Analysis of the candidate genes responsible for non-syndromic cleft lip and palate in Japanese people

Atsuko TANABE*, Shigeru TAKETANI†, Yoko ENDO-ICHIKAWA‡, Rikio TOKUNAGA†, Yutaka OGAWA* and Michiaki HIRAMOTO§
Department of Plastic & Reconstructive Surgery, Kansai Medical University, Osaka 570-8506, Japan, †Department of Hygiene, Kansai Medical University, Osaka 570-8506, Japan, ‡Department of Public Health, Kansai Medical University, Osaka 570-8506, Japan, and §Saiseikai Nakatsu Hospital, Osaka 531-0071, Japan

ABSTRACT
In order to assess the association of alleles for candidate genes with non-syndromic cleft lip and palate, DNA samples from 43 Japanese patients were compared with those from 73 control subjects with respect to the genes encoding transforming growth factor α (TGFα), TGFβ and γ-aminobutyric acid type A receptor β3 (GABRB3). The restriction fragment length polymorphisms of the 3′-non-coding region of the TGFα gene K-primer region were observed after digestion with NcoI and Hinfl. Allele 4 was the most common among cases of cleft lip with or without cleft palate, whereas allele 2 was the most common among controls. A significant difference was found in this region between groups with cleft lip (with or without cleft palate) and controls ($\chi^2 = 10.190; P = 0.017$). Three alleles of the TGFβ2 gene were tested, and allele 2 was the most common in both cases and controls. The proportion of allele 2 in the case group was greater than that in the control group, showing a significant difference between cases of cleft lip (with or without cleft palate) and controls ($\chi^2 = 19.208; P < 0.0001$). No significant differences in variants of TGFβ3 or GABRB3 between case and control populations were observed. Thus it is concluded that TGF genes play a role in craniofacial development, and that alleles of TGFα or/and TGFβ2 are associated with cleft lip and cleft palate in Japanese populations.

INTRODUCTION
Cleft lip and palate is one of the most common birth defects. The frequency of cleft lip and palate in Japanese people is about 1 in 500, which is higher than in Caucasian and Black races. Epidemiological studies suggest that exogenous factors and genetic factors play a role in the aetiology of cleft lip and palate, but its aetiology and pathogenesis remain obscure.

The mammalian palate begins to develop from projections of the paired maxillary processes of the first branchial arches, termed the palatal shelves (or lateral palatine processes) [1]. Initially, these shelves are in a vertical position on each side of the developing tongue, but, as the mandible grows, the tongue moves downward, and the shelves become horizontal and grow towards each other. Subsequently, the shelves grow sufficiently large to contact one another and begin to fuse. During fusion, the apposed epithelia form an epithelial seam, which undergoes apoptosis, migration and/or transformation, and results in mesenchymal continuity. Thus palate formation is complex, and there are numerous potential untoward possibilities, the most common being delayed shelf horizontalization and inadequate shelf growth [2,3]. Transforming growth factor α (TGFα) is a secretory protein that binds to the epidermal growth factor (EGF)
receptor, which has been localized to the palatal epithelium during palatal closure [4]. TGFβ may function as a normal embryonic version of EGF-related growth factor [5]. Sandgren et al. [6] reported that TGFβ is a potent epithelial mitogen, based on observations that epithelium hyperplasia was seen in many tissues of transgenic mice with excessive TGFβ production. The TGFβ gene family has biological activities that control cell proliferation, migration and differentiation, regulation of extracellular matrix deposition and epithelial/mesenchymal transformation. Immunohistochemical and in situ hybridization analyses have demonstrated high expression of TGFβ1, TGFβ2 and TGFβ3 during the development of palatal tissue [7–10]. In addition, transgenic mice lacking TGFβ2 or TGFβ3 in vivo develop a cleft palate [11–13]. Polymorphisms of TGFβ genes have been reported, and may play a role in disease phenotypes [9]. Furthermore, teratological and genetic studies have implicated γ-aminobutyric acid (GABA) in mouse craniofacial development [14]. Culiat et al. [15] reported the necessity of the GABA type A receptor β3 (GABRB3) gene for normal development of the palate. Subsequent studies [16–18] demonstrated that GABRB3-knockout mice showed the phenotype of a cleft palate, which was rescued by the GABRB3 gene.

Aetiological studies [19,20] have shown that cases of non-syndromic cleft palate are associated with allelic variations of TGFβ. In contrast, a failure to observe an association of TGFβ with non-syndromic cleft lip and cleft palate has been reported [21,22]. Furthermore, the association of the expression and allelic variations of TGFβ1, TGFβ2, TGFβ3 and GABRB3 with non-pathological cleft lip and palate has not been reported. To define further the involvement of genes that affect clefting aetiology, we investigated genetic variations of candidate gene loci, including the TGFβ gene (chromosome 2p11–13) [23], the TGFβ2 gene (chromosome 1q41) [24,25], the TGFβ3 gene (chromosome 14q24) [26] and the GABRB3 gene (chromosome 15q) [14,27], in a group of unrelated Japanese individuals with cleft lip and palate. We report here that certain alleles of TGFβ and TGFβ2 variants are associated with the manifestation of cleft lip and palate.

MATERIALS AND METHODS

Populations of patients and controls

The study subjects were all Japanese. The study was approved by the local ethical committee, and all subjects gave informed consent. Blood samples were collected from the patient and control groups. The patient group contained subjects with cleft lip (with or without cleft palate) or cleft palate only; subjects with chromosomal disorders, known teratogenic exposure and other recognized syndromes, as well as children with other structural anomalies and/or developmental delay, were excluded. Cases ranged in age from 0 years to 37 years, and were recruited through the Kansai Medical University Hospital, the Saiseikai Nakatsu Hospital or the Hikone City Hospital. Subjects who had no craniofacial anomaly or other congenital disease and no family history of craniofacial anomaly were used as controls. The controls were healthy volunteers ranging in age from 15 years to 60 years, and were recruited through the Kansai Medical University Hospital.

Molecular analysis

DNA was extracted from whole blood using a DNA preparation kit (BIO101 Inc., Vista, CA, U.S.A.). DNA samples were dissolved in 10 mM Tris/HCl, pH 7.5, containing 1 mM EDTA and stored at −20 °C.

The primer pairs for each of the TGFβ1, TGFβ2, TGFβ3 and GABRB3 genes were synthesized to amplify regions of the corresponding genes, and the conditions of PCR for the indicated DNA fragments are shown in Table 1. For analysis of the TGFβ K-primer region, the amplified DNA fragment was screened by digestion with the restriction enzymes HinfI and NcoI and by direct sequencing, using the method of Shiang et al. [20]. As shown in Figure 1(A), alleles 1 and 2 of the TGFβ K-primer region can be cut with NcoI (C/CATG), but not with HinfI. Allele 1 was distinguished from allele 2 by DNA sequencing. While no site digested with NcoI or HinfI was present in allele 3, allele 4 of the TGFβ K-primer region can be cut with NcoI and HinfI. After treatment of the fragment with restriction enzymes, DNA samples were analysed by electrophoresis using a 1% (w/v) agarose gel.

Amplified DNA fragments of the TGFβ P-primer region (Figure 1B) were screened by single-strand conformation polymorphism (SSCP) analysis and by direct sequencing [20].

The TaqI-primer region of TGFβ has two different types of polymorphism [28], whereby the sequences either contain a restriction site for TaqI (allele 2), due to insertion of TAAT, or lack this site (allele 1) (Figure 1C). The amplified DNA fragments of the TaqI-primer region of the TGFβ gene were then digested with TaqI at 65 °C for 2 h, and the digested DNAs were electrophoresed with a 2% NuSieve/agarose (3:1, w/w) gel (FMC Bioproducts, Rockland, ME, U.S.A.).

The TGFβ2 gene has an ACA repeat sequence in its 3′-non-coding region, and polymorphism is dependent on differences in ACA repeats [24]. The DNA sequence variants of the amplified DNA fragments of TGFβ2 were examined by SSCP analysis, and the number of ACA repeats was confirmed by DNA sequencing. Alleles 1, 2 and 3 of the gene contain seven, eight and nine ACA repeats respectively.

The TGFβ3 gene has a CA repeat sequence [22], and
Table 1  Primer pairs and PCR conditions for candidate genes

All PCR reactions were carried out for 40 cycles.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer pairs</th>
<th>PCR conditions</th>
<th>Size of product(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFα</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-primer</td>
<td>KF: 5'-GAG ACG GAC TCC TGT TCA CTT AGG-3'</td>
<td>94 °C, 1 min; 59 °C, 2 min; 72 °C, 1 min</td>
<td>345</td>
</tr>
<tr>
<td></td>
<td>KR: 5'-CAA GAG TGG CGA TAG CTT GGG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-primer</td>
<td>PF: 5'-CTC TTA AGT GCA CCT AGC T3'</td>
<td>94 °C, 1 min; 59 °C, 2 min; 72 °C, 1 min</td>
<td>345, 369</td>
</tr>
<tr>
<td></td>
<td>PR: 5'-GGT CTC TAA GCA GGA TGC TAC AG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TaqI-primer</td>
<td>TaqI: 5'-TCA CTT CCC CTT TTT CAT CTG-3'</td>
<td>92 °C, 1 min; 57 °C, 1 min; 72 °C, 1 min</td>
<td>174, 178</td>
</tr>
<tr>
<td></td>
<td>TaqIR: 5'-GGA GGC TCC TGT GAG TGT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFβ2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFβ2 primer region</td>
<td>CAAf: 5'-CGA CGA GCA TGA TGC TT-3'</td>
<td>94 °C, 1 min; 62 °C, 1 min; 72 °C, 1 min</td>
<td>322</td>
</tr>
<tr>
<td></td>
<td>CAAr: 5'-TAC GTA CAG CAA CTC CAC TT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFβ3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFβ3 primer region</td>
<td>F: 5'-GAA ACA GAT TCT GGC TCC TCA-3'</td>
<td>94 °C, 1 min; 57 °C, 1 min; 72 °C, 1 min</td>
<td>166–170</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AAC TGT GTT TAG CAC AGT GCC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GABRB3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GABRB3 primer region</td>
<td>CA: 5'-CTC TGC TTC CTG TTC TCT CTA ATA CAC-3'</td>
<td>94 °C, 1 min; 55 °C, 2 min; 72 °C, 2 min</td>
<td>181–201</td>
</tr>
<tr>
<td></td>
<td>GT: 5'-CAC TGT GCT AGT AGA TTC AGC TC-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A. Allele1  AACCA GGAGA CAGCTAC AAACCA GCCCA GACAC  TTCAAAA GCCGAC
Allele2  AACCA GGAGA CAGCTAC AAACCA GCCCA GACAC  TTCAAAA GCCGAC
Allele3  AACCA GGAGA CAGCTAC AAACCA GCCCA GACAC  TTCAAAA GCCGAC
Allele4  AACCA GGAGA CAGCTAC AAACCA GCCCA GACAC  TTCAAAA GCCGAC

B. Allele1  TGAAT TTCT CTTA TTTT TTTTA
Allele2  TGAAT TTCTT ..........A TTTTT TTTTA

C. Allele1  TCACT TCCCCTTTTT CATCTGAAA AGGAGAAT TTGCTCCTAGA
461 AAGGCTCTAA ATCCCTTAA AACCTTAGA TCTCCTAGA CATCTAAGT
511 TACCTTGTT TCTGGAAT TTTGCCAAAT ATCCATCGAG ATCAGGAGAT
561 GTGGGCCCCA GCTGGCAACT CAGAGCTTCC TCG
Allele 2  TCACT TCCCCTTTTT CATCTGAAA AGGAGAAT TTGCTCCTAGA
461 AAGGCTCTAA ATCCCTTAA AACCTTAGA TCTCCTAGA CATCTAAGT
507 TACCTTGTT TCTGGAAT TTTGCCAAAT ATCCAGGAAG ATCAGGAGAT
557 GTGGGCCCCA GCTGGCAACT CAGAGCTTCC TCG

Figure 1  Amplified DNA fragment sequences of the TGFα gene

(A) K-primer allele sequence. Underlined sequences show the restriction sites of Hinfl and NcoI. White letters show the polymorphism region. (B) P-primer allele sequence. Allele 2 lacks 4 bases (CTTT). (C) TagI-primer allele sequence. The primer regions are shown with bold letters, and polymorphic regions are shown with white letters. Allele 2 lacks 4 bases (TAAT), and is cleaved by TaqI.

Polymorphism is dependent on the frequency of CA repeats. DNA sequence variants of the amplified DNA fragments of the TGFβ3 gene were analysed by denaturing PAGE (5% gels).

Polymorphism of the GABRB3 gene depends on differences in CA repeats [14]. Amplified fragments of the GABRB3 gene were screened for DNA sequence variants by SSCP analysis.

© 2000 The Biochemical Society and the Medical Research Society
Statistical analysis
In statistical examination, $\chi^2$ analysis was performed at the significance level of $P < 0.05$, which was accounted for using the Bonferroni correction.

RESULTS

Classification of groups of cleft lip and palate
Based on the mechanisms of development of the lip and the palate, previous observations [29–31] showed that cleft lip occurs by a similar mechanism to cleft lip and palate, which develops by a different mechanism from cleft palate only. We obtained statistics on the basis of the classification proposed by The International Confederation for Plastic, Reconstructive, and Aesthetic Surgery and the American Cleft Palate–Craniofacial Association [32], and the classification for cleft lip and palate was the same as reported by Kernahan and Stark [33], as follows.

1. Cleft lip: clefts lying anterior to the incisive foramen, i.e. clefts occurring in the 'primary palate' as a result of failure of proper mesodermal penetration. Thus this group would include symptoms from minor cleft lip to whole premaxilla.
2. Cleft lip and palate: clefts lying posterior to the incisive foramen, i.e. those due to a failure of the fusion of the two palatal processes to form the secondary palate.
3. Cleft palate: clefts that combine these two important embryological events, i.e. failure of normal development of both the primary and the secondary palates.

Analyses were performed separately for cleft lip (with or without cleft palate) and cleft palate only, but we did not analyse the statistical significance of genetic variants for the cleft palate group, since the number of blood samples collected from this group was small ($n = 4$).

Association of TGF$\alpha$ gene variants with cleft lip (with or without cleft palate)
To examine the variants of the TGF$\alpha$ gene in patients and controls, the PCR products of the TGF$\alpha$ K-primer region were first analysed by use of restriction enzymes (NcoI and Hinfl) and by direct sequencing. Figure 2(A) shows the analysis of allelic variants of the TGF$\alpha$ K-primer region. Fragments of alleles 1 and 2, lane 2, homozygote of allele 1.

![Figure 2 DNA analysis of TGF$\alpha$ polymorphism](image)

(A) Analysis of K-primer region. After PCR of TGF$\alpha$ K-primer region, the amplified DNA fragment was treated with NcoI or Hinfl and the samples were then electrophoresed. DNA fragments treated with NcoI were: lane 1, allele 1, 2 or 4; lane 2, allele 3 (upper band) and allele 1, 2 or 4 (lower band); lane 3, allele 3. DNA fragments treated with Hinfl were: lane 1, allele 1, 2 or 3; lane 2, allele 1, 2 or 3 (upper band) and allele 4 (lower band); lane 3, allele 4.

(B) Analysis of TaqI-primer region. PCR products were treated with TaqI: lane 1, heterozygote of alleles 1 and 2; lane 2, homozygote of allele 1.

Table 2 Statistical analysis of TGF$\alpha$, TGF$\beta$2 and TGF$\beta$3 polymorphisms

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Allele</th>
<th>Control</th>
<th>CL + CLP</th>
<th>$\chi^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF$\alpha$ K-primer</td>
<td>1</td>
<td>28</td>
<td>18</td>
<td>10.190</td>
<td>0.017</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>69</td>
<td>26</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>12</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>29</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF$\alpha$ P-primer</td>
<td>1</td>
<td>68</td>
<td>44</td>
<td>1.623</td>
<td>0.203</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>22</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF$\alpha$ TaqI-primer</td>
<td>1</td>
<td>129</td>
<td>49</td>
<td>0.028</td>
<td>0.867</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>17</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF$\beta$2</td>
<td>1</td>
<td>27</td>
<td>5</td>
<td>19.208</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>84</td>
<td>71</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>27</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF$\beta$3</td>
<td>1</td>
<td>75</td>
<td>32</td>
<td>0.415</td>
<td>0.520</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>69</td>
<td>24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
K–primer region can be cut with NcoI (C/CATGG), whereas only the allele 4 fragment of the K–primer region can be cut with HindIII (G/ANTC). Direct sequencing was performed for groups which could not be distinguished on the basis of digestion with restriction enzymes (alleles 1 and 2). No restriction site for HindIII or NcoI was present in allele 3. From the results of this analysis, allele 4 was the most common among the cases of cleft lip (with or without cleft palate), whereas allele 2 was the most common among controls. A highly significant difference was observed in cases of cleft lip (with or without cleft palate) and controls ($\chi^2 = 10.190; P = 0.017$) (Table 2).

The PCR products of the P–primer region were next analysed by SSCP, and the differences were confirmed by direct sequencing. Two different alleles of the TGFβ3 P–primer region are shown in Figure 1(B). In both patient and control groups, the frequency of allele 1 in the TGFβ3 P–primer region was 3–4 times greater than that of allele 2 (Table 2). There was no significant difference in allele frequencies in the TGFβ3 P–primer region between cases and controls ($\chi^2 = 1.623; P = 0.203$).

The PCR products of the TaqI–primer region of the TGFβ3 gene were then analysed by digestion with TaqI. As shown in Figure 2(B), the variant allele 2 was digested with TaqI, but allele 1 was not. In the 104 subjects analysed, a heterozygote of alleles 1 and 2 (lane 1) and a homozygote of allele 1 (lane 2) were found. Allele 1 was predominant in both patient and control groups. No significant difference was observed between cases of cleft lip (with or without cleft palate) and controls ($\chi^2 = 0.028; P = 0.866$) (Table 2).

**Analysis of TGFβ genes in patients with cleft lip (with or without cleft palate)**

We next examined variations in the PCR products of the TGFβ2 gene by SSCP. Figure 3 shows SSCP analysis of the variation in the number of ACA repeats in the TGFβ2 primer region. There were three different alleles in the case and control groups under the conditions used. Allele 2 of the TGFβ2 gene, corresponding to eight ACA repeats, was most common in both the patient and control groups, and the proportion of allele 2 in the case group was greater than in the control group (Table 2). There was a significant difference in the proportion of allele 2 between cases of cleft lip (with or without cleft palate) and controls ($\chi^2 = 19.228; P < 0.0001$). In addition, the frequency of cases of cleft lip (with or without cleft palate) carrying allele 2 of the TGFβ2 gene plus allele 4 of the K–primer region of the TGFβ3 gene was much higher than in the control group ($P = 0.0117$).

The PCR products of the TGFβ3 primer region were analysed by SSCP and denaturing PAGE (results not shown). Lidral et al. [22] found three types of alleles of the TGFβ3 gene: the product of allele 1 was 166 bp in size, that of allele 2 was 168 bp and that of allele 3 was 170 bp. In the present study, we recognized two variations (alleles 1 and 2) of the TGFβ3 gene in Japanese people. In both groups, allele 1 of TGFβ3 was present in greater amounts than allele 2, and $\chi^2$ analysis of TGFβ3 variants between cases and controls showed no significant difference ($P = 0.520$) (Table 2).

**No association of GABRB3 gene variants with cleft lip (with or without cleft palate)**

Finally, the PCR products of the GABRB3 gene were analysed by SSCP. A total of 28 types of allele variants of the GABRB3 primer region, derived from differences in the number of CA repeats, were found, and statistical analysis of GABRB3 polymorphism was difficult due to these variations. However, no CA repeat variant was significantly more frequent in the patient group than among controls.

**DISCUSSION**

The prevalence of cleft lip and palate in humans is between 1 in 500 and 1 in 2000. Clinically it causes problems of ingestion, swallowing, dysarthria, occlusal disharmony, hypoplasia of the maxilla during growth and external appearance to differing degrees. Corrective surgery, orthodontic treatment and speech exercises are required to effect improvement. The burden on patients and their families is therefore heavy. In the present study, we selected as candidate genes members of the TGF family (TGFα, TGFβ2, and TGFβ3), for which local occurrence in the developmental stage of the palate has been demonstrated in studies on rodents [4,11–13], and the GABRB3 gene, which has been suggested to be involved in craniofacial development as a GABA receptor [16–18].

We found that there were significant differences in the polymorphism of the TGFα gene K–primer region and of the TGFβ2 gene between the patient group and a control group. These differences were not ascribed for mutations in these genes causing orofacial cleft, since the frequencies determined for these variant alleles permits the prediction of a Hardy–Weinberg equilibrium at each individual locus (results not shown). No significant differences
were observed between the case and control groups in the polymorphism of the TGF\(\alpha\) gene P-primer region, the TaqI-primer region of the TGF\(\beta 3\) gene, or the GABRB3 gene. The present results indicate that the TGF\(\alpha\) gene (K-primer region) and TGF\(\beta 2\) gene may be involved in the manifestation of cleft lip and palate in Japanese populations.

Several aetiological studies of cleft lip and palate have been performed to date. Ardinger et al. [19] reported that, in Caucasian patients having cleft lip with or without cleft palate, there was a significant difference in the TGF\(\alpha\) TaqI region between the patient group and a control group. Jara et al. [34] performed association studies between controls and patients with cleft lip and cleft palate in South America, and found a significant difference in the TGF\(\alpha\) BamHI region. On the other hand, Shiang et al. [20] reported that significant differences were found \((P = 0.02)\) in the TGF\(\alpha\) TaqI region and K-primer region when cleft palate only was designated as a case group of the American population, but no significant difference in the P-primer region of the TGF\(\alpha\) gene was observed. Hecht et al. [35] failed to find an association with TGF\(\alpha\) polymorphisms in a small number of cleft lip/palate groups \((n = 7)\). Hwang et al. [28] reported that no significant difference was found compared with controls \((P = 0.51)\) in the TGF\(\alpha\) TaqI region when cleft lip with or without cleft palate was designated as a case group. Like ours, their study was also performed with patients having cleft lip with or without cleft palate being employed as a case group; in addition, statistics were also taken separately from patients with cleft palate only, but no significant differences were found. Lidral et al. [22] reported no significant differences between patient and control groups with regard to TGF\(\alpha\), TGF\(\beta 2\) and TGF\(\beta 3\) genes in the Philippines. Although there were some variations in selection of cases, control and statistical procedures among the various studies that may explain these different results, it should be noted that TGF\(\alpha\) is one of several genes that affect palate development, and the influence of other genes may be dominant in some populations.

In a Japanese population, we found significant differences in TGF\(\alpha\) K-primer region and TGF\(\beta 2\) polymorphism between a patient group with non-syndromic cleft lip (with or without cleft palate) and a control group. The TGF\(\alpha\) K-primer region was identified with the 3'-non-coding region of TGF\(\alpha\) mRNA. The ACA repeat of the TGF\(\beta 2\) gene is also located in the 3'-non-coding region of TGF\(\beta 2\) mRNA. The functions of differences in the expression of the TGF\(\alpha\) and TGF\(\beta 2\) variants are unclear, but it is possible that the different sequences may contribute to the stabilities of TGF\(\alpha\) and TGF\(\beta 2\) mRNAs.

TGF\(\alpha\), which was thought to be a candidate gene related to cleft lip with or without cleft palate, is highly expressed on the palate epithelium during the embryonic stage, and seems to be involved in adhesion [19,20,34]. However, TGF\(\alpha\) knockout mice did not yield a phenotype with cleft palate [36]. Development may be different between mice and humans with regard to a defect of TGF\(\alpha\), but it is reasonable to conclude from the results of our experiments that variations in TGF\(\alpha\) expression may alter sensitivity to other environmental factors, thereby leading to development of cleft lip with or without cleft palate. In addition, if one of the TGF\(\beta\) isoforms (including TGF\(\beta 1\), TGF\(\beta 2\) and TGF\(\beta 3\)) is absent, other isoforms can compensate for their activity to some extent [8,9], but it is impossible to compensate for all activities. Therefore we propose that interactions between certain variations of TGF\(\beta 2\) and other factors may trigger the development of cleft lip and palate in humans. Although we examined genetic variations of GABRB3, another factor that may be involved in clefting defects [16–18], both the patient group and the control group showed more than 20 types of polymorphism of the gene, which were similarly distributed. Thus we obtained no evidence that GABRB3 is involved in these defects in our population, as no actual statistics were performed.

It is known that drugs, chemical substances, physical factors, environmental factors, infections and hereditary factors may be involved in the development of cleft lip and palate [3]. Furthermore, multiple hereditary factors complicated by other environmental factors may also cause sensitivity in individuals. We cannot exclude the possibility that other, as yet unidentified, polymorphisms in the TGF\(\alpha\) and TGF\(\beta\) genes are associated with orofacial cleft defects, since we analysed these genes in a relatively small case group \((n = 43)\). However, the present data indeed show that polymorphisms of the TGF\(\alpha\) and TGF\(\beta 2\) genes are associated with the development of cleft lip and palate in humans, and suggest that allele 1 of the TGF\(\alpha\) K-primer region as well as that of the TGF\(\beta 2\) gene increase the likelihood of a subject developing cleft lip and palate. Therefore the involvement of environmental factors in addition to the sensitivity conferred by genetic factors should be considered. We hope that the results of the present study will help to clarify the aetiology, gene diagnosis and gene therapy of oral clefts in the future.

ACKNOWLEDGMENTS

We thank the individuals who permitted their blood samples to be used in this study, and Dr M. Longaker (NYU Medical Center, New York, NY, U.S.A.) for his valuable advice. In addition, we thank the staff of Kansai Medical University Hospital, Saiseikai Nakatsu Hospital, Imagawa Hospital and Hikone City Hospital who assisted in collecting blood samples.
REFERENCES


13. Sanford, L. P., Ormsby, I., Gittenberger-de Groot, A. C. et al. (1997) TGFβ2 knockout mice have multiple developmental defects that are nonoverlapping with other TGFβ2 knockout phenotypes. Development 124, 2659–2670


22. Lidral, A. C., Murray, J. C., Buetow, K. H. et al. (1997) Studies of the candidate genes TGFβ2, MSX1, TGFα and TGFβ in the etiology of cleft lip and palate in the Philippines. Cleft Palate Craniofacial J. 34, 1–6


36. Zagzag, D., Proccaccino, F., Lakshmanan, J. et al. (1997) Mice lacking transforming growth factor alpha have an increased susceptibility to dextran sulfate-induced colitis. Gastroenterology 113, 825–832

Received 1 September 1999/29 February 2000; accepted 28 March 2000

© 2000 The Biochemical Society and the Medical Research Society