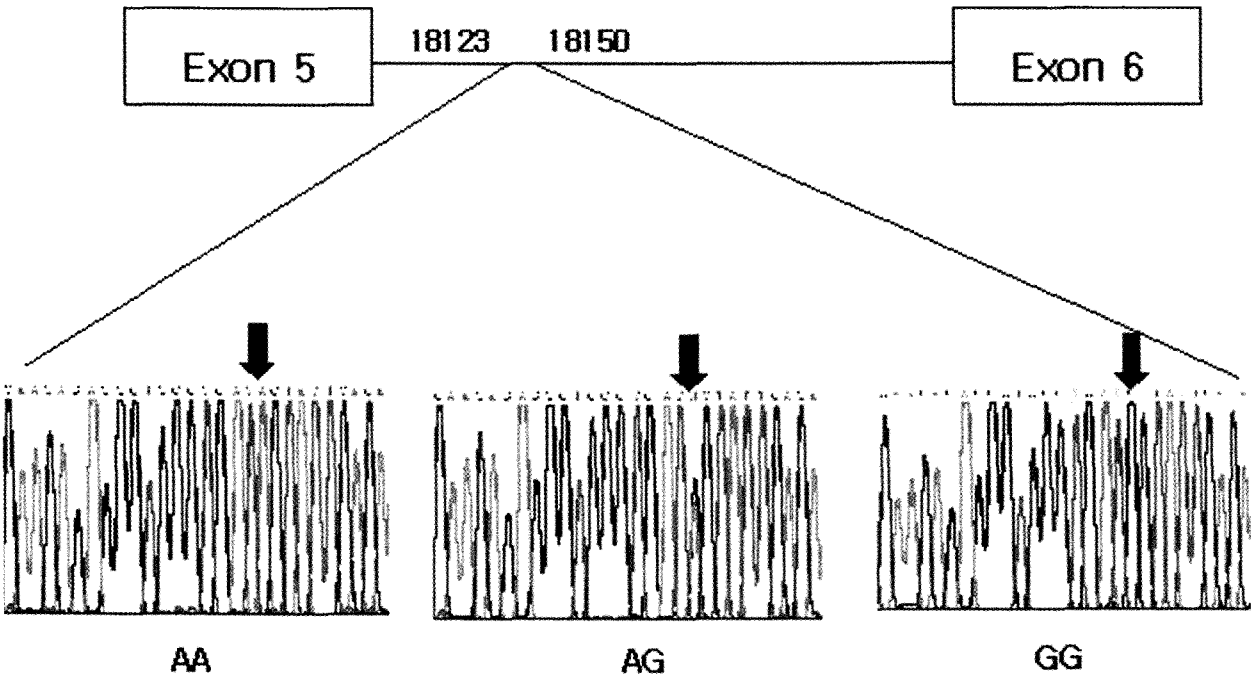

Fig. 1. The locus of single nucleotide polymorphism of TGF-β3 in this study. The accession number of this gene is AY140241.

N NaOH 40ml. Distilled water was added and the final volume of buffer A was set at 2 liters. Buffer B was 70% ethanol. After adding 200 l of buffer A to a PCR tube, it was vortexed and placed at room temperature for 5 minutes. The PCR tube was then centrifuged at 12,000 rpm for 15 seconds, liquid extracted, and the same procedure repeated. Buffer B then received the same treatment as buffer A and the same procedure was carried out. This procedure also repeated. Finally, the disc was dried in the PCR tube at room temperature.

When the disc was dried completely, the PCR was done. The primer was designed from a known sequence of TGF-β3 (AY140241) and was as follows. The forward primer was 5'-TGTCACTTTCCTTCCC TTCTTC-3' and the backward primer was 5'-TTCTTCCTGGAGATGTTTGTGA-3'. The PCR products were examined by direct sequencing.

Statistical analysis

The frequency of each allele was compared with an independent sample t-test. Stepwise logistical regression was then used to determine those alleles associated with nonsyndromic cleft lip and/or palate. The statistical significance of the coefficients in the logistic regression models was tested using Wald statistics and the $p<0.05$ level was used to determine which variables should be included in the regression model. Odds ratios and confidence intervals were calculated from the regression coefficients, and the statistical significance of the regression coefficients evaluated using the t-test (0.05 level). A p-value <0.05 was used as the criterion for including variables in the final logistics and linear regression models.

RESULTS

A single nucleotide polymorphism was observed in the intron 5 of TGF-β3. It was A18141G (Fig.1). The frequency of each allele was significantly different between Korean and Romanian samples from the affected families. Each frequency was summarized in

Table 1. The summary of samples

		Korea	Romania
Sex of patients	Male	17	11
	Female	11	8
Total number of samples		78	41
The number of family		26	18
The number of family that showed more than two patients		2	2
Disease category			
Cleft lip and palate		26	17
Cleft palate only		2	2

Table 2. The frequency of each allele type in Korean and Romanian family members of the nonsyndromic cleft palate

Type	Korean	Romanian
AA type	18/78(23.1%)	27/41 (65.9%)
AG type	27/78 (34.6%)	13/41 (31.7%)
GG type	33/78 (42.3%)	1/41 (2.4%)

Table 3. The frequency of each allele type in Korean and Romanian nonsyndromic cleft palate patients

Type	Korean	Romanian
AA type	5/28 (17.9%)	14/19 (73.7%)
AG type	4/28 (14.3%)	5/19 (26.3%)
GG type	19/28 (67.9%)	0/19 (0.0%)

Table 2. The AA type occurred in 18 out of 78 samples (23.1%) in the Korean population, but was seen in 27 out of 41 samples (65.9%) in the Romanian population. The AG type occurred in 27 (34.6%) Korean samples and 13 (31.7%) Romanian samples. The GG type was seen in 33 (42.3%) Koreans and in 1 (2.4%) Romanian. The difference between the groups was studied by independent sample t-test and it was significant ($p<0.001$).

When comparing clefts, the difference was also significant ($p<0.001$). The total number of clefts was 28 in Korea and 19 in Romania (Table 1). The AA type was 5 samples (17.9%) in Korean clefts, but 14 samples (73.7%) in Romanian. The AG type was 4 (14.3%) in Korean and 5 (26.3%) in Romanian. The GG type was 19 (67.9%) in Korean and none in Romanian clefts (Table 3).

Those people with G allele were 5.59 times more likely to have nonsyndromic cleft palate than those with A allele ($p=0.018$) in affected Korean families. However, this tendency was not observed in affected Romanian families.

DISCUSSION

The formation of the palate is a very important process in craniofacial development. TGF-β3 is a member of the transforming growth factor-β super-family. Members of this family have biological and cellular activities that control adhesion, proliferation, differentiation, and epithelial-mesenchymal transformation.[15] In a recent study, the exogenous addition of TGF-β3 could induce palatal fusion in the cleft palate model.[16] TGF-β3 executes an important role not only in the formation of the palate.[5,6,17,18] but also in cardiac morphogenesis, mammary gland development, and wound healing.[6]

EGFR has a close relationship to many factors related

to palatal formation, and experimentally induced EGFR deficiency mice showed a high frequency of cleft palate.[19] The chemical agents that induce cleft palate (like 2,3,7,8-tetrachloridibenzo-p-dioxin or retinoic acid) also act via EGFR.[20,21] As TGF-α is an embryonic homologue to EGF, it can bind EGFR.[22] Thus, there is some probability that TGF-α may involve palatogenesis.[22,23] However, some reported that TGF-β1 could regulate the expression of EGFR. The mechanism was that TGF-β1 could induce protein kinase C and this enzyme inactivated the action of EGFR.[24]

Clefts are a multifactorial disease. Thus, it would be difficult to find any single gene that was definitely related to the disease. However, statistical approaches searching for evidence of an unequal distribution of genetic variants for candidate genes in case and control populations can provide an identification of the role of a candidate gene in a particular disorder.[25] TGF-β3 could be a candidate gene for the cleft palate. In a study on the Japanese population, the TGF-β3 gene or an adjacent DNA sequence was reported as potentially contributing to the development of a cleft lip and palate.[10] And the homozygous TGF-β3 null mouse has unique and consistent phenotypic features that include 100 percent of animals with a cleft palate, and has no other concomitant craniofacial abnormalities.[5,6] Though there were negative results found in the Philippines,[11] they included the cleft lip only and they didn't compare the SNP between groups. The study of the Chinese population showed that there may be an autosomal recessive major locus for cleft lip with or without cleft palate.[26]

In this study, we compared the SfaN1 polymorphism frequency in Romanian clefts and Korean clefts. We excluded candidates with a cleft lip only. The frequency of recessive allele (GG) was significantly higher in the Korean clefts group than in the Romanian clefts group. Though we suppose that this recessive form allele may play some role in cleft formation, its low frequency in the Romanian population provides little chance to interact with environmental factors. However, the frequency of recessive allele in Korean patients was 0.70, and higher than in a previous report.[13] Though it will require further study, if the Korean common population shows a significantly lower frequency than the cleft patients,[14] this SNP can be supposed to be a contributing factor toward the disease. The frequency of recessive allele in the Korean common population was 0.16 (1 out of 41 cases) in our previous study[14] and this frequency was similar to that of the Romanian cleft palate. However, the recessive allele does not always result in disease. Some family members who didn't have a cleft also displayed the recessive allele. Thus, other factors like maternal smoking, drug intake, or nutrition may be also required to result in a cleft lip and/or palate.[1] To find how much GG allele increases the risk of cleft palate, combinational studies with environmental factors are required.

The functional meaning of SfaN1 polymorphism has not been clarified. As the location of SfaN1 polymorphism is in the intron, it is not related to the coding area. As it is close to the exon, it may influence gene splicing. There have been examples where an intron SNP can result in truncated protein via the hampering of normal gene splicing.[27] However, it is a crude assumption in SfaN1 polymorphism and thus should be confirmed experimentally.

In this study, we showed that the frequency of SfaN1 polymorphism in TGF-β3 was significantly different between the Korean cleft and Romanian cleft palate. To our best knowledge, this is the first comparative SNP study of cleft palate among different ethnic groups. In conclusion, a SNP associated with cleft palate in one ethnic group cannot guarantee its association in the other ethnic group. Thus, individual study for each ethnic group must be tried before its diagnostic use.

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국문초록

비증후군성 구개열에서 SfaN1 polymorphism발현빈도의 인종적 차이에 관한 연구

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비증후군성 구순구개열은 가장 빈도가 높은 선천성 기형 중의 하나로 특히 한국이나 일본과 같은 극동 지방에서 높은 발생율을 보이고 동유럽에서는 드물게 보고되고 있다. 이러한 인종에 따른 차이는 이 질환에 유전적인 배경이 있음을 의미한다. 본 연구의 목적은 한국인의 비증후군성 구개열과 연관이 있다고 알려진 SfaN1 단일 염기 다형성증의 발현빈도가 한국인 구개열 가족과 루마니아 구순구개열 가족 사이에 통계적으로 유의할만한 차이가 있는지를 알아보기 위하여 시행하였다.

한국인 26가족과 루마니아 18가족을 대상으로 하였다. 전체 인원수는 한국인의 경우 78명이었고 루마니아의 경우 41명이었다. 유전자 서열분석에 사용된 샘플은 각 참여자의 혈액이나 타액을 채취하여 분석하였다. SfaN1 단일 염기 다형성증은 TGF-β3 유전자의 5번 인트론에서 관찰된다 (A18141G).

결과를 보면 한국인과 루마니아인의 비증후군성 구개열 가족 사이에는 통계적으로 유의할만한 차이가 인지되었다. 전체 샘플 중에서 AA allele는 한국인에서는 18명 (23.1%)이었으나 루마니아는 27명 (65.9%)이었다. AG allele는 한국인에서는 27명 (34.6%)이었으나 루마니아에서는 13명 (31.7%)이었다. GG allele는 한국인에서는 33명 (42.3%)이었으나 루마니아에서는 1명 (2.4%)이었다. 두 집단 사이의 차이는 통계적으로 유의하였다 ($p<0.001$).

결론적으로 한국인과 루마니아인의 비증후군성 구개열 가족 사이에 SfaN1 단일 염기 다형성증의 발현빈도는 한국인에서 통계적으로 유의할만하게 높게 나타났으며, 이는 한국에서 루마니아보다 비증후군성 구개열의 빈도가 높게 나타나는 현상을 부분적으로 설명하여 주는 것으로 사료된다.

주요 단어 : Nonsyndromic cleft palate, SNP, TGF-β3, SfaN1