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RECENT ADVANCES IN THE UNDERSTANDING OF GENETIC CAUSES OF CONGENITAL HEART DEFECTS

Bruce D. Gelb

Departments of Pediatrics and Human Genetics, Mount Sinai School of Medicine, New York, NY

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1. ABSTRACT

The clinical approach to children with congenital heart defects (CHD) has been revolutionized during the past four decades by developments in diagnostics and therapeutics. In contrast, a profound understanding of the causes of the majority of CHD has only begun to emerge within the past few years. Prior epidemiological studies suggested that Mendelian disorders constituted a very small percentage of CHD and that polygenic inheritance was responsible for the majority of cases. Recent discoveries, largely achieved with molecular genetic studies, have provided new insights into the genetic basis of heart malformations. These studies have shown that CHD caused by single gene or single locus defects is more common than had been suspected. In addition, a higher percentage of heart malformations occur in the context of familial disease than was evident previously. In this review, molecular genetic studies of specific heart lesions and syndromes with CHD are reviewed. Progress on the Human Genome Project has accelerated identification of genes for Mendelian traits with heart defects, and it is anticipated that disease genes for most single gene traits will be known within a few years. Future challenges include utilizing this emerging genetic information to improve diagnosis and treatment of children with CHD, and harnessing the power of genomics to analyze isolated heart defects with complex inheritance patterns.

2. INTRODUCTION

Congenital heart defects (CHD) are present in nearly 1% of all newborns and continue to be a significant cause of death in infancy. A major goal for clinicians and basic scientists has been to understand the sources of these relatively common developmental errors. Most discussions of the topic divide potential etiologies between environmental and genetic causes. Exposure of the developing embryo to numerous environmental agents, including specific teratogens and infectious agents as well as certain maternal diseases, has clearly been demonstrated to cause heart malformations (1). In aggregate, however, environmental factors seem to account for only a small percentage of congenital heart disease. The relative constancy of the population prevalence of heart defects through time and across widely spread geographic regions provides further evidence that environmental effects are not a major source of congenital heart disease.

Genetic causes of congenital heart disease have traditionally been divided into three categories: gross chromosomal abnormalities, single gene defects, and complex inheritance (1). Aneuploidies, such as trisomy 21 and Turner syndrome, are clearly associated with congenital heart disease but account for less than 10% of all heart defects affecting live born infants. Single gene defects, also known as Mendelian disorders, are generally thought to account for approximately 3% of heart malformations. These monogenic disorders occupy a disproportionately important role in current molecular research because robust methodology exists for the isolation of Mendelian disease genes.

The focus of this review will be a discussion of recent progress made in cloning and studying gene defects associated with congenital heart disease in humans. Research with other species, such as *Drosophila*, zebrafish, *Xenopus*, chick, and mouse, to find cardiogenetic

Table 1. Clinical Features in Patients with 22q11 Deletions

Condition	%
Congenital heart defects	75%
Abnormal psychomotor development	68%
Hypocalcemia	60%
Cleft palate or velopharyngeal insufficiency	46%
Genitourinary abnormalities	36%
Skeletal abnormalities	17%

Data taken from Ref. 23. Immunologic status not included due to incompleteness of the data set.

genes is currently an area of particularly intense activity. This includes studies of normal cardiac embryology as well as identification of disease genes from animal models with congenital heart defects. While these effort are critical to our understanding of processes that are difficult to study in humans, those genes may or may not have equivalent roles or result in similar phenotypes in people. Thus, this review starts with molecular genetic studies in patients, and attempts to incorporate information from animals that illuminates the functions of human cardiogenetic genes or the pathogenesis of congenital heart disease.

3. CONOTRUNCAL HEART DEFECTS

3.1. 22q11 Deletion Syndromes

Conotruncal defects (truncus arteriosus, aorto-pulmonary window, interrupted aortic arch type B, tetralogy of Fallot, and conal septal ventricular septal defects) have been associated with both the DiGeorge syndrome (DGS, MIM# 188400) and velocardiofacial syndrome (VCFS, MIM# 192430). Recent molecular investigations have revealed that a high percentage of patients with both syndromes have microscopic or submicroscopic deletions at chromosome 22q11. It is now clear that these two syndromes are part of the clinical spectrum of a single disorder. While the acronym, CATCH22 (Cardiac defects, Abnormal facies, Thymic aplasia/hypoplasia, Cleft palate, Hypocalcemia) was proposed as a single name (2), some objections over its possible pejorative nature have prevented its widespread acceptance. The two phenotypes will be reviewed separately, reflecting their history and the independent molecular studies that were undertaken.

DiGeorge first described the association between thymic aplasia and absence of the parathyroid glands in certain immunologically impaired infants in 1965 (3). He attributed this combination of abnormalities to a common embryologic origin of the defective structures from the third and fourth pharyngeal pouches. The association with certain dysmorphic features and congenital heart disease in DGS were noted subsequently when Van Mierop and Kutsche delineated the congenital heart defects in a study of 161 autopsy cases of DiGeorge syndrome (4). They found an interrupted aortic arch (type B) in 32% of cases, truncus arteriosus in 23%, tetralogy of Fallot in 21%, isolated VSD in 6%, isolated right aortic arch 5%, transposition of the great vessels 4%, patent ductus arteriosus 3%, assorted other lesions 6%, and no heart defect in 3%.

Gross chromosomal abnormalities have been identified in a minority of patients with DGS but were crucial in identifying potential genetic loci containing genes responsible for this disorder. In 1981, de la Chapelle and co-workers reported a patient with an unbalanced translocation between chromosomes 20 and 22 resulting in monosomy for 22q11 (5). Using high resolution banding cytogenetic techniques, 18% of patients with DGS were shown to have chromosomal defects, primarily monosomy 22q11 but also monosomy 10p13 (6). Using DNA probes from the DGS region at 22q11 for dosage analysis or fluorescence in situ hybridization (FISH), several groups documented that approximately 90% of patients with DGS have deletions of 22q11 (7–10).

VCFS, first described by Shprintzen in 1978 (11), includes overt or submucosal cleft palate associated with hypernasal speech, facial anomalies including a squared nasal root with a narrow alar base, retrognathia, and malar flatness, as well as congenital heart defects (12). Essentially the same disorder, called the conotruncal anomaly face syndrome, was described independently in Japan by Takao and Kinouchi (13,14). The heart anomalies associated with VCFS, present in greater than 80% of cases, include ventricular septal defects (56% of patients), especially conal septal ventricular septal defects, tetralogy of Fallot (19%), and isolated right aortic arch (11%) (12,15). Prompted by the clinical overlap with DGS, evidence for 22q11 microdeletions was sought in VCFS patients (8,16–18). By high-resolution cytogenetic analysis, approximately 20% of VCFS patient had 22q11 deletions (16). Molecular analyses including FISH and marker haplotyping demonstrated 22q11 deletions in approximately 80% of cases (8,19). Considerable phenotypic variability was found within families transmitting these submicroscopic deletions (20,21). This variability included kindreds in which parents with VCFS produced offspring with DGS.

3.2. Clinical studies of Patients with 22q11 Deletions

The incidence of 22q11 deletions has been estimated at 1 in 4,000, making it the most common microdeletion syndrome identified to date (1,22). The actual prevalence may be higher, however, since a prospective population study has never been performed. The spectrum of clinical features associated with 22q11 deletions was defined in a European collaborative study of 558 patients (table 1) (23). Seventy-two percent of patients had de novo deletions, while the remainder had inherited deletions. Twenty-five percent of patients had no heart defects or trivial ones (e.g., isolated right sided aortic arch). Among those with significant heart lesions, the following were observed: tetralogy of Fallot with or without pulmonary atresia, 36%; ventricular septal defect, 19%; interrupted aortic arch, 19%; and truncus arteriosus, 12%. As reported in other studies, the cardiac phenotype varied substantially among affected sib pairs.

A critical issue is the relative importance of 22q11 deletions among all patients with conotruncal defects. Results of prospective studies of patient cohorts with conotruncal defects of varying sizes have been published (24–27). The group from the University of Pennsylvania studied 260 patients, the largest reported cohort (24). Performing FISH with the DNA probe used most commonly for DGS/VCFS molecular diagnostics (D22S75), they demonstrated that 18% of patients had deletions at 22q11. The frequency of 22q11 deletions varied significantly with the cardiac lesion: interrupted aortic arch type B, 50%; truncus arteriosus, 34%; tetralogy of Fallot, 16%; double outlet right ventricle, 5%; and

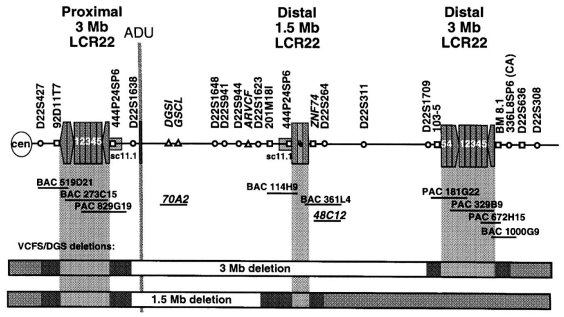

Figure 1. Schematic representation of the physical map of the 22q11 region associated with DGS/VCFS. The ordered PCR-based markers are indicated above the line representing chromosome 22q11. Polymorphic markers are denoted by circles, monomorphic markers by squares, and gene-based markers by triangles. The three low copy repeats (LCR22s) are indicated as shaded clusters. The proximal and distal 3 Mb LCR22s contain a set of genes or pseudogenes that are numbered [1= gamma-glutamyl transpeptidase (*GGT*)-related, 2=*GGT*, 3=*V7-Rel*, 4=*POM121L* and 5=*BCRL*]. Inverted sub-repeats consisting of anonymous genomic markers are indicated as inverted triangles I each LCR22. The bars underneath the physical map indicate the positions of the 3 and 1.5 Mb DGS/VCFS deletions. The positions of the breakpoints as determined by haplotype analysis are indicated as dark shaded regions in the bars. The region containing the 1.5 and 3 Mb chromosome breakpoints as determined by somatic hybrid analysis is indicated as a lightly shaded interval that extends from the LCR22 clusters to the bars. Reprinted with permission of Oxford University Press.

transposition of the great arteries, 0%. In addition, patients with lesions that included abnormal aortic arch sidedness and/or other vessel anomalies were noted to be at greater risk. For instance, the predicted probability of a 22q11 deletion for a patient with interrupted aortic arch and a right aortic arch with an aberrant left subclavian artery was 85%. Three smaller studies provided comparable results (25–27). One prospective study demonstrated that 9/17 dysmorphic patients with conotruncal defects harbored 22q11 deletions while 0/19 non-dysmorphic patients had deletions (25). Similarly, no 22q11 deletions were found among affected individuals from 16 families with multiple members with conotruncal heart defects but a non-syndromic appearance (28). These latter studies suggest that only dysmorphic patients with these heart defects require molecular cytogenetic screening.

The relevance of 22q11 deletions has been investigated among patients with specific cardiac anatomy. One study found an equal prevalence of 22q11 deletions among patients with tetralogy of Fallot with pulmonary stenosis, pulmonary atresia, or absent pulmonary valve syndrome (24). In contrast, another study reported deletions in 8 of 130 patients with tetralogy of Fallot with pulmonary stenosis (6%) compared to 12 of 22 patients with TOF with pulmonary atresia (55%) (29). A third report suggested that 22q11 deletions were common among patients with tetralogy of Fallot with absent pulmonary valve syndrome (30). Rare cases of transposition of the great vessels with 22q11 deletions have been reported (31,32). Increased prevalence of two unusual anatomic abnormalities, malposition of the branch pulmonary arteries and cervical aortic arch, has also been associated with 22q11 deletions (33,34).

3.3. Molecular Studies of 22q11Deletions and Disease Pathogenesis

Molecular studies of the DGS/VCFS region at 22q11 have been undertaken in order to identify gene(s) relevant to the phenotypes, to understand the variability in the phenotype, and to determine the etiology of the relatively common deletional events. Delineation of the extent of 22q11 deletions from 61 VCFS patients using polymorphic DNA markers spanning the commonly deleted region revealed that 82% of patients were deleted for two markers, compatible with a commonly deleted region (21). The phenotype could not be correlated with either the size of the deletion or, in familial cases, the parent of origin. The latter observation ruled out a role for imprinting in the expression of VCFS. Similar conclusions were drawn from molecular analyses of DGS patients and families (35).

The DGS/VCFS region has been mapped, cloned, and sequenced (19). Studies with VCFS patients with 22q11 deletion established that 90% have a similar 3-Mb deletion and 7% have a nested 1.5-Mb deletion (19). Breakpoint mapping of deletions and unbalanced translocations defined a minimal critical region of 250 kb (21,35–37). Mutation analyses of genes within this critical region in DGS/VCFS patients without 22q11 deletions have been negative (38–40). Moreover, DGS/VCFS patients with non-overlapping deletions within the 3-Mb common deletion region as well as one with a deletion telomeric to that region have been identified (41–43). These findings suggest that a genetic model of DGS/VCFS in which haploinsufficiency of genes due to deletion or disruption results in the observed phenotype is too simple. As proposed by Dallapiccola, Pizzuti, and Novelli (44), positional effects of these 22q11 abnormalities on genes in a larger chromosomal region are likely relevant in the molecular etiology of DGS/VCFS.

Morrow's group has investigated the molecular mechanism leading to the 3- and 1.5-Mb deletions at 22q11 (45,46). They showed that the common 3-Mb deletion region was flanked by 250-kb low-copy repeats containing five genes and pseudogenes (figure 1). The smaller 1.5-Mb deletion region shared the centromeric low-copy repeat region with the 3-Mb deletion and contained another low-copy repeat region without any genes at its telomeric end. It appears likely, therefore, that the two commonest deletions at 22q11 causing DGS/VCFS result from intra-chromosomal homologous recombination events. Interestingly, a third low-copy repeat resides telomeric to the distal portion of the 3-Mb deletion, suggesting a similar mechanism could explain the non-overlapping distal deletion (43).

Recently, Deepak Srivastava's research group identified a putative DGS/VCFS candidate gene, *UFD1L*, that mapped to the commonly deleted region, although not

in the minimal critical region (47). *UFD1L* is involved in the degradation of ubiquitinated proteins. The mouse orthologue, *Ufd1l*, was down regulated in mice lacking *dHAND*, a neural crest-related transcription factor. Analysis of 21 patients with DGS/VCFS without 22q11 deletions detected by *D22S75* revealed one individual with a lesion deleting exons 1-3 of *UFD1L*, leading the authors to conclude that UFD1L might be the DGS/VCFS gene (47). Analysis of 42 additional DGS/VCFS patients lacking 22q11 deletions by a consortium of laboratories, however, failed to reveal any deletions or point mutations of *UFD1L* (48). Thus, the apparent involvement of *UFD1L* in a pathway relevant to neural crest development makes it an excellent positional candidate for DGS/VCFS, but additional studies are needed prior to concluding whether or not it plays a central role in disease pathogenesis.

3.4. DGS/VCFS Region at 10p13

While 22q11 microdeletions have been the focus of the molecular studies of DGS/VCFS to date, it has been recognized for some time that genetic heterogeneity exists for both syndromes- that is, genetic defects at other loci can also cause DGS and VCFS. Peter Scambler and colleagues (49) studied the locus at chromosomal band 10p13 that had previously been identified in DGS patients with gross chromosomal defects (6,50), performing FISH analyses with several large-insert genomic clones from that region. Using three patients with DGS associated with 10p terminal deletions and one VCFS patient with an interstitial 10p deletion, the smallest region of overlap among these deletions was defined. Subsequent FISH analysis of five patients with VCFS without 22q11 deletions were normal at 10p13. Nevertheless, these molecular tools can now be used to determine the prevalence of 10p13 submicroscopic deletions among DGS and VCFS patients. Although the majority of DGS/VCFS patients have 22q11 deletions, the smaller subset associated with 10p13 deletions may prove to have specific phenotypes.

4. SECUNDUM ATRIAL SEPTAL DEFECTS

Secundum atrial septal defects (2^{o} ASDs) are generally sporadic, but may be inherited in an autosomal dominant manner. Two Mendelian disease entities have been delineated: 2^{o} ASD with atrio-ventricular (AV) conduction defects and 2^{o} ASD with normal conduction. The Seidmans and co-workers at Harvard assembled four families inheriting 2^{o} ASD with AV conduction defects. In addition to 2^{o} ASDs present in 27 of 33 affected individuals, tetralogy of Fallot, ventricular septal defect, subvalvular aortic stenosis, and mitral valve abnormalities were also observed (51). All affected individuals, plus one with no structural abnormalities, had AV conduction delay. After performing a genome scan with the largest kindred, this trait was linked to a locus at chromosomal band 5q35 (51). An excellent positional candidate gene was *NKX2-5*, a transcription factor containing a homeobox element with a known role in cardiogenesis in other species. Three *NKX2-5* mutations were identified among the four families inheriting 2^{o} ASD with conduction delay: two nonsense defects, including one within the homeobox domain, and a missense change that altered a conserved residue (Thr178Met) (51).

NKX2-5 belongs to a family of homeodomain-containing transcription factors that are homologues of the *Drosophila* gene, *tinman*. *tinman* plays a central role in dorsal mesoderm formation, and flies lacking it fail to form the dorsal vessel, the rough insect equivalent of the vertebrate heart (52,53). In the mouse, the *NKX2-5* gene is expressed in the precardiac mesoderm and then in the myocardium throughout development (54,55). Mice homozygous for an insertion mutation of *NKX2-5* had normal cardiac development through the linear tube stage, but died at day 8.5 *post coitum* (*p.c*) due to heart defects with abnormal cardiac looping (56). Heterozygotes were not noted to have any cardiac defects. There are several closely related *NKX*2 genes in vertebrate genomes and the functions of their protein products are divided in a species-specific fashion. Thus, the critical understanding of the manner in which the molecular lesions of *NKX2-5* in humans results in 2^{o} ASDs with AV conduction defects is not available currently and may prove somewhat difficult to infer from murine models.

Secundum ASDs with normal AV conduction can be inherited as an autosomal dominant trait with incomplete penetrance. Affected individuals may have aneurysm of septum primum rather than interatrial communications, and bicuspid aortic valves have also been observed in some patients. A partial genome scan performed with a four-generation kindred inheriting this disorder resulted in assignment of a 2^{o} ASD locus to the telomeric region of chromosome 5p (57). Analysis with an unrelated family inheriting 2^{o} ASD with normal AV conduction resulted in significant negative LOD scores, excluding linkage to the 5p locus. Prior to the availability of polymorphic DNA markers, linkage of 2^{o} ASD (AV conduction status was not noted) to the HLA region of chromosome 6p was published (58). No further information about this linkage is available. Thus, there appear to be at least three loci for autosomal dominant 2^{o} ASD. The identification of one or more of these disease genes will provide insights into normal and perturbed atrial septation.

5. HOLT-ORAM SYNDROME

Holt and Oram first described this prototypical heart-hand syndrome in 1960 in four generations of a family with 2^{o} ASDs and thumb anomalies (59). This syndrome is an autosomal dominant trait with a high degree of penetrance but variable expression. While 2^{o} ASDs are the most commonly observed heart anomaly in Holt-Oram syndrome (HOS), the range of cardiac phenotypes includes normal, isolated first-degree atrioventricular block, ostium primum ASDs, isolated ventricular septal defects, tetralogy of Fallot, aortic stenosis, mitral valve prolapse, and hypoplastic left heart syndrome (60–62). Limb abnormalities affect structures derived from the embryonic radial ray (radius, carpal, and thenar bones), unilaterally or

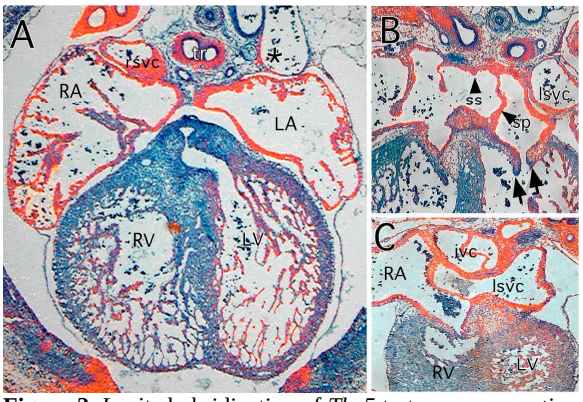

Figure 2. *In situ* hybridization of *Tbx5* to transverse section of an E13.5 mouse embryo. Hybridization of ^{33}P-labeled *Tbx5* probe produces a red signal. A. Transverse section showing a four-chamber view of the heart. The asterisk indicates the junction of the left superior vena cava (LSVC) with the accessory hemizygous vein and the left superior intercostal vein. B. Close-up of a more posterior section reveals expression in both the septum primum (sp) and the septum secundum (ss) as well as in the LSVC and the atrioventricular valves (arrows). C. A section more posterior than that in *B* shows *Tbx5* expression mainly in the atria and left ventricle (LV), as well as in the inferior vena cava (ivc) (67). Printed with permission from *Developmental Biology*.

bilaterally, with severity ranging from triphalangeal thumb to phocomelia.

HOS was mapped to chromosomal band 12q24 using genetic linkage analysis, but was shown to be genetically heterogeneous (63). The HOS disease gene, *TBX5*, was identified by positional cloning (64,65). *TBX5* is a transcription factor containing a T-box DNA-binding domain, which belongs to a family of homologous genes identified in several species that have importance during development. Several nonsense and insertion *TBX5* mutations, some occurring early in the coding sequence, were identified. These findings suggested that the pathogenetic mechanism was haploinsufficiency.

Recently, Basson and co-workers identified a larger number of HOS mutations and established some genotype-phenotype correlation (66). They documented that truncation mutants resulted in severe cardiac and skeletal malformations. A missense mutation (Gly80Arg) affecting the amino region of the T-box resulted in severe cardiac defects but mild skeletal anomalies, whereas two missense mutations which altered single residues at the carboxy-terminal region of the T-box (Arg237Gln and Arg237Trp) resulted in severe skeletal malformation with mild cardiac phenotype. Since all three of these missense mutations are predicted to affect DNA binding, the authors proposed that the *TBX5* protein binds different targets in developing heart and limb.

During mouse and chick development, *Tbx5* is expressed in the developing heart, eye, and forelimbs (67–69). More in-depth analysis of expression during cardiogenesis in the mouse revealed expression in the cardiac crescent at day 8.0 *p.c.* At day 8.25 *p.c.* during formation of the linear heart tube, *Tbx5* is highly expressed in the posterior portion of the heart, which is destined to become the atria and sinus venosa, and expressed more weakly in the myocardium. At days 8.5-9.0 *p.c.* as ventricular looping occurs, the expression of *Tbx5* includes the future left ventricle but not the right, a discrepancy that persists throughout the horizon of cardiac development. During septation at day 13.5 *p.c.*, *Tbx5* is expressed in septum primum and secundum in the atria but primarily on the left side of the developing ventricular septum (figure 2). At this stage, *Tbx5* is also expressed on the atrioventricular valves. Since this expression pattern matched the sites of the vast majority of the cardiac defects observed in HOS patients, it was concluded that the deleterious effects of *TBX5* mutations were likely to be direct. The relatively global effects of *Tbx5* on cardiac development were highlighted by overexpression of a dominant negative mutant in developing *Xenopus* embryos which led to an absence of the heart (70).

6. ATRIOVENTRICULAR SEPTAL DEFECTS

Atrioventricular septal defects (AVSDs), or endocardial cushion defects, comprise a spectrum of heart defects ranging from ostium primum atrial septal defects to complete atrioventricular canal defects. AVSDs present frequently in the context of trisomy 21, but can also occur as an isolated abnormality. Kindreds inheriting AVSDs as an autosomal dominant trait have been identified. Linkage of non-syndromic AVSDs to chromosome 21 was excluded by two groups (71,72). Sheffield and co-workers subsequently performed a genome scan with a four-generation kindred and achieved linkage to a locus at chromosomal bands 1p21-p31. Since the current critical region is 12 cM, it is likely that additional kindreds and/or fortuitous chromosomal lesions will need to be identified in order to permit cloning of this AVSD gene.

Efforts to identify the gene(s) responsible for AVSD and other heart defects associated with trisomy 21 are also underway. While the vast majority of individuals with the clinical features of Down syndrome have complete trisomy 21, a minority of patients have duplications of portions of chromosome 21. Molecular and cytogenetic mapping of small duplications has permitted assignment of various aspects of the Down syndrome phenotype to specific chromosomal regions (73,74). In this manner, the AVSD critical region has been assigned to 21q22.1-qter which is a 4-5 Mb interval (73,74). This region has been mapped in large insert genomic clones, which will facilitate future efforts to isolate AVSD candidate genes (75).

7. LATERALITY DEFECTS

Heterotaxy syndromes occur both sporadically and familially with apparent autosomal recessive, autosomal dominant, or X-linked inheritance. The X-linked form of heterotaxy includes complex cardiac defects in association with asplenia or polysplenia. Other midline

malformations observed in this syndrome include arhinencephaly, cerebellar hypoplasia, and sacral agenesis. Initially, Brett Casey and colleagues mapped the gene for X-linked situs defects to Xq24-q27.1 (76). A submicroscopic interstitial deletion was identified in one family inheriting this disorder (77). Positional cloning techniques were then used to identify genes mapping into the deleted region (78). One such gene was *ZIC3*, a transcription factor containing five zinc fingers. The mouse orthologue, *Zic3*, is expressed in the primitive streak at day 7.0 *p.c.* and in several developing structures that are relevant to this disorder, such as the cerebellum and olfactory bulb (79,80). Expression studies with *Xenopus* embryos revealed that *Zic3* plays a significant role in neural and neural crest development (81). Mutation analysis of the *ZIC3* gene revealed two nonsense defects, both predicting truncation of the protein prior to the second zinc finger, and two missense changes affecting conserved residues in the first or second zinc finger domains. Additional studies are needed to delineate the precise role of *ZIC3* in the development of the left-right axis as well as the perturbations in the downstream pathways induced when it is deficient.

The development of left-right asymmetry in developing embryos has been an active area of research in recent years. Using a variety of animal model systems, investigators have identified several genes in pathways leading to that asymmetry (well reviewed in Refs. 82–84). There are significant differences in the roles of these genes in different species (e.g., altered role of the genes, *Sonic hedgehog* and *Fibroblast growth factor 8*, in chick and mouse development (85)). While the precise role of these genes in the development of laterality during human embryogenesis is not well established, Casey's group screened patients with heterotaxy for mutations in several candidate laterality genes. Using this approach, three missense changes were identified in the activin receptor type IIB (*ACVR2B*) among 126 sporadic and familial cases of heterotaxy (86). One mutation, R40H, was recurrent while the other, V494I, was found once. Neither change was detected among 200 control chromosomes, and both altered residues that were highly conserved among species ranging from goldfish to human. Similarly, mutation screening of *LEFTY A* in this patient cohort revealed a nonsense and a missense mutation in heterozygosity in two patients with bilateral left sidedness (87). No *LEFTY B* mutations were identified. Thus, all of the mutations in laterality genes among heterotaxy patients have been found in heterozygosity. It is unclear whether these mutations have dominant-negative effects or result in haploinsufficiency. Alternatively, there is the possibility that these patients are double heterozygotes who also inherited mutations in other laterality genes in heterozygosity. Complex genetic inheritance causing heterotaxy has precedence in mice in which double heterozygotes for targeted disruptions of *nodal* and either *HNF3b* or *Smad2* have the disease (88,89). Since the pathways leading to left-right asymmetry are complex with many contributing genes, more thorough analyses will be required before conclusions are drawn about the relevance of double heterozygosity as a cause of heterotaxy in patients.

8. PATENT DUCTUS ARTERIOSUS

Patent ductus arteriosus (PDA) has been used as the model of polygenic inheritance in congenital heart disease, based in large part on the thesis work of Zetterqvist (90). While sibling recurrence rates appear to be ~3%, PDA can be inherited in a Mendelian fashion as an isolated trait or in the context of a syndrome. One PDA syndrome, named Char syndrome (MIM# 126830), includes craniofacial abnormalities and defects of the 5th finger (91,92). Recently, Satoda and co-workers performed a genome scan using two unrelated, multi-generational families inheriting Char syndrome (93). The disorder was linked to chromosome 6p12-21.1 and a 3.1-cM critical region was defined by haplotype analysis. A mouse model of PDA was created by targeted gene disruption of the prostaglandin receptor gene, EP4 (94). Homozygote mice die in the early neonatal period, but have no other defects. To date, no defects in the human EP4 gene have been identified among patients inheriting isolated PDA.

9. FAMILIAL SUPRAVALVULAR AORTIC STENOSIS AND WILLIAMS SYNDROME

Supravalvular aortic stenosis (SVAS) is an unusual form of CHD presenting in three ways: 1) an autosomal dominant familial arteriopathy with SVAS, main and branch pulmonary artery stenoses, and obstructions of various large and small systemic arteries (MIM# 185500), 2) a sporadic arteriopathy indistinguishable from the inherited form (which presumptively represents *de novo* mutations), and 3) Williams syndrome (MIM# 194050) which includes the same arteriopathy accompanied by hypersocial personality, developmental delay, characteristic dysmorphic facial features, and infantile hypercalcemia (95). Williams syndrome is generally sporadic but can be transmitted from affected parent to child.

A series of molecular genetic investigations, principally by Mark Keating's group at the University of Utah, have revealed that the SVAS arteriopathy results from elastin gene defects. First, genetic linkage analyses of three families with autosomal dominant SVAS localized the SVAS gene to the long arm of chromosome 7 (96,97). The elastin gene was known to lie in the SVAS critical region and became the leading candidate gene. Subsequently, another family with autosomal dominant SVAS was found to have a balanced translocation between chromosomes 7 and 6 that co-segregated with SVAS (98). DNA sequencing of the breakpoint revealed that the translocation disrupted the elastin gene (99). Molecular analysis of two families inheriting SVAS demonstrated large deletions affecting the elastin gene (97,100). Final proof that the culprit gene was elastin, and not some other gene in the region affected by chromosomal rearrangements, was obtained when point mutations in the elastin gene were identified in seven, unrelated patients with SVAS (101). Gene defects included two nonsense changes and a single base pair deletion that caused a frameshift leading to a premature stop codon. No genotype-phenotype correlation could be made because

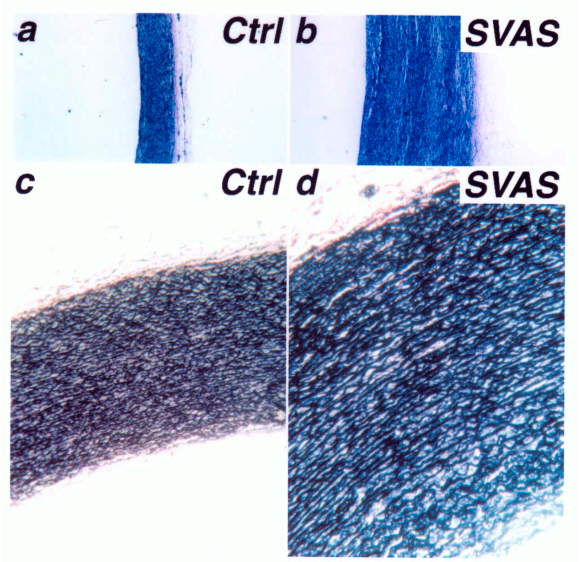

Figure 3. Elastin Van Gieson's stain of descending aortae cross-sections from a control (A and C) and a human with supravalvular aortic stenosis SVAS (B and D). Descending aortae were examined 1.0 cm distal to the left subclavian artery and were free of discrete stenosis. Low magnification of aortic sections (A and B) demonstrates the marked increase in thickness of SVAS samples. Higher magnification (C and D) shows 2.5-fold more elastic lamellae in the aortae of an individual with SVAS (104). Printed with permission from the *Journal of Clinical Investigation.*

intrafamilial variation was as great as interfamilial variation.

Since the arteriopathy associated with Williams syndrome is clinically and histologically indistinguishable from that found in familial SVAS, elastin gene defects were investigated among patients with Williams syndrome. FISH studies revealed haploinsufficiency at the elastin locus in nine individuals with Williams syndrome, including two rare pairs of affected parent and child (102). Moreover, gene dosage data using cosmids flanking the two ends of the elastin gene revealed that the entire gene was deleted. Subsequent FISH studies using an elastin probe have revealed submicroscopic deletions in 96% of patients with classic Williams syndrome (n=114) (103).

The pathogenesis of the arteriopathy has been investigated using a mouse model with a null elastin gene mutation (104). The aortas and pulmonary arteries of *Eln* +/- mice were grossly normal. The elastic lamellae in the aorta were ~50% thinner than those in controls, but vessel extensibility was normal. This was accounted for by the increase in the number of lamellae (10.5 $\pm$ 0.5 in *Eln* +/- mice compared to 8.4 $\pm$ 0.5 in controls) and accompanying layers of smooth muscle. Similar increases were observed in the pulmonary arteries. To determine if this vascular phenotype was present in humans lacking one elastin allele, the investigators studied aortic sections from SVAS patients at sites distant from discrete stenoses (figure 3) (104). It was observed that, compared to controls, 2.5-fold more elastic lamellar units were present in SVAS aortas (152 $\pm$27.6 vs. 62 $\pm$8.7). The authors suggested that the more dramatic changes in SVAS patients compared to elastin-deficient mice might be attributable to greater wall stress in larger arteries. They proposed that the profound increase in smooth muscle outstrips the blood supply from the vaso vasorum, leading to medial necrosis and fibrosis. This hypothesis is consistent with the histology of SVAS stenotic lesions, which features fibrosis, disruption of the elastic fibers, and hypertrophy of the vascular smooth muscle (105,106).

10. ALAGILLE SYNDROME

Alagille syndrome (MIM 118450), also known as arteriohepatic dysplasia, consists of neonatal cholestasis due to paucity of intrahepatic bile ducts, facial dysmorphism, vertebral anomalies (butterfly vertebra, hemivertebra), eye defects (anterior chamber anomalies, especially posterior embryotoxon, and retinal pigmentary abnormalities), and congenital heart disease (107). Greater than 95% of patients with Alagille syndrome have cardiovascular defects such as peripheral pulmonic stenosis, valvular pulmonary stenosis, tetralogy of Fallot, coarctation of the aorta, and atrial septal defects. This disorder is inherited in an autosomal dominant fashion. Estimates of the percentage of cases resulting from *de novo* mutation events has varied, but was recently suggested to be ~70% (108). The phenotype of Alagille is highly penetrant but also highly variable. Partial Alagille syndrome has been recognized in infants with paucity of intrahepatic bile ducts and three out of four other major criteria.

The Alagille syndrome locus was assigned to chromosomal band 20p11.2-p12 based on the discovery of a patient with a deletion of that region (109). Subsequently, other patients with similar deletions were identified and linkage analysis of a three-generation kindred using markers from 20p11.2-p12 was confirmatory (110). Two groups independently identified the Alagille syndrome disease gene as *JAGGED1* (111,112). *JAGGED1* encodes a transmembrane protein that is a ligand for *Notch* proteins, which are also membrane spanning and have critical roles in cell fate determination. More than 100 mutations causing Alagille syndrome have been identified (108,111–114). All but one mutation have affected the large extracellular domain (figure 4). No phenotype-genotype correlation can be made from these molecular analyses.

The mechanism by which *JAGGED1* mutations cause Alagille syndrome is uncertain, although the more likely one is haploinsufficiency. Since large deletions affecting the *JAGGED1* gene are found in some patients, it seems clear that loss of one allele is sufficient to result in this disorder. A possible second pathogenetic mechanism is a dominant negative effect. To date, the effects of missense and nonsense mutations identified in Alagille patients on *JAGGED1* protein function have not been studied formally. It is possible that these gene alterations result in unstable transcripts and/or protein products that have no function or are removed rapidly, resulting *de facto* in haploinsufficiency.

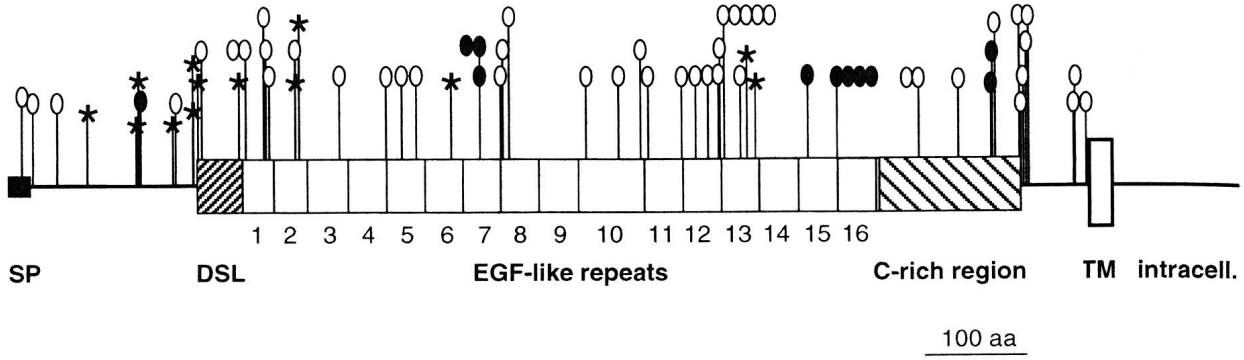

Figure 4. Position of 69 mutations in the *JAGGED1* protein identified in patients with Alagille syndrome. AP, signal peptide; C-rich region, cysteine-rich region; TM, transmembrane domain; intracell., intracellular region. Oval, premature stop codon; filled oval, splice-site mutation; *, amino acid substitution (108). Printed with permission from W.B. Saunders and Company.

However, truncation mutants of two *Drosophila* homologues of *JAGGED1, Delta* and *Serrate*, function in a dominant negative manner with respect to *Notch* signaling, presumably through secretion of truncated proteins that lack the anchoring transmembrane and intracellular domains (115). Since several of the*JAGGED1* mutations are predicted to cause similar truncations, it remains possible that some mutants may act in this dominant negative fashion. Thus, *JAGGED1* mutations may cause Alagille syndrome by more than one mechanism.

With the identification of *JAGGED1* as the Alagille gene, efforts are in progress to establish the full range of phenotypes that can be seen when the gene is defective. Since liver involvement can be subtle or apparently absent in some "affected" members in families inheriting Alagille syndrome, it seemed likely that congenital heart defects would be the presenting feature in patients with new *JAGGED1* mutations or in families lacking an individual with severe liver involvement (precluding the diagnosis of Alagille syndrome). Nancy Spinner's group at the University of Philadelphia recently reported two such cases (116). In one instance, a 3 1/2-year-old girl presented with peripheral pulmonic stenosis, but no other medical problems. In the three preceding generations on her mother's side, individuals had pulmonic stenosis or ventricular septal defect. When investigated by a clinical geneticist, the proposita was found to have the typical facial features of Alagille syndrome, posterior embryotoxon, and moderately elevated liver enzymes. A frameshift mutation of *JAGGED1* was identified in this girl and her mother. The second patient, a 5 1/2-year-old girl, presented with tetralogy of Fallot. She had suggestive facial features, a butterfly vertebra, posterior embryotoxon, but normal liver function tests. High-resolution chromosome analysis revealed a *de novo* interstitial deletion of 20p11.23-p12. Population-based studies are needed in order to document the proportion of congenital heart defects that are attributable to *JAGGED1* mutations as well as to establish the prevalence of those gene defects among patients with specific cardiovascular anomalies.

11. PERSPECTIVE

The discoveries from molecular studies of inherited syndromic and isolated heart lesions discussed in this review represent the first success stories in understanding the genetic causes of congenital heart disease. These research efforts have already taught us much about the subject, but considerably more work needs to be done. After accounting for the percentages of congenital heart defects attributable to environmental causes, aneuploidy, and Mendelian disorders, greater than 80% of cases remain unexplained.

In the 1960s, James Nora proposed that polygenic inheritance was important for the majority of congenital heart disease (117). Bolstering this view were data showing that sibling recurrence rates for most cardiac lesions were 2-3%, typical for traits thought to be inherited in a complex manner. During the ensuing thirty years, several developments have forced a re-evaluation of that view. With the survival of patients with significant congenital heart defects into adulthood, Ruth Whittemore was able to document that recurrence rates were far higher if a parent, particularly the mother, had been born with a heart anomaly (118). Reclassification of heart lesions, such as was done for the Baltimore-Washington Infant Study, revealed that sibling precurrence rates for left heart flow lesions approached values expected for single-gene defects, while rates for other lesions, such as muscular ventricular septal defects, were near population prevalence (119,120). These findings and others were inconsistent with critical predictions arising from the polygenic inheritance model, leaving its validity in question.

The available molecular data suggest novel paradigms that broaden our understanding of the genetic basis of congenital heart disease. As reviewed above, several syndromes that frequently include heart anomalies have been found to result from submicroscopic, interstitial chromosomal deletions. Molecular cytogenetic studies have proven that the frequency of previously unsuspected

deletions can be quite high. Moreover, these small deletions often occur *de novo* but will be passed on as Mendelian traits, explaining in part the difference in recurrence rates for siblings versus offspring. *De novo* point mutations also may constitute a high proportion of lesions affecting some disease genes associated with Mendelian disorders. Founding mutational events for Mendelian syndromes can be obscured further by phenotypic variability, so that the cardiac defect may be the only obvious manifestation (e.g., *JAGGED1* mutations). Thus, some as-yet-unknown percentage of congenital heart disease is genetic, but not familial. Since DGS/VCFS alone accounts for 2-3% of all congenital heart defects, it is clear that prior estimates that 3% of all heart anomalies had a simple genetic basis were too low.

The second lesson learned from molecular analyses is that congenital heart disease is more often familial than had been recognized. Prior failure to appreciate inheritance patterns resulted from a combination of widely variable expression of disease components and incomplete penetrance. More thorough clinical evaluation for subtle phenotypes as well as mutation analyses have shown that propositi with congenital heart defects may have affected family members with minimal to no cardiac phenotype (e.g., inherited 2^{o} ASD traits).

The third paradigm to emerge from the molecular analysis of congenital heart defects is that complex inheritance may be oligogenetic (meaning the trait is determined by few genes) rather than polygenic. As was discussed in the section on heterotaxy, evidence is emerging that double heterozygosity can result in complex heart defects. While the risk that siblings inherit both mutations is 1/4, disease recurrence rates may be lower due to incomplete penetrance (e.g., if gene defects result in a randomization of laterality, then genotypically affected individuals with situs solitus will appear to be normal). Thus, observation of low recurrence risks does not dictate the involvement of large numbers of disease genes.

At this juncture, we cannot judge which genetic mechanisms will prove most important for the etiology of apparently sporadic, isolated heart defects. Basic research on the mechanisms underlying cardiac development and progress on the Human Genome Project are accelerating dramatically studies about the genetic causes of congenital heart defects. It can safely be predicted that the disease genes for nearly all Mendelian syndromes will be known within 10 years, and most within five. As many inherited syndromes with complex phenotypes include heart defects (see Appendix in Ref. 1 for a comprehensive list), the list of cardiac disease genes is certain to grow substantially. The number of Mendelian forms of isolated heart defects, while fewer in number, will also be readily analyzed by positional candidacy approaches. Identification and analysis of new submicroscopic chromosomal lesions will also become simpler using whole genome analyses. The task ahead will be to use creatively the emerging genomic technologies that will permit rapid whole genome analysis and mass sequencing to improve diagnostics and genotype-phenotype correlation as well as to analyze congenital heart defects with apparently complex inheritance. It is also important to bear in mind that not all heart malformations without environmental cause will have genetic ones- some fraction may be attributable to random failures in an intricate process carried out with high, but imperfect, fidelity.

Geometric increases in this molecular information will present new challenges to physicians and other health professionals who care for patients with heart malformations and counsel their families. The difficulties in interpreting the data and in making it understandable to the public will require creative new approaches. As genetic information becomes part of the currency of the field of pediatric cardiology, sensitivity will be required to potential risks affecting privacy, insurability, and employability.

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Send correspondence to: Bruce D. Gelb, M.D., Mount Sinai School of Medicine, One Gustave Levy Place, Box 1498, New York, NY 10029, Tel:212-659-6705, Fax: 212-849-2508, E-mail: gelb@msvax.mssm.edu

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