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Linkage analysis between BCL3 and nearby genes on 19q13.2 and non-syndromic cleft lip with or without cleft palate in multigenerational Japanese families

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OBJECTIVE: To investigate the linkage between candidate genes on chromosome 19 and cleft lip with or without cleft palate in Japanese using a parametric method.

MATERIALS AND METHODS: After informed consent was obtained, blood samples were drawn from 90 individuals in 14 families, 30 of whom were affected, and genomic DNAs were extracted. PCR-amplified products using four microsatellite markers, D19S178, BCL3, APOC2[007/008] and APOC2[AC1/AC2] located in 19q13.2, were separated by 8% polyacrylamide gel electrophoresis. Linkage analysis was carried out using the MLINK and LINKMAP programs, and logarithm of odds (LOD) scores were calculated for each family.

RESULTS: Before undertaking linkage analysis, we analyzed 74 healthy Japanese subjects and found racial differences in that the observed number of alleles and their heterozygosity were lower in Japanese than in Caucasians, and that both populations tended to show a different allele distribution. In 14 families, two-point maximum LOD score (Z_{max}) for BCL3 was 0.341 and multi-point Z_{max} was less than −2 excluding linkage. But in 9 families with left and bilateral CL/P, two-point Z_{max} for APOC2[AC1/AC2] was 1.701 and multi-point Z_{max} at APOC2 locus was 1.909.

CONCLUSION: The LOD score was relatively high but provided no evidence of linkage for CL/P to BCL3 and nearby genes in Japanese subjects.

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Keywords: cleft lip with or without cleft palate; Japanese; microsatellite; BCL3; linkage analysis

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Introduction

Recent studies have reported on the relationship between cleft lip and/or palate (CL/P) and candidate genes (chromosomal regions) as TGFA (2p13) (Ardinger *et al*, 1989; Ozawa *et al*, 1996; Lidral *et al*, 1997), HOX7 or MSX1 (4p16.3–p16.1) (Ozawa *et al*, 1996; Lidral *et al*, 1997, 1998; Sato, 2000), F13A1 (6p25.3–p24.3) (Eiberg *et al*, 1987; Scapoli *et al*, 1997), TGFB3 (14q24) (Lidral *et al*, 1997, 1998; Sato, 2000), and RARA (17q12) (Chenevix-Tranch *et al*, 1992; Maestri *et al*, 1997), suggesting associations with these congenital anomalies. However, other studies excluded such associations (Hecht *et al*, 1991a,b; Vintiner *et al*, 1992, 1993; Stein and Hecht, 1995; Wyszynski *et al*, 1997a; Machida, 1998), leaving the responsible genes unidentified.

On the other hand, some studies (Stein *et al*, 1995; Wyszynski *et al*, 1997b; Martinelli *et al*, 1998) demonstrated an association of the BCL3 gene on the long arm of chromosome 19 with cleft lip with or without cleft palate (CL/P). Although these studies suggested strong candidate genes in Caucasians, there is no report concerning such genes in oriental peoples.

The aim of this study is to investigate the linkage between CL/P and the BCL3 and nearby genes in Japanese families with multiple CL/P patients.

Materials and methods

Cases and controls

The families of patients with CL/P were studied in detail who visited the Department of Oral and Maxillofacial Surgery in Niigata University Dental Hospital. And 14 families consisting of 90 members including at least parent–child or sib pairs with CL/P were investigated. Of these 90 individuals, 30 had CL/P, and 60 were unaffected. The affection status, classifying cleft type and laterality in the same family, is shown in Table 1. Patients with an additional, diagnosed or suspected anomaly or syndrome were excluded from this study.

Table 1 Summary and grouping of affection status by cleft type and laterality in 14 multigenerational Japanese families

Affection status	*Family*	*Affected*	*Unaffected*	*Total*	*Grouping*
Cleft type					
CL + CL	0	0	0	0	
CL + CLP, CL + CL + CLP	7	16	26	42	Group 1
CLP + CLP	7	14	34	48	Group 2
Laterality					
L + L	5	10	20	30	Group 3a
L + B, L + L + B	2	5	5	10	Group 3b
B + B	2	4	6	10	Group 3c
L + R, L + L + R	2	5	11	16	
B + R	3	6	18	24	Group 4
R + R	0	0	0	0	
Total	14	30	60	90	

CL, cleft lip; CLP, cleft lip and palate; L, left; B, bilateral; R, right.

Families including patients with cleft palate only (CPO) were excluded, because this anomaly has been considered genetically different from CL/P (Fogh-Andersen, 1942). This study was reviewed and approved in advance by the Ethics Committee of Niigata University School of Dentistry. After detailed explanations of the study, written informed consent was obtained from the families concerned, and blood was collected from as many family members as possible. In addition, to obtain information about the polymorphism of each genetic marker in a Japanese population, blood was collected from 74 unrelated healthy Japanese.

Isolation of genomic DNA

Heparinized fresh peripheral blood was diluted with ten volumes of 0.2% NaCl for hemolysis. Nucleated cells were collected by centrifugation and rinsed twice with 0.2% NaCl solution. The cells were suspended in TNE buffer (20 mM Tris–HCl, 100 mM NaCl, and 1 mM EDTA, at pH 7.5), containing 0.5% sodium dodecyl sulfate (SDS) and digested with 0.2 mg ml^{-1} of protenase K (Merck Co., Darmstadt, Germany) at 56°C overnight. The DNA was extracted twice with phenol, once with phenol/chloroform and precipitated with ethanol. The DNA pellet was dissolved in TE buffer (10 mM Tris–HCl and 1 mM EDTA, at pH 7.5).

Microsatellite markers

Dinucleotide repeat markers were analyzed at D19S178 (Weber *et al*, 1993), BCL3 (St George-Hyslop *et al*, 1992), APOC2[007/008] (Weber and May, 1989) and APOC2[AC1/AC2] (Smeets *et al*, 1989; Fornage *et al*, 1992) loci on 19q13.2. BCL3 gene is well known as proto-oncogene (Ohno *et al*, 1990), and APOC2[007/008] and APOC2[AC1/AC2] loci exist into apolipoprotein C-II gene (Ashworth *et al*, 1995). The order of these markers is cent-D19S178-BCL3-APOC2-tel on 19q13.2. Primer sequences, allele sizes and linkage-mapping information are available from the Genome Database (http://www.gdb.org/) for all markers.

PCR protocol and genotyping

Each amplification samples contained 50 ng of genomic DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1 unit of AmpliTaq Gold™ (Applied Biosystems, Foster City, CA, USA), 0.2 μM of each oligonucleotide primer, and 200 μM of each deoxyribonucleoside triphosphates (dNTPs) in a total volume of 25 μl. Thermal cycling was performed on GeneAmp® PCR System 9700 (Applied Biosystems) using the following conditions: initial denaturation at 95°C for 10 min; 40 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min; and a final extension of 72°C for 10 min. Eight microliters of PCR products were mixed with an equal volume of formamide loding buffer, heat denatured and electrophoresed on 8% 29:1 acrylamide–bisacrylamide denaturing sequencing gel (8 M Urea). DNA bands were visualized by silver staining (Bio-Rad, Hercules, CA, USA).

Linkage analysis

Individual information from each family was input in pedfile.dat, and information about the polymorphism of each genetic marker and various parameters in the Japanese control individuals was input in datafile.dat. Two-point linkage analysis was performed on UNIX using MLINK (version 5.10) and FASTLINK (version 4.0P) of the LINKAGE package (Lathrop *et al*, 1984) to calculate logarithm of odds (LOD) scores at various recombination fractions (θ) in all families under autosomal dominant models with penetrance values of 99.9, 80, 60, and 30% and an affected-only model. Under the affected-only model, to avoid confounding by penetration values and the mode of inheritance, data were input on the assumption that the phenotypes of members other than the patients were unknown. Multi-point linkage analysis was performed using LINKMAP (version 5.10) of the LINKAGE package to calculate LOD scores under autosomal dominant models. Marker distances for the multi-point map were set at 1.1 cM between D19S178 and BCL3 and at 2.5 cM from BCL3 to APOC2.

Results

Allele frequency and heterozygosity

The PCR-amplified alleles at each locus ranged in length from approximately 100–160 bp, showing polymorphism. Polymorphism analysis of 74 healthy Japanese showed that the D19S178, BCL3, APOC2[007/008], and

Table 2 Allele frequency distribution and observed heterozygosity (HET%) for four microsatellite loci investigated in 74 healthy unrelative Japanese individuals

Alleles	*D19S178*	*BCL3*	*APOC2 [007/008]*	*APOC2 [AC1/AC2]*
1	0.797	0.149	0.034	0.027
2	0.142	0.007	0.014	0.007
3	0.034	0.818	0.108	0.101
4	0.027	0.027	0.068	0.007
5			0.608	0.027
6			0.149	0.216
7			0.014	0.412
8			0.007	0.182
9				0.014
10				0.007
HET%	0.284	0.324	0.500	0.662

APOC2[AC1/AC2] loci had 4, 4, 8, and 10 alleles, respectively, and heterozygosities of 0.284, 0.324, 0.500, and 0.662, respectively. Alleles were numbered 1, 2, 3, and so 4th in increasing order of PCR fragment length. The allele frequencies at each locus are shown in Table 2.

Two-point linkage analysis

In 14 families, the LOD scores were negative at the D19S178, APOC2[007/008], and APOC2[AC1/AC2] loci for all penetrance values, and −2 or lower at recombination fractions below 0.050 or 0.010, excluding linkage. In contrast, at the BCL3 locus, as the recombination fraction was decreased, LOD scores for each penetrance were increased to positive numbers, showing a maximum at a recombination fraction of 0.000. Although BCL3 showed the highest LOD score ($Z_{\max}$) of 0.341 for a penetrance of 99.9%, this score was less than +3, which indicates linkage (Table 3).

In addition, 14 families were classified by affection status (Table 1) and we found higher LOD scores in nine families with left and bilateral CL/P (group 3c) than other groups. In this group, the LOD scores were positive numbers at the four loci for all penetrance values, and in particular, $Z_{\max}$ for APOC2[AC1/AC2] was 1.701 ($\theta = 0.000$) when penetrance was set at 99.9% (Table 4).

Multi-point linkage analysis

A multi-point map was calculated under an autosomal dominant model in 14 families and the LOD scores were −2 or lower at the D19S178, BCL3, APOC2[007/008], APOC2[AC1/AC2] and nearby loci, excluding linkage. When 14 families were classified into the group by cleft type (Figure 1) and laterality (Figure 2), group 1, 2, and 4 showed excluding linkage. But families without right lateral CL/P (group 3) showed positive LOD scores at all loci. In particular, group 3c showed a relatively high value ($Z_{\max} = 1.909$) at the locus of 3.6 cM from D19S178 (equivalent to APOC2).

Discussion

It has been described that the development of CL/P was frequently explained by the multifactorial/threshold model (Fraser, 1970; Carter, 1976; Carter *et al*, 1982;

Table 3 Two-point LOD scores in 14 Japanese families calculated under autosomal dominant and affected-only models

	LOD score at recombination fraction (θ) of							
Marker	*0.000*	*0.010*	*0.050*	*0.100*	*0.200*	*0.300*	*0.400*	Z_{max} *(θ)*
Penetrance = 99.9%								
D19D178	−12.580	−1.625	−0.902	−0.581	−0.267	−0.107	−0.025	0.000 (0.500)
BCL3	0.341	0.325	0.267	0.203	0.104	0.041	0.009	0.341 (0.000)
APOC2[007/008]	−16.063	−3.141	−1.341	−0.697	−0.224	−0.066	−0.013	0.000 (0.500)
APOC2[AC1/AC2]	−15.977	−3.049	−1.230	−0.574	−0.115	−0.003	0.004	0.006 (0.360)
Penetrance = 80%								
D19S178	−7.899	−1.690	−0.965	−0.640	−0.318	−0.147	−0.049	0.000 (0.500)
BCL3	0.323	0.308	0.251	0.189	0.094	0.036	0.008	0.323 (0.000)
APOC2[007/008]	−7.293	−1.147	−0.502	−0.266	−0.097	−0.046	−0.025	0.000 (0.500)
APOC2[AC1/AC2]	−7.105	−0.959	−0.314	−0.088	0.033	0.021	−0.007	0.036 (0.230)
Penetrance = 60%								
D19S178	−7.268	−1.754	−1.024	−0.695	−0.362	−0.180	−0.068	0.000 (0.500)
BCL3	0.298	0.284	0.230	0.171	0.083	0.031	0.006	0.298 (0.000)
APOC2[007/008]	−6.497	−1.049	−0.432	−0.225	−0.095	−0.060	−0.038	0.000 (0.500)
APOC2[AC1/AC2]	−6.240	−0.794	−0.191	−0.010	0.050	0.009	−0.021	0.052 (0.180)
Penetrance = 30%								
D19S178	−6.771	−1.844	−1.105	−0.766	−0.417	−0.218	−0.088	0.000 (0.500)
BCL3	0.261	0.248	0.199	0.146	0.069	0.024	0.004	0.261 (0.000)
APOC2[007/008]	−6.055	−1.174	−0.531	−0.304	−0.149	−0.097	−0.058	0.000 (0.500)
APOC2[AC1/AC2]	−5.723	−0.848	−0.235	−0.050	0.011	−0.024	−0.041	0.012 (0.180)
Affected-only								
D19S178	−6.464	−1.926	−1.177	−0.828	−0.462	−0.248	−0.103	0.000 (0.500)
BCL3	0.229	0.217	0.173	0.125	0.057	0.019	0.003	0.229 (0.000)
APOC2[007/008]	−5.883	−1.362	−0.681	−0.418	−0.216	−0.135	−0.075	0.000 (0.500)
APOC2[AC1/AC2]	−5.495	−0.984	−0.345	−0.137	−0.046	−0.060	−0.058	0.000 (0.500)

Table 4 Two-point LOD scores in nine families with left and bilateral cleft lip with or without cleft palate (group 3c)

	LOD score at recombination fraction (θ) of							
Marker	*0.000*	*0.010*	*0.050*	*0.100*	*0.200*	*0.300*	*0.400*	*Z_{max} (θ)*
Penetrance = 99.9%								
D19D178	0.046	0.045	0.038	0.030	0.017	0.008	0.002	0.046 (0.000)
BCL3	0.218	0.206	0.163	0.118	0.053	0.017	0.003	0.218 (0.000)
APOC2[007/008]	1.474	1.422	1.217	0.976	0.556	0.244	0.059	1.474 (0.000)
APOC2[AC1/AC2]	1.701	1.647	1.437	1.179	0.704	0.322	0.080	1.701 (0.000)
Penetrance = 80%								
D19S178	0.046	0.045	0.038	0.030	0.017	0.008	0.002	0.046 (0.000)
BCL3	0.230	0.218	0.173	0.126	0.057	0.019	0.003	0.230 (0.000)
APOC2[007/008]	1.294	1.247	1.064	0.850	0.482	0.212	0.051	1.294 (0.000)
APOC2[AC1/AC2]	1.549	1.500	1.305	1.068	0.633	0.287	0.070	1.549 (0.000)
Penetrance = 60%								
D19S178	0.046	0.045	0.038	0.030	0.017	0.008	0.002	0.046 (0.000)
BCL3	0.230	0.218	0.173	0.126	0.057	0.019	0.003	0.230 (0.000)
APOC2[007/008]	1.148	1.106	0.942	0.752	0.427	0.188	0.046	1.148 (0.000)
APOC2[AC1/AC2]	1.429	1.384	1.203	0.984	0.581	0.262	0.064	1.429 (0.000)
Penetrance = 30%								
D19S178	0.046	0.045	0.038	0.030	0.017	0.008	0.002	0.046 (0.000)
BCL3	0.224	0.216	0.169	0.122	0.055	0.018	0.003	0.224 (0.000)
APOC2[007/008]	0.987	0.951	0.812	0.650	0.372	0.166	0.041	0.987 (0.000)
APOC2[AC1/AC2]	1.305	1.263	1.099	0.898	0.530	0.238	0.058	1.305 (0.000)
Affected-only								
D19S178	0.046	0.045	0.038	0.030	0.017	0.008	0.002	0.046 (0.000)
BCL3	0.218	0.206	0.163	0.118	0.053	0.017	0.003	0.218 (0.000)
APOC2[007/008]	0.873	0.842	0.723	0.582	0.338	0.153	0.038	0.873 (0.000)
APOC2[AC1/AC2]	1.224	1.186	1.032	0.844	0.499	0.224	0.054	1.224 (0.000)

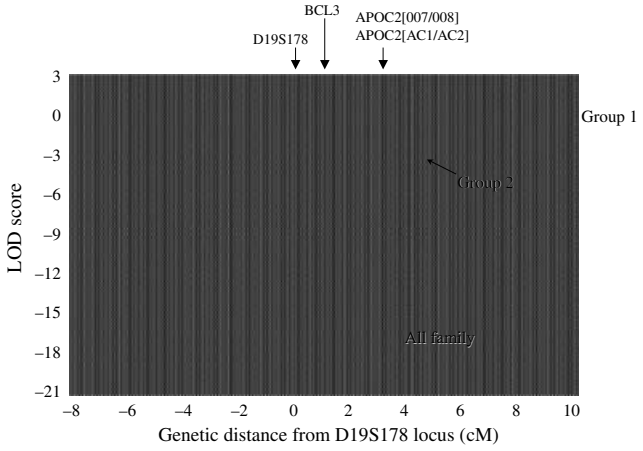

Figure 1 Multi-point map calculated under autosomal dominant model (cleft type)

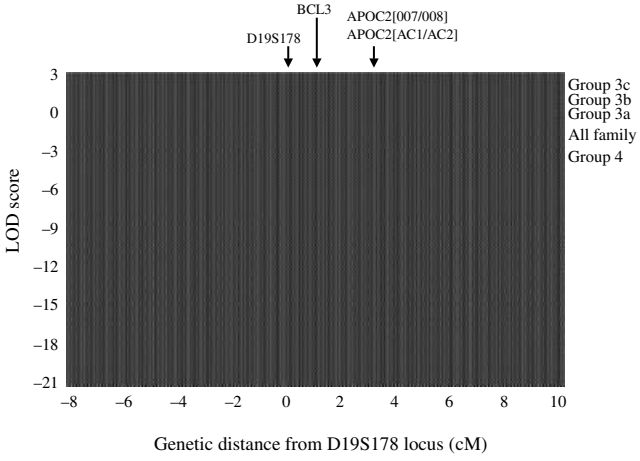

Figure 2 Multi-point map calculated under autosomal dominant model (laterality)

Mitchell and Risch, 1992), and CL/P was thought to develop when the disease liability exceeded a certain threshold as a result of interaction between polygenes of small but additive effect and many environmental factors. However, in the 1980s, statistical genetic studies of Caucasian families with CL/P showed that the major-gene model, under which a few genes of major effect control the development of a congenital disease, fitted data for CL/P in Caucasians (Marazita, Spence and Melnick, 1984, 1986; Chung *et al*, 1986; Temple *et al*, 1989; Hecht *et al*, 1991a). Later studies suggested several candidate genes as the major genes, and from the latter half of the 1980s onward, molecular genetic studies began to report on the association between this condition and candidate genes (Eiberg *et al*, 1987; Ardinger *et al*, 1989; Hecht *et al*, 1991b, 1993; Chenevix-Tranch *et al*, 1992; Vintiner *et al*, 1992, 1993; Stein and Hecht, 1995; Stein *et al*, 1995; Ozawa *et al*, 1996; Lidral *et al*, 1997, 1998; Maestri *et al*, 1997; Scapoli *et al*, 1997; Wyszynski *et al*, 1997a,b; Machida, 1998; Martinelli *et al*, 1998; Sato, 2000).

In 1995, Stein *et al* (1995) analyzed 39 Caucasian families with multiple occurrence of CL/P for linkage to 22 candidate gene loci related to craniofacial

development, and reported that 17 families showed linkage to the BCL3 gene, regarding it as one of the major genes. Later, Wyszynski *et al* (1997b) analyzed Americans and Mexicans, and Martinelli *et al* (1998) analyzed north-eastern Italians, for linkage to the BCL and nearby loci, and reported evidence of linkage.

The BCL3 gene was cloned as a gene located in the vicinity of the point of t(14;19)(q32.3; q13.2) translocation of chromosome 19, which was observed in certain patients with B cell chronic lymphocytic leukemia (B-CLL) (Ohno *et al*, 1990). Bcl-3 protein is present in the nucleus, has strong affinity for the transcription factors p50 and p52, and functions as a transcription activator by inhibiting their binding to DNA (Bours *et al*, 1993; Fujita *et al*, 1993). Stein *et al* (1995) have speculated that mutations in the BCL3 gene increase its binding to p50, inhibiting the expression of genes that play important roles in the development of mesodermal tissues, thereby developing CL/P.

It is also considered the BCL3 gene to be a candidate for the major genes, and first analyzed Japanese families with multiple occurrence of CL/P for linkage to the BCL3 and nearby loci under models of inheritance with various penetrance values. Although parametric linkage analysis using the LINKAGE package requires the input of information about the polymorphism of genetic markers, no such information about the genetic markers used in this study has been reported in the Japanese population. Before undertaking linkage analysis, we analyzed 74 healthy Japanese for the number of alleles and their frequencies and heterozygosity at each genetic marker locus, and found race differences in that the observed number of alleles and their heterozygosity were lower in Japanese than in Caucasians, and that both populations tended to show a different allele distribution (Smeets *et al*, 1989; Weber and May, 1989; Fornage *et al*, 1992; St George-Hyslop *et al*, 1992; Weber *et al*, 1993; Wyszynski *et al*, 1997b).

In linkage analysis, a LOD score of $+3$ or higher is accepted as indicating linkage, and -2 or lower excludes linkage (Strachan and Read, 1999). In 14 families, two-point LOD scores lower than -2 for D19S178, APOC2[007/008], and APOC2[AC1/AC2] ($\theta < 0.050$ or 0.010), excluded the presence of the responsible gene at any of these marker locations. Z_{max} of 0.341 ($\theta = 0.000$) for penetrance of 99.9% for BCL3 neither indicated nor excluded linkage, and multi-point LOD scores lower than -2 for all markers and nearby loci indicated no evidence of linkage for CL/P. On the other hand, when 14 families were classified by affection status, the group of nine families with left and bilateral CL/P showed a high LOD score in two-point and multi-point linkage analysis. Two-point Z_{max} of 1.701 for APOC2[AC1/AC2] and multi-point Z_{max} of 1.909 at APOC2 locus showed relatively high values, but still were lower than $+3$. Regarding the laterality of CL/P, it has been widely recognized that CL/P occurs frequently on the left side irrespective of the race, and infrequently on the right side or both sides (Fogh-Andersen, 1942); however, its etiology remains unknown. The results of this study suggest that left and bilateral-sided CL/P and right-sided CL/P differ in genetic origin, but it is necessary to analyze more families or collect data on large families with many patients to increase the information available for re-evaluation. It is thus desirable to increase the number of evaluable cases by conducting a multicenter study in the future.

Regarding genetic heterogeneity in CL/P in Japanese and Caucasians, Chung *et al* (1986) reported that CL/P unaccompanied by other anomalies occurred more frequently in Japanese than in Caucasians, but more frequently in Caucasian siblings than in Japanese siblings, indicating race differences in genetic factors between Japanese and Caucasians, and that the major-gene model best fitted data from Caucasians, whereas the multifactorial/threshold model fitted data from Japanese.

Reported Caucasian studies (Stein *et al*, 1995) analyzed many large families with many patients within the same family. In contrast, in the present study of 14 families, we observed the occurrence of CL/P over three generations in only one family or the occurrence in a parent–child pair and a cousin in only one family. The remaining 12 families had only affected parent–child or sib pairs, and included many small families. These findings were probably due to the lower sibling morbidity in Japanese than that in Caucasians, as described above, presumably reflecting genetic heterogeneity in Japanese and Caucasians.

The LINKAGE package used in the present study is classified as parametric linkage analysis software, and is essentially suited for analyzing large families with many patients, but is thought to perform poorly in detecting linkage in small families (Strachan and Read, 1999). This analysis method therefore involving a small number of large Japanese families with CL/P patients presumably led to poor detection of linkage. On the other hand, non-parametric linkage analysis with GENEHUNTER (Kruglyak *et al*, 1996) and a genetic association study using the transmission disequilibrium test (TDT) (Spielman, McGinnis and Ewens, 1993) seemed useful in analyzing Japanese CL/P patients, because collecting data on many small families consisting basically of the patient and the parents is sufficient.

In addition to TGFA and F13A1 already reported as candidate genes by linkage and association studies, recent studies have detected genome-wide polymorphic microsatellites, and suggested linkage to new genetic regions (Prescott *et al*, 2000). To further search for candidate genes for CL/P in Japanese, it is necessary to perform a similar whole genome scan, non-parametric linkage analysis, and an association study for detailed analysis of the genetic regions thereby suggested.

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