Molecular Genetic Analysis of the DiGeorge Syndrome among Korean Patients with Congenital Heart Disease

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The DiGeorge syndrome (DGS) is a developmental defect of the third and fourth pharyngeal pouches, which is associated with congenital heart defects, hypoparathyroidism, cell-mediated immunodeficiency, velo-pharyngeal insufficiency and craniofacial dysmorphism. The aetiological factor in a great majority of DGS cases is monosomy for the chromosomal region 22q11. To analyze DGS at the molecular level, a new molecular probe (DGCR680) encompassing the ADU balanced translocation breakpoint was prepared. When 13 Korean patients with DGS-type congenital heart disease were analyzed with this probe, 9 turned out to have a deletion at this locus, and all of them except one exhibited a typical facial dysmorphism associated DGS. Though only 9 independent patients were detected to have a deletion at the locus using the commercial probe N25 (D22S75), which maps at about 160 kb from the ADU breakpoint to the telomeric end, results from fluorescence in situ hybridization revealed a deletion in all cases tested at this locus. Two patients who had a deletion at the locus D22S75 but not at DGCR680 did not exhibit any DGS-type facial abnormalities. This result implies that the 680 bp probe covering the ADU translocation breakpoint might be a candidate for a molecular marker that can distinguish a specific phenotype, such as facial features associated with the DiGeorge syndrome. This study also suggested that systematic approaches with several small DNA probes along the DGCR could help to dissect the complex phenotypes associated with the DiGeorge syndrome, such as cardiac defects, abnormal faces, thymic hypoplasia, cleft palate, and hypocalcemia, etc.

Keywords: Conotruncal Anomaly Facial Syndrome (CTAFS); Diagnosis; DiGeorge Syndrome.

Introduction

The DiGeorge syndrome (DGS) is a developmental field defect of the third and fourth pharyngeal pouches which associates hypoplasia or aplasia of the thymus and parathyroids, conotruncal cardiac malformations and facial abnormalities (Muller et al., 1988). Although the aetiology of DGS is heterogeneous, about 90% of the patients exhibit monosomy at the 22q11 chromosomal region. Such monosomy is known to be associated with other phenotypes including the velocardiofacial (Shprintzen) syndrome (VCFS), conotruncal anomaly facial syndrome (CTAFS), and sporadic or familial cardiac defects, implying that these represent variants of the same disorder generated by the common cause (Demczuk and Aurias, 1995). Therefore, the defects caused by the deletions in chromosome 22 are all represented by the acronym CATCH22, which stands for cardiac defects, Abnormal faces, Thymic hypoplasia, Cleft palate, and Hypocalcemia (Wilson et al., 1993).

Cytogenetic as well as high resolution banding techniques have revealed that most patients with DGS have

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Abbreviations: CTAFS, conotruncal anomaly facial syndrome; DGS, DiGeorge syndrome; VCFS, velocardiofacial (Shprintzen) syndrome.

unbalanced translocation involving chromosome 22, or interstitial deletions within this chromosome. An analysis of numerous patients with different deletion endpoints showed that there is a region, so-called the DGCR (DiGeorge syndrome critical region), which is about 1.5 Mb, is deleted in the majority of the patients. The extent of the deletion, however, does not seem to correlate with the severity of clinical features, implying that one or perhaps a few genes play a major role in its pathogenesis. Moreover, a balanced translocation which disrupted sequences within the shortest region of the deletion overlap for the DiGeorge syndrome has been reported in one patient — ADU (46,XX,t(2;22)(q14.1;q11.1) — and the same translocation was present in her mother (VDU), who was mildly affected (Augusseau *et al.*, 1986). These findings strongly suggested that the 22q11 deletion phenotype could be determined by a single gene. The DGS phenotype in ADU and VDU has made this balanced translocation point the target for the positional cloning approaches in the hope of isolating the critical gene(s) responsible for DGS.

Thus far, several cDNA and molecular probes for diagnosis have been isolated (Funke *et al.*, 1997; 1998; Goldmuntz *et al.*, 1996; Halford *et al.*, 1993; Lindsey *et al.*, 1996; McKie *et al.*, 1997; Sutherland *et al.*, 1996; Wadey *et al.*, 1995). However, none of them encompasses the ADU breakpoint except *rnex40*, DGCR3 (Budarf *et al.*, 1995) and DGCR5 (Sutherland *et al.*, 1996). Since both DGCR3 and DGCR5 have not been proven to have protein encoding potential, we attempted to isolate a real transcript using a new DNA probe which covers the ADU breakpoint based on previously published sequences (Budarf *et al.*, 1995). During the course of this study, we found that the probe we generated was an efficient molecular marker for diagnosing DGS, since it hybridized with an unexpected band which could be used as an internal control for dosage analysis. In this study, 13 Korean patients having the DGS-type congenital heart disease were analyzed with the generated probe, and compared with the fluorescence *in situ* hybridization data prepared with commercial probe N25 (D22S75), which maps at about 160 kb from the ADU breakpoint toward the telomeric end.

Materials and Methods

Patient selection and clinical evaluation Thirteen Korean patients with DGS-type congenital heart disease (CHD) were selected for molecular studies (Ten patients were evaluated at the Seoul National University College of Medicine, and three were evaluated at the Sejong Hospital in Seoul, Korea). Eight out of the 13 patients studied had the characteristic facial features for DGS (Table 1), consisting of hypertelorism, antimongoloid slant, bloated eyelids, recurrent otitis media, prominent nose with squared nasal roof, deficient malar area, retruded mandible, short philtrum, small mouth, A-shaped mouth, nasal voice, high arched palate, narrow palpebral fissures, asymmetric low-set malformed ears and long face.

DNA probes Two primers (5′ primer, B1: 5′-GGCTCT AGGGATCAGCACAGCCAGC, and 3′ primer, B2: 5′-CTCCCATTTTACAGTAGGAGGCTGG) were designed to be located at both the 5′ and 3′ sides of the ADU balanced translocation point based on the nucleotide sequences around the breakpoint (Budarf *et al.*, 1995), and were synthesized chemically. The 680 bp fragment of DGCR680 was obtained by amplification of the human genomic DNA using B1 and B2 as PCR primers, and then it was used as a DNA probe after subcloning it into the pBluescript SK (pDGCR680) followed by sequencing.

Southern analysis The genomic DNA purified from the leukocytes of 13 patients and four normal controls was digested with *Hind*III, and then Southern hybridization was conducted according to the standard procedure with ^{32}P-labelled DGCR680 as a probe. In brief, all prehybridization and hybridization reactions were carried out in 0.5 M Sodium phosphate buffer (pH 7.2) with 7% SDS, 1 mM EDTA and 1% BSA at 55°C (Sambrook *et al.*, 1989). To decrease the background generated by the human repetitive sequences, membranes were prehybridized with sonicated human placental DNA. Membranes were then washed with 2× SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0), 1× SSC and 0.2× SSC containing 0.1% (w/v) SDS at 65°C for 30 min each.

Dosage analysis To determine the copy number, a quantitative analysis of Southern blot bands was performed. The DGCR680 probe recognized an 18 kb band in the 22q11 chromosomal region and a 16 kb *Hind*III fragment as well. Hybridization signals on the filter were quantitated by a Phosphor-Imager S425 system (Molecular Dynamics), equipped with ImageQuant software (version 3.3) which measured the radiation energy directly. The signal strengths were normalized and the ratios were calculated by following the basic protocol published previously (Driscoll *et al.*, 1992). In brief, the standard hybridization signals were calculated by taking the ratio of the radiation energy emitted by the 18 kb fragment to that of the control 16 kb fragment. The copy number was designated as twice the mean of the ratios of standard signals. It was assumed that one or two copies of a locus were present. Values less than 1.5 were defined as hemizygosity, whereas values greater than 1.5 were not. All studies were performed in triplicates.

FISH analysis Human metaphase chromosomes were prepared from lymphoid cell lines and/or peripheral blood by standard methods. The hybridization mixture containing the digoxigenin-labeled D22S75 (N25) DGCR probe, along with the D22S39 control probe (Oncor, Gaithersburg, MD, USA) was placed on the denatured chromosome slides following the manufacturer's instructions. After overnight hybridization at 37°C, the slides were incubated with anti-digoxigenin fluorescein in 1× PBD/5%BSA at 37°C for 1 h and rinsed. For amplification, the slides were treated with rabbit anti-sheep antibodies at 37°C for 30 min and incubated with FITC-rabbit antibody at 37°C for 30 min. The chromosomes were then counterstained with propidium iodide in an antifade solution.

Table 1. Summary of molecular analysis and clinical findings of DGS patients.

Patient No.	Sex	Age (yr.)	Types of cardiac anomaly[a]	Typical faces	Others	Molecular Dosage analysis	Deletion[b] FISH
1	M	0.2	TOF, MAPCA	+	Mother (TOF)	+	+
2	F	2.0	PA	−		−	NT
3	F	7.0	TOF, MAPCA	+		+	NT
4	M	13.0	TOF	−		+	+
5	F	1.0	TOF	+/−	Mother (CATCH22 questionable)		NT
6	M	2.0	TOF, PDA MAPCA	+		+	+
7	F	2.6	TOF, MAPCA	+		+	+
8	M	0.7	TOF, MAPCA	+/−		+	+
9	F	1.8	ASD	−		−	+
10	M	0.6	TOF, MAPCA	+		+	+
11	M	17.0	TOF, MAPCA	+	Cleft palate, Maternal aunt (CHD)	+	+
12	M	3.0	TOF, MAPCA	+		+	+
13	M	5.0	TOF, MAPCA	+		+	NT

[a] tertalogy of fallot (TOF), artrial septal defect (ASD), multiple anomalous pulmonary collateral arteries (MAPCA), patent ductus arteriosus (PDA), pulmonary atresia (PA).

[b] Dosage analysis (+: deleted/ −: not deleted), NT (not tested).

Results and Discussion

The most dramatic and clinically important issue for the genetic origin of congenital heart disease has been the recent discovery of the association of conotruncal defects with the deletion within the chromosomal region, 22q11. The acronym CATCH22 (Cardiac defects, Abnormal faces, Thymic hypoplasia, Cleft palate, and Hypocalcemia from deletions in chromosome 22) was coined to encompass the spectrum of clinical manifestations in patients with deletions in this locus (Wilson et al., 1993). DGS, VCFS, and CTAFS belong to this syndrome having the chromosome 22q11 deletion and overlapping clinical features (Driscoll et al., 1995). Thus far, several cDNA and molecular probes used to diagnose CATCH22, including DGS, have been isolated and generated. However, none of

them encompasses the ADU breakpoint. Therefore, we generated a new probe which covers the ADU breakpoint. Two oligonucleotide primers, B1 and B2, originally designed to isolate a critical gene for DGS encompassing the ADU breakpoint, were designed to be located at both the 5′ (centromeric) and 3′ (telomeric) side of the balanced translocation breakpoint (Budarf et al., 1995), respectively, and were chemically synthesized to perform PCR using the human genomic DNA as a template (Fig. 1). The resulting 680 bp fragment (DGCR680) was cloned into the EcoRV restriction endonuclease site in the pBluescript SK vector (pDGCR680), and used as the hybridization probe to diagnose DGS among patients having congenital heart disease.

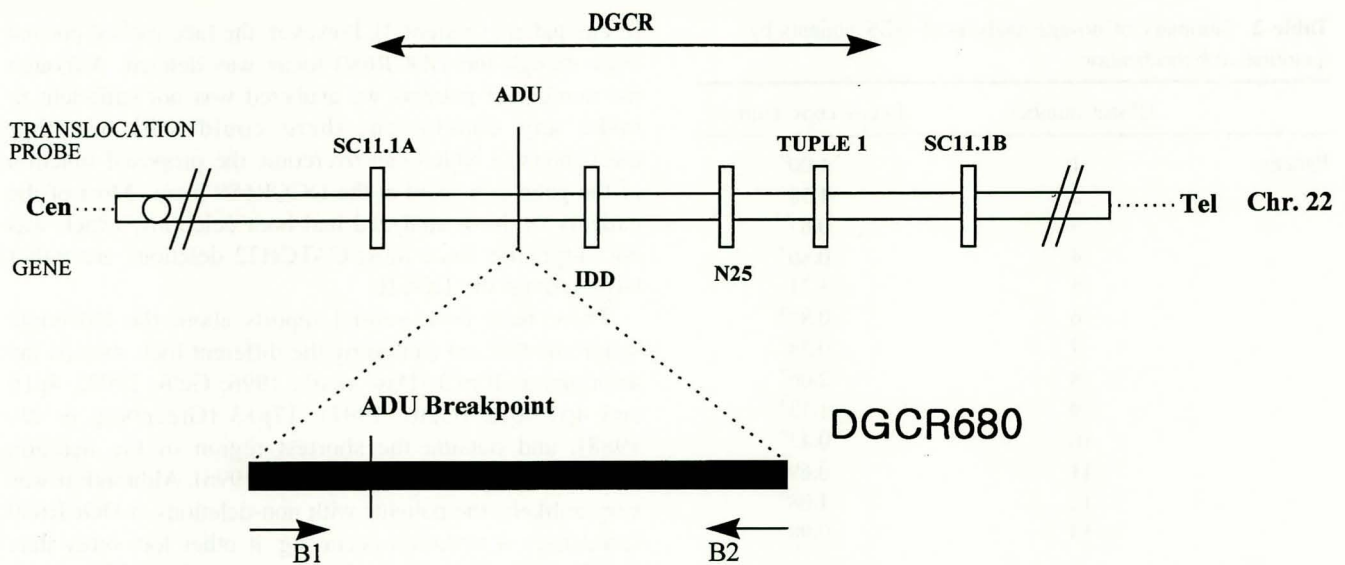The DNA from the 13 patients and four normal controls were completely digested with the restriction

Fig. 1. Schematic diagram of 22q11 showing relative order of probes. The vertical line indicates the ADU balanced translocation breakpoint, and the empty rectangles designate the loci. The black rectangle shows the DGCR680 probe, under which the primers B1 and B2 are marked. The DiGeorge syndrome critical region (DGCR) is shown by a double-headed arrow above the schematic chromosome 22.

endonuclease, *Hin*dIII, and subjected to Southern hybridization with DGCR680 as a hybridization probe. Surprisingly, the probe hybridized with two specific bands: one was the 18 kb fragment located in the DGCR, and the other was the unidentified 16 kb fragment. Furthermore, the relative band intensities of the two bands were the same in all four normal controls, whereas in the 13 patients, the intensities varied consistently, where the intensity of the upper band was weaker than the lower band. The 16 kb band was located neither in the PAC16K23 nor in the PAC1L11 region (constructed by Pieter J. DeJong), which covers about 200 kb around the ADU breakpoint (data not shown). A primitive study with panel blot hybridization implied that the 16 kb band was not from the chromosome 22, although the exact locus was not identified. Therefore we regarded the 16 kb band as being the internal control of two copies lying outside the DGCR.

Figure 2 shows the Southern blot data where the intensity of the 18 kb fragment is not reduced relative to that of the 16 kb fragment in normal controls, whereas the
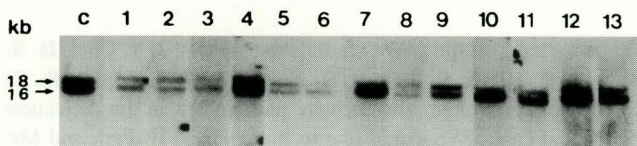
relative intensity of the 18 kb fragment was reduced in patients 1, 3, 4, 6, 7, 10, 11, 12, and 13. The copy number of the DGCR locus was determined by the relative band intensities quantitated by the PhosphorImager directly on the membrane, considering the the 16 kb fragment as a diploid control. Since one or two copies of the locus are assumed to be present, the dosage was calculated following the standard protocol (Driscoll *et al.*, 1992), in which values less than 1.5 were considered as heterozygosity (see Materials and Methods). As shown in Table 2, 9 out of 13 patients had monosomy at the chromosomal locus 22q11.

Since most patients with DGS are known to be associated with a large locus deletion at around 22q11, a commercial D22S75 DiGeorge chromosome region probe was applied to check the monosomy at this locus, mapped at about 160 kb from the ADU breakpoint toward the telomeric end. Nine patients (patients 1, 4, 6, 7, 8, 9, 10, 11, and 12) were randomly chosen and fluorescence *in situ* hybridization (FISH) was individually performed (Fig. 3). Surprisingly, the deletions were detected in all nine cases. Patients 8 and 9 were particularly interesting since they did not have any deletion at the locus DGCR680 whereas the locus D22S75 was deleted. Also, both patients did not exhibit any typical facial dysmorphism. Furthermore, patients 2 and 5, who did not have monosomy at the DGCR680 locus, did not exhibit the typical facial dysmorphism either, although the deletion at the D22S75 locus was not checked due to the failure of FISH. Meanwhile, the patients who had deletions at the DGCR680 locus (patients 1, 3, 6, 7, 10, 11, 12, and 13) did exhibit the typical DGS-type face, such as hypertelorism, narrow eye fissures, bloated eyelids, a flat nasal bridge, a

Fig. 2. Southern blot analysis of patients having congenital heart disease. Normal control (C) and the patient numbers are shown at the top.

Table 2. Summary of dosage analysis of DGS patients by quantitative hybridization.

	Object number	Locus copy number[a]
Patient	1	1.00[b]
	2	1.58
	3	0.67
	4	0.86[b]
	5	1.71
	6	0.89[b]
	7	0.38[b]
	8	2.06[b]
	9	1.73[b]
	10	0.43[b]
	11	0.69[b]
	12	1.08[b]
	13	0.98
Normal	14	2.01
	15	2.01
	16	2.29
	17	1.76

[a] Values less than 1.50 are consistent with a deletion (see Materials and Methods).
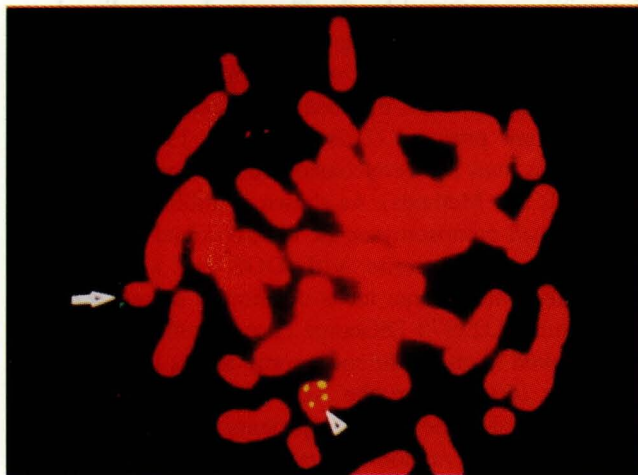[b] Deletion confirmed by FISH.

Fig. 3. Confirmation of deletion with fluorescence *in situ* hybridization (FISH) on the metaphase chromosome using the N25 (D22S75) probe. The internal control probe (D22S39) are shown on the long arms in both of the chromosome 22 while the DGS probe (D22S75) is hybridized to only the normal chromosome 22 (arrowhead). One single hybridization signal indicates a deletion of chromosome 22q11.2 in one of the chromosomes (arrow).

small mouth, and deformed ear lobe, etc. (Table 1). This result may imply that the DGS-type face could be associated with the gene located at the DGCR680 locus, strictly dissecting the complex phenotypic criteria of DGS.

In one patient (patient 4), however, the face looked normal even though the DGCR680 locus was deleted. Although the number of patients we analyzed was not sufficient to make any conclusion, there could still be other mechanism(s) which can overcome the proposed function of the gene(s) located at the DGCR680 locus. Most of the patients we have analyzed had both deletions, which was not surprising since most CATCH22 deletions are rather large around the DGCR.

There have been several reports about the DiGeorge syndrome that are caused by the different loci, such as the deletions at 10p13 (Daw *et al.*, 1996; Gelb, 1997), 4p16 and 4p13-q12 (Gelb, 1997), 17p13 (Greenberg *et al.*, 1988), and outside the shortest region of the deletion overlap in 22q11 (Kurahashi *et al.*, 1996). Although it was very unlikely, the patients with non-deletions at DGCR680 could have a mutation occurring at other loci other than DGCR, regardless of the deletion at the D22S75 locus. It is also possible that a small rearrangement, small deletions or point mutation could have occurred in the region of DGCR680.

All in all, the aetiology of DGS seems to be heterogeneous, not involving a single locus. However, the 680 bp probe encompassing the ADU breakpoint turned out to be interesting since it seemed to be able to dissect the complex phenotype caused by the large deletions in most patients. Thus far, it has been estimated that the minimum prevalence of 22q11 deletions among birth defects was 1/4000–1/5000, and this deletion seems to be involved in at least 5% of newborns with heart defects (Wilson *et al.*, 1994), which occur in approximately 8 out of every 1000 livebirths (Goldmuntz *et al.*, 1993). DGS, VCFS, and CTAFS belonging to the syndrome with chromosome 22q11 deletion and overlapping clinical features (Driscoll *et al.*, 1995), have been reported to have deletions within the chromosomal region 22q11 with frequencies of 89%, 81%, and 84%, respectively (Driscoll *et al.*, 1995; Matsuoka *et al.*, 1994). The probe we generated in this study seemed to be a candidate molecular marker to distinguish the specific phenotype, such as facial features associated with the DiGeorge syndrome. This study implies that systematic approaches with several small DNA probes along the DGCR could dissect the complex phenotypes associated with CATCH22 and would help to isolate the specific gene related to the specific phenotype such as cardiac defects, abnormal faces, thymic hypoplasia, cleft palate, and hypocalcemia, etc.

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