ORIGINAL ARTICLES

Microdeletions of chromosomal region 22q11 in patients with congenital conotruncal cardiac defects

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Abstract

Congenital conotruncal cardiac defects occur with increased frequency in patients with DiGeorge syndrome (DGS). Previous studies have shown that the majority of patients with DGS or velocardiofacial syndrome (VCFS) have a microdeletion within chromosomal region 22q11. We hypothesised that patients with conotruncal defects who were not diagnosed with DGS or VCFS would also have 22q11 deletions. Seventeen non-syndromic patients with one of three types of conotruncal defects most commonly seen in DGS or VCFS were evaluated for a 22q11 deletion. DNA probes from within the DiGeorge critical region were used. Heterozygosity at a locus was assessed using restriction fragment length polymorphisms. Copy number was determined by dosage analysis using Southern blot analysis or fluorescence in situ hybridisation of metaphase spreads. Five of 17 patients were shown to have a 22q11 deletion when evaluated by dosage analysis. This study shows a genetic contribution to the development of some conotruncal cardiac malformations and alters knowledge regarding the risk of heritability of these defects in certain cases.

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Congenital heart disease (CHD) occurs in approximately 8 in 1000 livebirths and is one of the most common major congenital anomalies.¹ The majority of congenital heart defects occur as isolated malformations. Studies to elucidate an aetiology have proven to be difficult. Epidemiological studies have indicated a variable but increased risk of recurrence in families with one affected proband and a higher recurrence risk if the mother is affected.²⁻⁴ This suggests a genetic predisposition to cardiac malformation which may be influenced by in utero environment or genetic background. However, the genetic contribution remains to be defined.

Congenital cardiac defects are frequently observed in association with other malformations and as a feature of well defined genetic

syndromes. In some syndromes, specific types of CHD are overrepresented as compared to their incidence in the general population. For example, over 40% of patients with Down's syndrome (trisomy 21) have CHD; approximately 40% of those with CHD will have a complete common atrioventricular canal, which is an otherwise uncommon lesion in the non-syndromic patient.⁵ However, a narrowly defined chromosomal region has not been linked to the development of this particular type of CHD.

Conotruncal cardiac defects are one feature of DiGeorge syndrome (DGS) where they are seen in association with thymic and parathyroid gland hypoplasia or aplasia.^{6,7} The types of conotruncal defects, particularly interrupted aortic arch type B (IAA), truncus arteriosus (TA), and tetralogy of Fallot (TOF), are overrepresented in this subgroup of patients as compared to their incidence in the general population.⁸ TOF is also one of the most common cardiac defects seen in velocardiofacial syndrome (VCFS), which is characterised by CHD, palatal abnormalities, learning disabilities, and a characteristic facies.⁹⁻¹² Clinical overlap between DGS and VCFS has been suggested by several investigators since features of DGS, such as hypocalcaemia and immunodeficiencies, have been reported in patients with VCFS.^{13,14}

Recent molecular studies have shown microdeletions of chromosome 22, within 22q11, in the majority of patients with DGS and VCFS, and in some familial cases of congenital heart disease.¹⁵⁻²¹ This deleted region, the DiGeorge critical region (DGCR), presumably encodes for genes necessary for normal development of the derivatives of the third and fourth pharyngeal pouches: the thymus, parathyroid glands, and conotruncus. Based on these findings, we hypothesised that non-syndromic patients with conotruncal cardiac defects might also have deletions within the DGCR or in a partially overlapping region. To test this hypothesis, we have evaluated 17 patients with conotruncal cardiac malformations for microdeletions of 22q11 using restriction fragment length polymorphism (RFLP) and dosage analysis.

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Table 1 Clinical and molecular findings of patients with CHD.

Patient	CHD	Dysmorphology	Family history	DNA studies
CHD-1	TA	Pointed superior helix, long slender fingers		Deleted
CHD-2	TOF	Bulbous tip of nose, high arched palate	Sib with TGA	Not deleted
CHD-3	TOF	Simple ears, long slender fingers	Mother with TOF	Deleted
CHD-4	TA/IAA	Necropsy: simple ears, micrognathia		Deleted
CHD-5	IAA	Necropsy: normal		Not deleted
CHD-7	TA	Bulbous nose tip, squared helix, small palate, almond shaped eyes		Deleted
CHD-8	TOF	Pierre Robin sequence (includes cleft palate)		Not deleted
CHD-9	TOF	Narrow palate		Not deleted
CHD-10	IAA	Not evaluated		Not deleted
CHD-11	TOF	Bulbous nose tip, depressed nasal bridge, wide nipples	Sib with CHD & features of VCFS	Not deleted
CHD-12	TOF	Tracheal stenosis, bulbous nose tip, prominent nasal bridge, posteriorly rotated ears, high arched palate		Not deleted
CHD-13	TOF	Cleft lip/palate, posteriorly rotated ears, imperforate anus, 14 pairs of ribs	Normal dizygotic twin	Not deleted
CHD-14	TOF	Left epicanthic fold, wide nipples		Not deleted
CHD-15	IAA	Not evaluated		Not deleted
CHD-16	TA	Dead, no necropsy		Not deleted
CHD-17	TOF	Prominent nasal bridge, high arched palate, long slender fingers, trigonencephaly, eye dysmorphism*		Deleted
CHD-18	TOF/PA	Not evaluated		Not deleted

TA = truncus arteriosus, TOF = tetralogy of Fallot, IAA = interrupted aortic arch type B, PA = pulmonary atresia. *Epicanthic folds, hypertelorism, upward slanting palpebral fissures, V shaped eyebrows.

Materials and methods

PATIENT SELECTION AND CLINICAL EVALUATION

Seventeen patients with conotruncal cardiac defects admitted to the cardiology service of The Children's Hospital of Philadelphia and one patient evaluated at the Washington University School of Medicine were selected for molecular studies. A brief synopsis of the salient clinical features is shown in table 1. The study included patients with the three most common forms of conotruncal defects seen in DGS and VCFS.^{8,9} Four patients had TA, three had IAA, and 10 had TOF. Patients with the other cardinal features of DGS, including neonatal hypocalcaemia, documented immune deficiency, or absent thymus, were excluded from the present study. Fourteen of the 17 patients had only CHD and no other congenital defects. Three patients have other significant malformations; CHD-12 has tracheal stenosis, CHD-8 has the Pierre Robin sequence with cleft palate, and CHD-13 has a cleft lip and palate, imperforate anus, and 14 pairs of ribs. Fourteen of the 17 patients had no previous family history of CHD. One patient with TOF (CHD-3) has a mother with TOF; two patients (CHD-2, CHD-11) have older sibs with different types of conotruncal defects. The majority of the patients were recruited into the study as infants: 11 patients were less than 6 months old, three were between 6 and 12 months old, and three were between 1 and 2 years old.

Eleven patients entered into this study were examined by a clinical geneticist who was not informed of the laboratory results before the

examination. Two patients, who were not seen by a geneticist, underwent a necropsy. Four patients were not examined by either a geneticist or at necropsy; consent for necropsy was denied by one family and three patients were unavailable for follow up.

LABORATORY EVALUATION

Genomic DNA

Lymphoblastoid cell lines were established by Epstein-Barr virus transformation of peripheral blood lymphocytes obtained from each cardiac patient. DNA was isolated by phenol:chloroform extraction and ethanol precipitation.²² When available, parental whole blood was obtained for DNA extraction using similar methods.

DNA probes

Three DNA probes, N25 (D22S75), 160b (D22S66), and R32 (D22S259), previously mapped to 22q11 and shown to be deleted in patients with DGS and VCFS, were used in the present study.^{17,18} The probes N25 and R32 are the most proximal and distal markers delineating the DGCR, respectively. The probe 160b is a representative internal marker from the DGCR as shown in fig 1. The markers 160b and R32 were isolated from flow sorted chromosome 22 libraries.²³ The clone N25 was isolated from a *NotI* linking library.²⁴ Both N25 and R32 detect a two allele *TaqI* polymorphism whereas 160b is not polymorphic. N25 and 160b detect single *HindIII* 2.6 kb and 3.0 kb bands, respectively. R32 detects a single *EcoRI* 7.1 kb band.

Southern analysis

DNA was digested with restriction enzymes as recommended by the manufacturer (New England Biolabs), separated by agarose gel electrophoresis, and transferred to Hybond N+ (Amersham) using the method of Southern.²⁵ DNA probes were labelled with [α^{32} P]dCTP using the random primer method.²⁶ These probes were preannealed with sonicated placental DNA.²⁷ Hybridisation was performed at

Figure 1 Schematic diagram of 22q11, showing relative order of probes in proximal 22q11 (Driscoll et al, 1992). Probe names are shown on the top line and D numbers on the bottom. The order for 22/34, H98, H11, and N25 was established by genetic mapping in CEPH pedigrees (Fibison et al, 1990). The jagged lines represent the most frequently observed deletions for DGS and VCFS patients. Probes which are delineated by the black circles are in the VCFS/DGS critical region.

65°C with Church's buffer (0.5 mol/l NaHPO₄, 7% SDS, 1 mmol/l EDTA). Filters were washed twice with 2 × SSC, 0.1% SDS at room temperature, and twice with 0.1 × SSC, 0.1% SDS at 65°C. They were exposed to Kodak XAR-5 film at -70°C from three to 10 days.

Dosage analysis

Southern blots of DNA from patients and normal controls were cohybridised with the test probe and one of two control probes, pG14E1.9 or H2-27, which map to chromosome 13 (ATCC, Rockville, MD). Control probe pG14E1.9 recognises a 7.4 kb *Hind*III band and was used with N25 and 160b. All studies were performed in triplicate. Hybridisation signal was quantitated by an imaging system that directly measures counts per minute (cpm) (AMBIS). This method avoids non-linearity problems encountered in densitometric measurements of autoradiograms. Standard hybridisation signals were calculated by taking the ratio of cpm of the test probe to the control probe. Copy number is designated relative to a normal subject. Values less than or equal to 1.4 are consistent with a deletion, whereas ratios greater than or equal to 1.6 represent a normal copy number. Those values falling between 1.4 and 1.6 are indeterminate. In such cases, copy number at that locus was determined by fluorescence in situ hybridisation (FISH).

Cosmids to loci in the DGCR were isolated from a flow sorted, chromosome 22 specific cosmid library (kindly provided by P de Jong) for use in FISH studies. The cosmid cos82 (kindly provided by H Vissing) maps to the distal long arm of chromosome 22 and was used as the control probe. Using modifications of the technique of Lichter *et al.*,²⁸ metaphase spreads of lymphoblastoid cell lines were cohybridised with biotinylated-11-d-UTP labelled test and cos82 probes, and visualised with fluoresceinated avidin. Twenty metaphase spreads showing hybridisation to both chromatids of both chromosome 22 homologues with cos82 were scored for copy number at the test locus.

Results

RFLP ANALYSIS

Thirteen of the 17 patients were evaluated by RFLP analysis using both N25 and R32. Parental DNA was available in three of these cases and family based RFLP studies could be completed. In each family studied, the proband and parents showed single and identical RFLP bands; the studies were therefore uninformative. These patients required further evaluation by dosage analysis to determine the number of copies at each locus.

In the absence of parental DNA, RFLP analysis was performed as a screen to determine whether the patient was heterozygous and therefore not deleted. Ten patients were studied: one patient was heterozygous at D22S75 (N25) and a second patient was

Figure 2 Autoradiogram of Southern blot of three patients (CHD-16, 17, and 18) and one normal control. The blot was cohybridised with test probe N25 and control probe H2-27. By visual inspection, the intensity of the test to control probe signal for CHD-17 is markedly diminished as compared to that of the normal control. As described in the text, quantitation of the hybridisation signal confirmed the presence of a single copy.

heterozygous at D22S259 (R32). Thus, RFLP studies identified two patients without evidence of a deletion at these loci. Since the RFLP studies were largely uninformative, all patients were subsequently studied by dosage analysis.

DOSAGE ANALYSIS

Dosage studies were performed on all 17 patients at both loci D22S75 (N25) and D22S259 (R32). Fig 2 is an example of an autoradiogram of a Southern blot of three patients and one control. The blot was cohybridised with test probe N25 and control probe H2-27 in order to determine copy number at the test locus D22S75 (N25). By visual inspection, in patient CHD-17 the intensity of the 2.6 kb band recognised by N25 is markedly reduced compared to the control probe, in contrast to the same comparison in the normal subject. Quantitation of the hybridisation signals confirmed the presence of a single copy of N25 in CHD-17. A total of four patients, two with TA (CHD-1, CHD-7), one with TA/IAA (CHD-4), and one with TOF (CHD-3), had a single copy of both N25 and R32 consistent with a deletion of the entire region from N25 to R32. Table 2 summarises these results. A fifth patient with TOF (CHD-17) had a single copy of N25, but equivocal results by dosage analysis at locus D22S259 (R32) (copy number 1.58). More recently, we have used FISH of metaphase spreads to determine copy number.²⁹ FISH of metaphase

Table 2 Summary of dosage analysis of CHD cell lines by quantitative hybridisation.

Patient	Probe		
	N25	160b	R32
CHD-1	0.98*	—	1.02
CHD-2	2.21	—	1.64
CHD-3	0.84	—	1.06
CHD-4	0.90*	—	0.62
CHD-5	1.80	—	2.16
CHD-7	0.93	1.02	1.27
CHD-8	1.83	1.82	2.63
CHD-9	1.73	2.12	2.33
CHD-10	1.51†	2.06	2.89
CHD-11	1.83	1.95	2.37
CHD-12	1.62	2.00	2.65
CHD-13	1.75	2.29	2.08
CHD-14	1.98	2.26	2.26
CHD-15	1.73	2.29	2.32
CHD-16	1.77	2.11	2.55
CHD-17	0.82*	—*	1.58*
CHD-18	1.60	1.92	1.95

These values represent locus copy number, standardised from quantitative analysis of the hybridisation signals obtained with the test probe relative to those obtained with a control probe. They were obtained by taking the mean of three independent ratios of patient to control. Values less than 1.40 are consistent with a deletion. See text.

* Deletion confirmed by FISH.

† Not deleted by FISH.

spreads from the cell line CHD-17 was performed using N25, 160b, and R32 cosmid (fig 3). Hybridisation to a single chromosome 22 for each test probe was observed in 20 metaphase spreads confirming the presence of a deletion of all three loci. Thus, a total of five patients had deletions spanning the DGCR (N25 to R32).

Eleven patients had two copies of both loci by dosage analysis and hence are not deleted for the most proximal and distal markers within the DGCR. One patient with IAA (CHD-10) had no evidence of deletion at locus D22S259 (R32) but equivocal findings by dosage analysis at locus D22S75 (N25) (copy number 1.51). FISH with an N25 cosmid confirmed the presence of two copies of this locus in the cell line from this patient. Ten of the 12 non-deleted patients were further evaluated by dosage analysis using the probe 160b

which maps between N25 and R32 to determine whether they have a smaller deletion within the DGCR. None was found to have evidence of a deletion at this locus (table 2).

CLINICAL EVALUATION

A total of 13 patients, including the five patients with 22q11 deletions, was examined. None of these patients was determined to have other malformations or clinical features suggestive of a well described genetic syndrome. Careful examination by the dysmorphologist showed that 10 of the 11 patients had mild dysmorphic features, as outlined in table 1. The most common dysmorphic features included a bulbous tip of the nose (4) or prominent nasal bridge (3), a high arched palate (4) or cleft palate (2), abnormally formed ears (7), or abnormal fingers (5) including long slender fingers (3). Thus, while the deletion positive patients all had subtle dysmorphic features, many of the non-deleted patients showed similar findings.

Discussion

The aetiology of CHD is presumably heterogeneous and may reflect the interaction between genetic predisposition and environmental factors. Epidemiological studies and reports of families with multiple affected sibs point to a genetic predisposition.^{13 30 31} Other investigations implicate environmental factors. For example, infants of diabetic mothers, who may be exposed in utero to raised serum glucose levels, are at increased risk for CHD, particularly for conotruncal defects.³² Similarly, specific teratogens and infections, such as retinoic acid and rubella, respectively, are associated with the development of CHD.³³

This study shows that five of 17, or 29%, of non-syndromic patients with conotruncal lesions have microdeletions of 22q11. This suggests a genetic contribution to the development of at least a proportion of conotruncal malformations. Four of the five deletion positive patients do not have a family history of CHD. The mother of the fifth patient, CHD-3, had TOF. We have not had the opportunity to study the mother to determine whether she also carries a 22q11 deletion which would have been inherited by CHD-3. In a recent study, Wilson *et al*²⁰ reported 22q11 deletions in patients with familial CHD. Two of their patients had features suggestive of DGS in contrast to our study where patients with features of DGS were excluded. Our results should alert the cardiologist to suspect 22q11 microdeletions not only in patients with DGS or familial CHD, but also in those with a conotruncal defect, no family history of CHD, and no obvious genetic syndrome.

The results of this study suggest that a subgroup of infants with apparently isolated forms of conotruncal defects are deleted for 22q11. Such patients may represent a form of VCFS that will only be recognised in an older child. Subtle dysmorphic features were

Figure 3 FISH of biotinylated cos82 and N25 cosmids to metaphase chromosomes from CHD-17. Signal from cos82 on the distal long arm of 22 is seen on both homologues whereas signal from cosmid N25 is seen on only one homologue. This is consistent with a microdeletion at the corresponding locus in the homologue with the arrow. Also note absence of one signal in the interphase nucleus located above and to the left of the arrow.

observed in several deletion positive patients in this study. However, the features were not characteristic of a well known genetic syndrome and further they were not apparent during early infancy when the patients were first ascertained for the study. While the features of DGS are recognisable in infancy, the characteristic speech difficulties, learning disabilities, and distinctive facial features of VCFS are not readily apparent at birth. Thus, the diagnosis of VCFS may be delayed or overlooked. Perhaps this subgroup of our patients with conotruncal defects, subtle dysmorphic features, and microdeletions of 22q11 spanning the DGCR are at increased risk for the learning and speech disabilities seen in patients with VCFS. Long term follow up of these children will determine whether they develop these additional difficulties.

Recent molecular studies using the same markers used in this study have shown that the majority of patients with DGS and VCFS have microdeletions of 22q11.^{17,18,21} Therefore, the deleted region in DGS, VCFS, and in the five patients with conotruncal defects overlaps, suggesting a common genetic aetiology for these disorders. Variations in the endpoints of the deletions as well differences in the genetic background or in utero environment may account for differences in the phenotypes observed in these three groups of patients. Perhaps a 'cardiac critical region' lies within the DGCR and contains a gene(s) critical to the development of the conotruncus. Studies are in progress, using markers which flank the DGCR, to delineate the deletion boundaries in these patients and to determine whether specific deleted regions correspond to a specific clinical feature such as CHD.

The proportion of patients with genetic alterations in 22q11 may be underestimated in the study. For example, some of the non-deleted patients may prove to have smaller deletions within the DGCR. So far, we have not detected smaller deletions between N25 and R32, but the region spans at least 1 Mb and only a single internal marker, 160b, was used in the present study. Studies are in progress with additional markers to determine whether the non-deletion patients have smaller, internal deletions. Moreover, it is possible that different types of genetic alteration within the same chromosomal region can produce related phenotypes. Thus, not only the 22q11 microdeletions but also point mutations within essential genes in this region may contribute to the development of conotruncal malformations. Kirby and associates have used the chick embryo to show that neural crest cell migration is critical to the normal development of the conotruncus.³⁴⁻³⁶ Thus, the genes in the DGCR may play a major role in neural crest cell migration, such that loss of a single copy may disrupt normal migration. As the genes are identified in the DGCR, and their role in developmental pathways is characterised, DNA sequencing could be used to look for point mutations in candidate genes in non-deleted patients.

The identification of molecular genetic alterations in association with CHD will enable us to provide accurate genetic counselling to the family and proband. For example, identification of a de novo deletion implies that the parents of an affected child have a negligible risk of recurrence. However, the affected subject with a microdeletion has a 50% risk of transmitting the deletion bearing chromosome to his or her offspring. Expression might be variable depending upon the genetic and environmental background, but it is possible that the offspring of an affected parent could have a conotruncal defect as well as additional phenotypic features of DGS or VCFS. While fetal echocardiography can be used to evaluate the at risk fetus, molecular-cytogenetic studies of amniocytes or chorionic villi may be used at a much earlier time in the pregnancy to determine whether the fetus has inherited the deletion bearing chromosome. As more children with complex CHD survive to a reproductive age, accurate genetic counselling becomes increasingly important.

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