Part XI

Current Genetics in Congenital Heart Diseases

Perspective

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Congenital heart disease (CHD) occurs in nearly 1 % of all live births and is the major cause of infant mortality and morbidity; about three per 1,000 live births will require some intervention during the first year of life. Additionally, 95 % of CHD patients survive to adulthood in these days, resulting in a growing population of adult CHD. Despite their clinical importance, the underlying genetic etiology of most CHD remains unknown, so-called "multifactorial" disease.

Identifying genetic cause of CHD is important not only to well understand the disease but also to enhance current knowledge about the molecular biology and genetics involved in the human cardiovascular development. Such knowledge may lead to new preventive and/or therapeutic strategies. Identification of disease genes would benefit the genetic counseling for CHD that is particularly important for the growing population of adult CHD. Deeper understanding of factors and pathways involved in differentiation of the cardiac stem cell and morphogenesis of the cardiovascular system would also provide the development of regenerative therapy for CHD.

During the last two decades, linkage analysis has been used to successfully identify disease genes involved in isolated CHD or genetic syndromes where CHD is part of the phenotype. However, this traditional approach is not generally suitable for CHD because it requires many large families with multiple affected individuals. During the same period, some of syndromes with chromosomal abnormalities have been well studied to identify disease genes in syndromic CHD. CHD is commonly a

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characteristic part of the clinical spectrum in a significant number of syndromes caused by chromosomal abnormalities, including submicroscopic deletions or duplications. To date, these genetic approaches have led to the identification of more than 50 human genes although they have limitation where large familial cases and chromosomal abnormalities account for relatively small portion of CHD.

Recently, a technique of genome-wide association studies (GWAS) has provided common genetic variations that can influence population-attributable risks of certain types of CHD although the information cannot be directly related to the affected individual. Finally, application of the next-generation sequencing (NGS) technologies is revolutionary in the field of genetics in CHD. In contrast to GWAS, the results of NGS are directly applicable to the affected individual. NGS can be targeted or nontargeted. Exome sequencing and whole-genome sequencing, scanning the whole exome of ~20,000 base pairs and the whole genome of ~3,000,000,000 base pairs, respectively, are well suited to the study of complex, heterogeneous diseases such as CHD and the current best technique for discovery of novel genetic causes for CHD. The biggest obstacle during any NGS analysis is, however, to single out the causal variant from the thousands of variants identified during sequencing. Follow-up animal studies, particularly in mice, for candidate genes discovered by genetic analyses have been successful in validating the candidates and uncovering the function of their gene products for the cardiovascular development. More recently, fine mapping of genomic copy number variants (CNVs) by NGS in patients with isolated or syndromic CHD has been used to identify candidate disease genes.

In this part, authors describe the current advance in genetics in CHD using linkage analysis, chromosomal studies, and CNVs studies by NGS, combined with animal experiments that verified novel genetic causes of CHD and provided new insights into the molecular and functional analyses of the cardiovascular development. Current understandings about molecular pathways associated with CHD involve numerous transcription factors and cofactors, including chromatin modifiers, and signaling molecules from ligands to receptors.

Genetic Discovery for Congenital Heart Defects

51

Bruce D. Gelb

Abstract

Congenital heart disease (CHD) behaves like a complex genetic trait in most instances. Recent advances in genomics have provided tools for uncovering genetic variants underlying complex traits that are now being applied to study CHD. Massively parallel DNA sequencing has shown that de novo mutations contribute to ~10 % of severe CHD and implicated chromatin remodeling in pathogenesis. Genome scanning methods for copy number variants (CNVs) identify likely pathogenic genomic alterations in 10 % of infants with hypoplastic left heart syndrome and related single ventricle forms of CHD. The growth and neurocognitive development of children with CHD and those CNVs is worse, and clinical examination is relatively insensitive for detecting those CNVs. In sum, new opportunities for preventing and ameliorating CHD and its comorbidities are anticipated as its genetic architecture is elaborated through the use of state-of-the-art genomic approaches.

Keywords

Congenital heart disease • Copy number variants • Exome sequencing • Genetics

51.1 Introduction

Congenital heart defects (CHD), with an estimated incidence of 2–3 % when bicuspid aortic valve (BAV) is included, are widely believed to have strong genetic underpinnings. Epidemiologic studies have shown considerable consistency in the

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distribution of CHD lesions across time and geographic location. The landmark study by Ruth Whittemore, in which she examined recurrence risks for offspring of women with CHD, revealed a 16 % rate with a 60 % concordance in the form of CHD between mother and child [1]. Estimates of heritability for BAV and hypoplastic left heart syndrome (HLHS) are 89 % and 95 %, respectively [2, 3]. Recent studies of CHD from Denmark, where the highly organized medical system enables population studies, have provide estimates of the relative risks of various forms of CHD among first-degree relatives that significantly increased, often to >5 [4]. Taken as a whole, these epidemiologic findings point to genetic defects contributing importantly to CHD etiology.

Identification of the precise mutations has been challenging [5]. We have known for some time that a modest percentage of CHD (~5 %) is attributable to aneuploidies such as trisomy 21. With the advent of molecular genetic approaches, point mutations with apparently strong effects have been identified in rare families inheriting CHD in Mendelian or near-Mendelian fashion. More recently, the role of larger genomic events generating pathologic copy number variants (CNVs) for CHD has become apparent. This started with the recognition of 22q11 deletions underlying DiGeorge, Takao conotruncal face, and velocardiofacial syndromes. Several surveys have implicated a wide range of gain and loss CNVs in various forms of CHD.

Based on the author's oral presentation at the 2013 Takao Symposium, two recent studies that further elaborate the genetic etiology of CHD will be reviewed here.

51.2 De Novo Mutations

Through recent advances in molecular genetic technologies, it is now possible to sequence the roughly 1 % of the human genome that contains the coding regions for all genes (called the exome), representing approximately 180,000 exons and 30 megabases (Mb), in a relatively rapid and affordable manner. While exome sequencing was initially used to discover mutations underlying Mendelian disorders, current efforts are increasingly focusing on unraveling complex genetic traits.

The Pediatric Cardiac Genomics Consortium (PCGC) [6], a National Heart, Lung, and Blood Institute-funded research enterprise, recently completed a first-of-kind study to determine the role of de novo mutations in the etiology of severe forms of CHD [7]. Exome sequencing was performed for 362 parent-offspring trios, in which the offspring had a sporadic conotruncal defect, left ventricular outflow track obstructive lesion, or heterotaxy, and compared to comparable data from 264 control trios. While the overall rate of de novo point and small insertion/deletion (indel) changes was equivalent between CHD cases and controls, there was an excess burden of protein-altering mutations in genes highly expressed during heart development (odds ratio (OR) of 2.53). Excess mutations had a role in 10 % of CHD cases and led to the estimate that ~400 genes underlie these birth defects. After filtering to retain variants most likely to be deleterious (nonsense,

splice site, and frameshift defects), the burden among CHD cases increased, attaining an OR of 7.50.

Next, the PCGC investigators asked whether the burden of de novo protein-altering mutations among the CHD cases preferentially targeted particular biologic processes [7]. Indeed, they observed a highly significant enrichment of mutation among genes encoding proteins relevant for chromatin biology, specifically the production, removal, or reading of methylation of Lys4 of histone 3 (H3K4me) (Fig. 51.1). The phenotypes of the eight subjects harboring H3K4me de novo mutations were diverse, both with respect to the form of CHD and the involvement of extracardiac tissues. In addition, two independent de novo mutations were found in *SMAD2*, which encodes a protein with relevance for demethylation of Lys27 of histone 3 (H3K27me). SMAD2 contributes to the development of the left-right body axis; both subjects harboring *SMAD2* mutations had dextrocardia with unbalanced complete atrioventricular canal defects with pulmonic stenosis. While the contribution of chromatin remodeling to cardiovascular development generally and

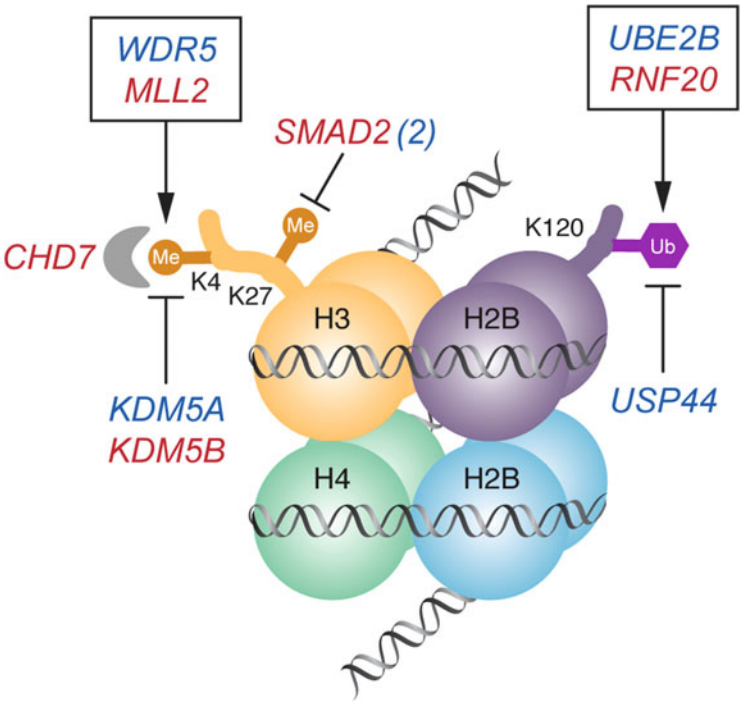

Fig. 51.1 De novo mutations in the H3K4 and H3K27 methylation pathways. Nucleosome with histone octamer and DNA, H3K4 methylation bound by CHD7, and H3K27 methylation and H2BK120 ubiquitination is shown. Genes mutated in CHD that affect the production, removal, and reading of these histone modifications are shown; genes with damaging mutations are shown in red, and those with missense mutations are shown in blue. SMAD2 (2) indicates there are two patients with a mutation in this gene. Genes whose products are found together in a complex are enclosed in a box (Reprinted without modification from Ref. [7])

certain rare genetic syndromes with CHD like Kabuki syndrome had been recognized previously, this study exposed a far broader role in CHD pathogenesis. The finding also suggests a fascinating potential link to other birth defects as de novo chromatin remodeling mutations have also been implicated in autism [8].

51.3 Copy Number Variants

CNVs, which are gains or losses of DNA ranging in size from 1 kb to several Mbs, affect roughly 10 % of the human genome [9]. CNVs are typically detected on a genome-wide basis using SNP microarrays or array comparative genomic hybridization (aCGH). Although differentiating pathogenic CNVs from benign polymorphic one remains challenging, it has become clear that pathologic CNVs contribute significantly to the pathogenesis of CHD as rare large CNVs are observed in 5–15 % of affected individuals [10–14].

To address the issue of how pathogenic CNVs affected outcomes for children with CHD, Carey and colleagues studied children who had previously been subjects in one or both of two clinical trials undertaken by the National Heart, Lung, and Blood Institute-funded Pediatric Heart Network [15]. The two studies were the Infants with Single Ventricle (ISV) and Single Ventricle Reconstruction (SVR) trials, both focusing primarily on infants with HLHS. Carey et al. used aCGH to identify CNVs in 223 subjects from the ISV and SVR trials for whom genomic DNA was available. Because the DNA samples were procured late in those trials, subjects who died earlier, particularly perioperatively, could not be studied. For the CNV work, CNVs were $\geq$300 kb and not identified as genetic polymorphisms based on existing databases.

Carey and co-workers observed pathogenic CNVs in 13.9 % of the children, which was significantly more than the 4.4 % rate in the controls (preexisting data from blood samples from subjects with solid cancers from The Cancer Genome Atlas project) [15]. For the CHD cases, there was a roughly 4:1 ratio of duplications to deletions and the median sizes were 674 kb and 1.5 Mb, respectively. Parental genomic DNAs were available for 12 of the subjects with pathogenic CNVs; analysis of those showed that slightly more than have of the pathogenic CNVs were inherited.

For the ISV and SVR studies, careful assessment of growth (weight-, height- and head circumference-for-age z-scores) and neurocognitive function (Mental Developmental Index (MDI) and Psychomotor Developmental Index (PDI) measured with the Bayley Scales of Infant Development II) were determined at 14 months of age [15]. Comparison of the CHD subjects harboring pathogenic CNVs to those who did not revealed that the former were significantly shorter by an average of 0.65 z-score. Subgroup analysis showed that those with deletion CNVs had significantly lower PDI scores.

Among the 31 pathogenic CNVs found among the CHD subjects, 13 had previously been associated with genomic disorders. The children harboring these known CNVs had the worst outcomes with globally reduced neurocognitive development (MDI and PDI) as well as the slowest growth (Table 51.1).

Table 51.1 Fourteen-month outcomes for subgroups based on genetic examination

	N^a	MDI	PDI	Weight *Z*	Length *Z*	HC *Z*
CNV- Syndrome- Dysmorphic- Extracardiac-	69	89.1	77.5	−0.71	−1.13	−0.34
		(18.0)	(20.2)	(1.07)	(1.32)	(1.24)
CNV+	14	85.4	65.1*	−0.94	−1.61	−0.04
		(20.1)	(17.6)	(0.88)	(1.08)	(1.38)
CNV+ or syndrome	18	83.2	67.9*	−1.19*	−1.99*	−0.16
		(18.7)	(19.4)	(1.11)	(1.73)	(1.27)
Dysmorphic/extracardiac	29	89.3	78.1	−0.63	−1.18	−0.10
		(18.1)	(20.0)	(1.25)	(1.30)	(1.28)
CNV syndrome	98	89.4	77.8	−0.73	−1.18	−0.25
		(17.5)	(19.8)	(1.10)	(1.43)	(1.32)

All data shown as mean (standard deviation)
MDI mental developmental index, *PDI* psychomotor developmental index, *Weight Z* weight-for-age Z score at 14 months, *Length Z* length-for-age Z score at 14 months, *HC Z* head circumference Z score at 14 months
*$p < 0.05$ compared to CNV/syndrome/dysmorphic/extracardiac
[a]Size of each cohort. Incomplete data for outcomes resulted in lower Ns. Reprinted without modification from Ref. [15]

Finally, Carey and co-workers looked at the sensitivity of clinical examination in detecting children with CHD and pathogenic CNVs, which was possible for the subjects from the SVR study [15]. Of 116 children examined, 3.4 % were diagnosed with a defined genetic syndrome, none associated with a pathogenic CNV, and 25 % had one or more dysmorphic features and/or extracardiac malformations, which were not enriched among those with CNVs. Most strikingly, more than 70 % of the children with CNVs previously associated with genomic disorders had no dysmorphic feature or extracardiac anomaly. Taken as a whole, this analysis showed that clinical examination was relatively insensitive for determining which children with CHD harbored pathogenic CNVs.

The findings from this study support the routine use of CNV testing in newborns with single ventricle forms of CHD to enable better prognostication and early intervention. Similarly, the poorer linear growth associated with all pathogenic CNVs, the worse neurocognitive outcomes with deletions, and particularly the globally poor outcomes with CNVs associated with known genomic disorders could impact clinical trial outcomes depending on the designated endpoints.

51.4 Future Directions

Recent advances in genomics have enabled the elucidation of the architecture of CHD genetics. As that project proceeds, the next challenge will be translating those gene discoveries into actionable clinical approaches. Improved prognostication with respect to the heart disease and extracardiac comorbidities can be used most immediately. Finding strategies that reduce CHD incidence or alter its natural

history will require the elucidation of pathogeneses, and a careful balancing of potential benefits with adverse effects as fundamental biological process like chromatin remodeling will probably predominate.

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52 Evidence That Deletion of ETS-1, a Gene in the Jacobsen Syndrome (11q-) Cardiac Critical Region, Causes Congenital Heart Defects through Impaired Cardiac Neural Crest Cell Function

Maoqing Ye, Yan Yin, Kazumi Fukatsu, and Paul Grossfeld

Abstract

Jacobsen syndrome (11q-) is a rare chromosomal disorder characterized by multiple problems including congenital heart defects, behavioral problems, intellectual disability, dysmorphic features, and bleeding problems. Septal defects, including double outlet right ventricle (DORV), are among the most common CHDs that occur in 11q-. One possible mechanism underlying the CHDs and other problems in 11q- is a defect in neural crest cell function. The E26 avian leukemia 1, 5′ domain (ETS-1) gene is a member of the ETS-domain transcription factor family. ETS-1 is deleted in every 11q- patient with CHDs, and gene-targeted deletion of the ETS-1 gene in C57/B6 mice causes DORV with 100 % penetrance. Normal murine cardiac development requires precisely regulated specification of the cardiac neural crest cells (cNCCs). To begin to define the role of ETS-1 in mammalian cardiac development, we have demonstrated that ETS-1 is strongly expressed in mouse cNCCs during early heart development. Sox10 is a key regulator for the neural crest cell gene regulatory network. It is also an early marker for NCCs, and its expression can facilitate the analysis of cNCC function during embryonic development. We have demonstrated that loss of ETS-1 causes decreased migrating Sox10-expressing cells in E10.5 C57/B6 mouse embryos. These results suggest a NCC migration defect in ETS-1 mutants. Our data support the hypothesis that ETS-1 is required for specification and migration of cNCCs and for regulating a cNCC-specific gene regulatory network that is required for normal cardiac development.

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Keywords
Jacobsen syndrome • Cardiac neural crest • ETS-1 • Double outlet right ventricle • Genetic modifier

52.1 Introduction

Congenital heart defects (CHDs) are the most common birth defect in live-born infants, occurring in 0.7 % of the general population. Although there are numerous genetically engineered mouse models for CHDs, only a small number of these genes are currently associated with CHDs in humans.

Conotruncal defects (CTDs), including double outlet right ventricle (DORV), are among the most common CHDs in the general population and usually require surgical repair to ensure a normal life expectancy. Little is known about the molecular and cellular mechanisms underlying the development of CTDs in humans. Normal murine cardiac development requires precisely regulated specification of the cardiac neural crest cells (cNCCs) and subsequent migration to the developing outflow tract. In animal models, impairment of NCCs causes CTDs [1].

The 11q terminal deletion disorder (11q-, Jacobsen syndrome) (OMIM # 1477910) is caused by heterozygous deletions in distal 11q (Fig. 52.1).

Fifty-six percent of patients have CHDs (Table 52.1). Septal defects, including DORV, account for about half of all CHDs that occur in 11q- patients.

As shown in Fig. 52.2, we have identified a Jacobsen syndrome cardiac "critical" region in distal 11q containing only five known genes, including the ETS-1 transcription factor.

The ETS-1 gene is a member of the ETS-domain transcription factor family. ETS factors have important roles in a host of biological functions, including the regulation of cellular growth and differentiation as well as organ development.

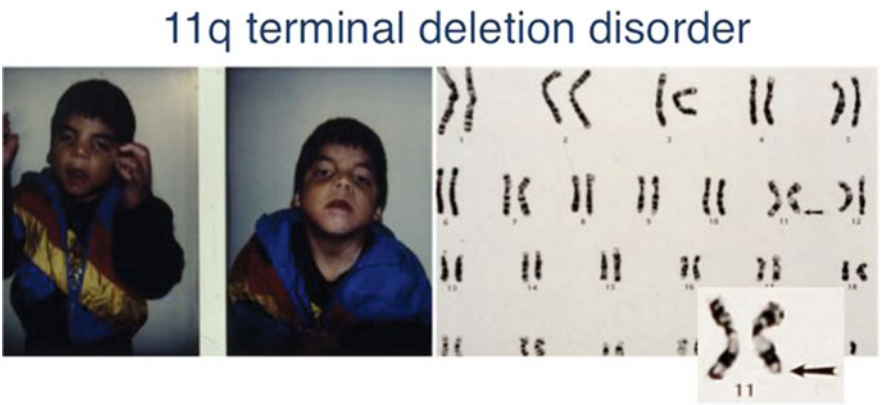


Fig. 52.1 Patient with Jacobsen syndrome. Karyotype demonstrates large terminal deletion in 11q

Table 52.1 CHDs in 11q-

Left-sided/flow lesions (two-thirds)
Hypoplastic left heart syndrome[a]
Shone's complex
Coarctation
Bicuspid aortic valve
Aortic valve stenosis
Mitral valve stenosis
Ventricular septal defect
Less common heart defects (one-third)
Secundum atrial septal defect
Aberrant right subclavian artery
Atrioventricular septal canal defect
D-transposition of the great arteries
Dextrocardia
Left-sided superior vena cava
Tricuspid atresia
Type B interruption of the aortic arch/truncus arteriosus
Pulmonary atresia/intact ventricular septum
TAPVR
Ebstein anomaly
Tetralogy of Fallot

[a]~10 % born with HLHS; ~1–2 % of all HLHS pts

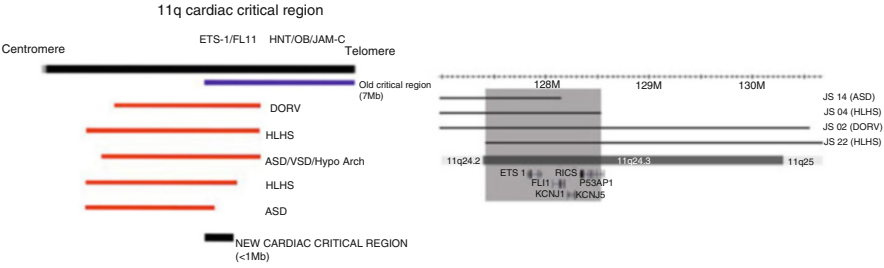


Fig. 52.2 Cardiac "critical" region in 11q, defined by region of overlap between smallest terminal deletion and interstitial deletions in patients with Jacobsen syndrome clinical phenotype, including congenital heart defects

Until recently, nothing was known about the function of ETS-1 in mammalian heart development or its possible role in causing human congenital heart disease.

Although little is known about the mechanisms underlying ETS-1 in mammalian heart development, recent studies in the ascidian *Ciona intestinalis* have demonstrated that ETS-1 regulates two critical aspects of heart development: heart progenitor cell migration and heart cell differentiation. Interestingly, loss of ETS-1 abolishes normal heart cell migration during development, resulting in an ectopically located heart chamber. Taken together, these results indicate that ETS-1

is required for normal cell migration in heart development, although the mechanism (s) underlying these cell migration defects remains to be elucidated.

52.2 Evidence for a Role for ETS-1 in the Cardiac Neural Crest in Mice

52.2.1 Expression of ETS-1 in Cardiac Lineages During Murine Heart Development

To begin to define the role of ETS-1 in mammalian cardiac development, we have performed in situ hybridization studies on mouse embryos and have shown that ETS-1 is strongly expressed in murine cNCCs as well as the endocardium during early heart development (Fig. 52.3).

52.2.2 ETS-1 Mutant Mice Have a Double Outlet Right Ventricle (DORV) Phenotype

To determine if loss of ETS-1 causes congenital heart defects, we have analyzed gene-targeted ETS-1 deletion mice. As shown in Fig. 52.4, ETS-1 homozygous null mice in a C57/B6 background exhibit DORV with 100 % penetrance, resulting in perinatal lethality [2].

52.2.3 Lost of ETS-1 Causes Decreased Expression of Sox10

We have previously demonstrated that ETS-1 expression is expressed in cNCCs and endocardium during murine embryonic development. Sox10 is a key regulator in the NCC gene regulatory network. It is critical for migration and specification of NCC fate. To examine the role of ETS-1 in murine cardiac NCC migration, we examined Sox10 expression in ETS-1−/− mutant and control C57/B6 embryos at E10.5 by using whole-mount in situ hybridization analysis. Expression of Sox10 in the NCCs in the pharyngeal arch region and dorsal root ganglia was reduced in ETS-1−/− mutant embryos, suggestive of a cNCC migration defect as shown in Fig. 52.5 (left). The result was confirmed independently by quantitative RT-PCR analysis (right).

52.3 Establishment of an Explanted cNCC "Ex Vivo" Culture System

We hypothesized that defects in cNCCs migration should be able to be reproduced in an "ex vivo" culture system. Toward that end, we have utilized an explanted culture system to observe cNCC migration [3]. Mouse embryos were collected from

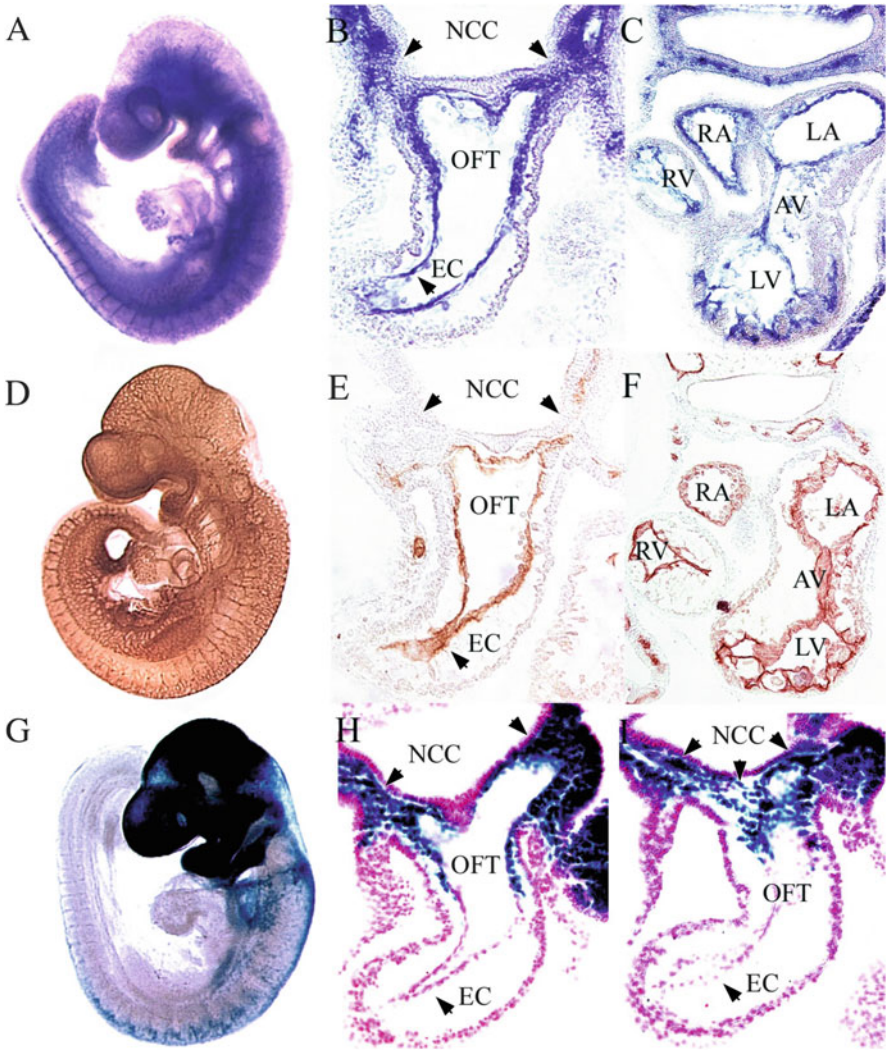


Fig. 52.3 Expression of ETS-1 in the heart in ED9.5 embryos: in situ hybridizations are shown in (**a**) (whole mount) and in sections (**b**) (anterior coronal section) and (**c**) (posterior coronal section). Immunohistochemistry indicating endothelial expression using a PECAM (CD31) antibody is shown in (**d**) (whole mount) and in sections (**e**) (anterior coronal) and (**f**) (posterior coronal). LacZ staining of neural crest using a Wnt1-Cre; ROSA26 LacZ indicator strain is shown in (**g**) (whole mount), (**h**) (anterior coronal), and (**i**) (posterior coronal)

C57/B6 background embryos at E8.5, coinciding with the onset of cNCC migration toward the heart. E8.5 embryos were collected and treated by dispase to dissociate the tissue gently. After treatment, neural tubes from somite one to three region (cardiac neural crest) were dissected out and cut into 100×300 um pieces. Each

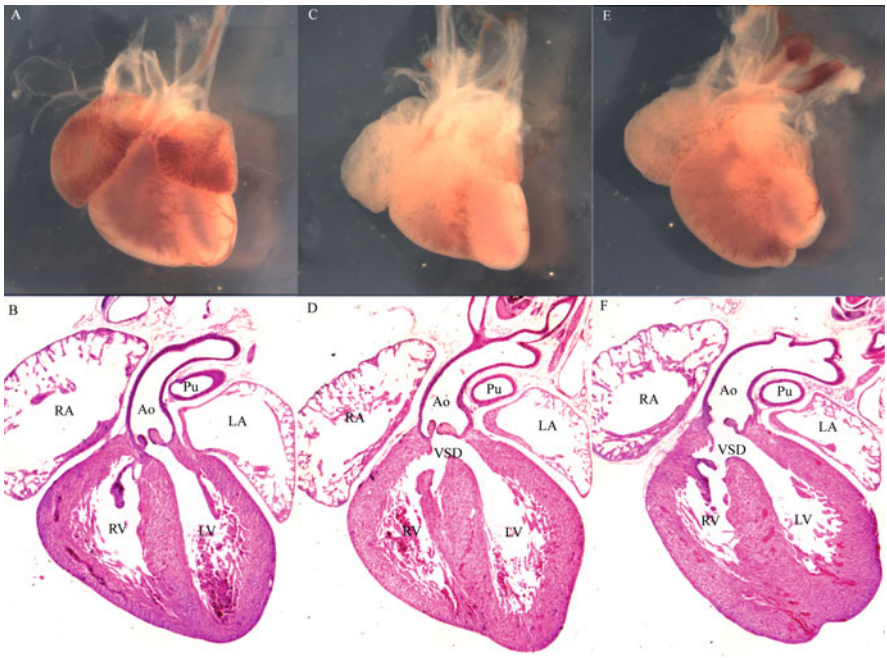


Fig. 52.4 Gene-targeted knockout of ETS-1 in C57/B6 E16.5 mice, showing double outlet right ventricle with normally related great arteries. Wild type is shown in (**a**) and (**b**); two mutant hearts are shown in panels (**c–f**). *RA* right atrium, *LA* left atrium, *RV* right ventricle, *LV* left ventricle, *Pu* pulmonary artery, *Ao* aorta, *VSD* ventricular septal defect

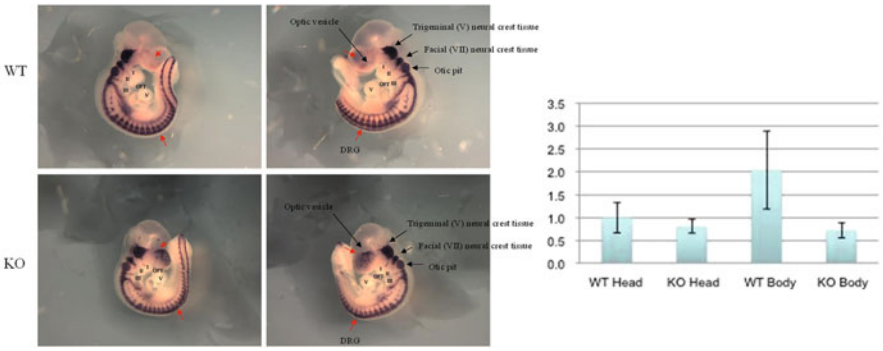
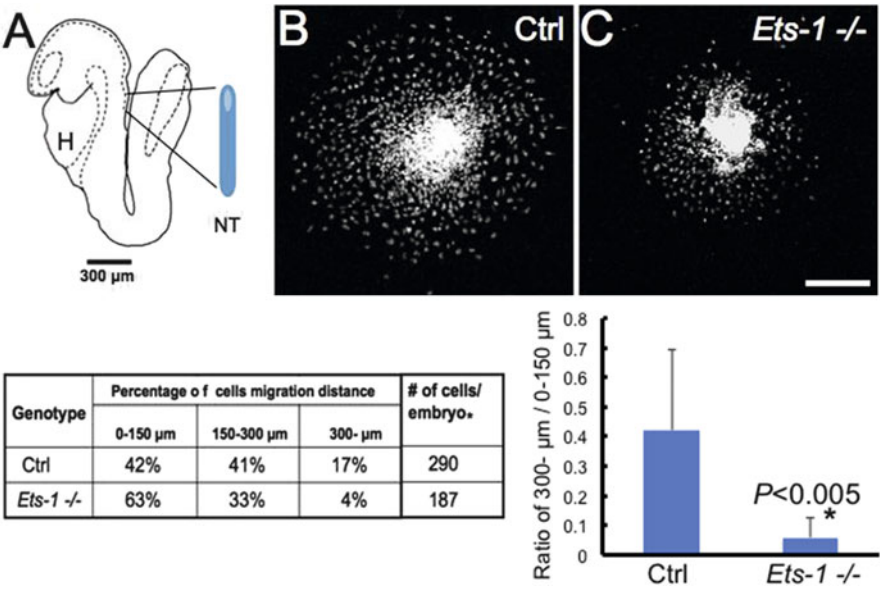


Fig. 52.5 Whole mount study demonstrating decreased Sox10 expression in E10.5 ETS-1−/− C57/B6 embryos (left, lower panel) compared to wild type (left, upper panel). Real-time quantitative PCR demonstrates decreased Sox10 expression in the body of E10.5 embryos in ETS-1−/− embryos, compared to wild type (right)

piece was placed on fibronectin-coated glass bottom slides and incubated in culture media in 5 % CO_2 and 21 % O_2. After 24 h incubation, we performed DAPI staining and obtained images of the migrating cells. Representative results from WT and ETS-$1^{-/-}$ mice are shown in Fig. 52.6.

52.3.1 Loss of ETS-1 in C57/B6 Mice Causes Decreased NCC Numbers and Decreased Migration

To analyze the migration distance using our ex vivo system, we counted the number of migrating cells in each explanted culture. The migration distance was divided into three distanced from the neural tube edge: 0–150 μm, 150–300 μm, and over 300 μm edge. The total number of cells that had migrated for each distance was manually counted, and the percentage of the total for each migration distance was determined. As shown in Fig. 52.6, cNCCs from ETS-1−/− C57/B6 embryos were fewer in number and had decreased migration distance. The percentage of total cells migrating >300 μm 24 h after explantation in ETS-1−/− mutant embryos is fourfold lower than control. The cell number per embryo was also significantly decreased in ETS-1−/− mutants.



Genotype	Percentage o f cells migration distance			# of cells/ embryo.
	0-150 μm	150-300 μm	300- μm	
Ctrl	42%	41%	17%	290
Ets-1 -/-	63%	33%	4%	187

Fig. 52.6 Cardiac neural crest cells migration in explant culture. (*A*) Schematic of E8.5 embryo. *Blue bar* shows the neural tubes somite one to three region. *H* heart, *NT* neural tube. (*B*) Representative images with DAPI staining of 24 h cultured cardiac neural crest cells in control (*B*) and ETS-1−/− mutants (*C*). *Scale bar*, 300 μm. Both images were taken by an inverted confocal microscope FV-1000 using a 10× objective lens. (*D*) Quantification of migration distances, demonstrating impaired migration ability in ETS-1−/− cells compared to wild type

We then calculated the ratio of 300+ μm over 0–150 μm of cultured migration cells. As shown in Fig. 52.6, the ratio was significantly reduced in ETS-1−/− mutants.

52.4 Cardiac Neural Crest Cell Number and Migration Are Preserved in ETS-1−/− Mice in an FVBN-1 Background

The cardiac phenotype in ETS-1 knockout mice is dependent on the genetic background. In contrast to C57/B6 mice, ETS-1−/− mutant mice in an FVBN-1 background have normal hearts. Consistent with a neural crest cell autonomous mechanism for causing DORV in the C57/B6 strain, ex vivo studies demonstrate normal cNCC numbers and migration in ETS-1−/− FVBN-1 embryos (data not shown).

52.5 Summary, Future Directions, and Clinical Implications

Using human and murine genetics systems, we have identified the ETS-1 transcription factor gene as the likely causative gene for CHDs in Jacobsen syndrome. Our expression data implicate an important role for ETS-1 in the cardiac neural crest during murine heart development. Based on the known function of the ETS-1 homologue in the ascidian *Ciona intestinalis* and using an ex vivo cell migration system, we hypothesize that ETS-1 is essential for early cNCC fate determination and migration in mammalian heart development. Future studies will include performing in vivo real-time imaging and lineage fate mapping studies in the neural crest to delineate how loss of ETS-1 causes decreased cNCCs in the developing heart [4], whether there is a NCC-autonomous mechanism and whether there is a migration defect. Importantly, loss of ETS-1 in FVBN-1 mice does not cause congenital heart defects, suggesting the presence of a genetic modifier(s) that can prevent the development of CHDs in the absence of ETS-1. To address this, determination of a neural crest cell autonomous mechanism would implicate a neural crest cell-specific modifier. Identification of such a genetic modifier could have important implications for the prevention of certain congenital heart defects.

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Notch Signaling in Aortic Valve Development and Disease

53

Vidu Garg

Abstract

Bicuspid aortic valve (BAV) is the most common type of cardiac malformation with an estimated prevalence of 1 % in the population. BAV results in significant morbidity usually during adulthood due to its association with aortic valve calcification and ascending aortic aneurysms. Mutations in the signaling and transcriptional regulator, *NOTCH1*, are a cause of bicuspid aortic valve in non-syndromic autosomal dominant human pedigrees. The Notch signaling pathway is critical for multiple cellular processes during both development and disease and is expressed in the developing and adult aortic valve consistent with the cardiac phenotypes identified in affected family members. Recent work has begun to elucidate the molecular mechanisms underlying the link between Notch1 signaling and the development of BAV and valve calcification. Using in vitro approaches, loss of Notch signaling has been shown to contribute to aortic valve calcification via Runx2-, Sox9-, and Bmp2-dependent mechanisms. In addition, Notch1 signaling has been shown to be responsive to nitric oxide signaling during this disease process. A new highly penetrant mouse model of aortic valve disease using *Notch1* haploinsufficient mice that are backcrossed in an endothelial nitric oxide synthase (*Nos3*)-*null* background was generated. *Notch1* and *Nos3* compound mutant mice ($Notch1^{+/-}$;$Nos3^{-/-}$) display a nearly 100 % incidence of aortic valve malformations, most commonly BAV. The aortic valves of adult mutant mice are thickened and have associated stenosis and regurgitation. Based upon the initial discovery of *NOTCH1* mutations in

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humans with aortic valve disease, subsequent studies have provided significant molecular insights into BAV-associated diseases.

Keywords
Congenital heart defect • Bicuspid aortic valve • Aortic valve calcification • Notch signaling

53.1 Introduction

Congenital heart disease (CHD) is the most common type of birth defect, with an estimated incidence that ranges from 6 to 19 per 1,000 live births [1]. Even with recent improvements in the care of children, CHD remains a leading cause of infant mortality [2]. The etiology for the majority of cases of CHD remains unknown despite advances in cardiac developmental biology and genetics [3]. While the role of nongenetic causes, such as infectious agents and teratogens, appears to play a causative role in a minority of cases CHD, the role of genetic factors in CHD has become an area of robust investigation. Numerous etiologic genes for CHD have been identified using conventional linkage or candidate gene sequencing approaches and more recently using array-based methodologies or whole exome/genome sequencing [4].

Within CHD, bicuspid aortic valve (BAV) is the most common congenital cardiac malformation with an estimated prevalence of 1 % in the population. BAV occurs when the aortic valve has only two cusps instead of the normal three [5]. BAV is a common cause of adult valve disease as it is often asymptomatic during childhood. With BAV, the normally thin aortic valve cusps often prematurely calcify leading to valvar thickening and stenosis [5]. BAV may also present with aortic regurgitation and affected individuals are at increased risk for infective endocarditis. BAV is also associated with ascending aortic dilation/aneurysm and may result in the development of aortic dissection [5].

Since early case reports described families with multiple members with BAV nearly four decades ago, several population-based studies have demonstrated a strong genetic component in BAV. This chapter will review how the identification of mutations in *NOTCH1* in families with inherited BAV have led to an increased understanding of the role of Notch signaling in aortic valve calcification and the generation of novel mouse model of BAV with associated valve disease.

53.2 *NOTCH1* Mutations and Aortic Valve Disease

We reported a novel genetic etiology of non-syndromic BAV in humans in 2005 [6]. Using a positional cloning approach, a large family with 11 members affected with autosomal dominant aortic valve disease was studied. The primary cardiac malformation in affected family members was BAV. Seven members had

developed calcification of the aortic valve including four who required surgical valve replacement. The disease locus was mapped to chromosome 9q34, and subsequent sequencing of a candidate gene, *NOTCH1*, identified a nonsense mutation in affected family members. In a smaller unrelated family, a *NOTCH1* frameshift mutation segregated with a similar aortic valve phenotype. Observations of missense *NOTCH1* mutations in a subset (~5 %) of individuals with BAV have also been reported with supporting functional data indicating impaired Notch signaling [7, 8]. These publications suggested that *NOTCH1* haploinsufficiency was a cause of BAV in humans.

NOTCH1 encodes a single-pass transmembrane receptor and functions in a highly conserved pathway, which plays critical roles in cell fate determination during organogenesis. In mammals, there are four NOTCH receptors (NOTCH1-4), and they interact with two families of ligands (Jagged 1 and 2 and Delta 1, 3, and 4) [9]. Other Notch family members have been linked to human disease as heterozygous mutations in *NOTCH3* have been identified in CADASIL syndrome, while mutations in *JAGGED1* and *NOTCH2* are found in Alagille syndrome. Targeted disruption of *Notch1* in mice results in embryonic lethality secondary to vascular defects prior to cardiac valvulogenesis [10]. Each Notch family member has a distinct expression pattern, and *Notch1* is expressed not only in the endocardium but also the outflow tract cushion mesenchyme during development consistent with the valve phenotype seen in the affected family members [6, 11]. In addition, Notch1 mRNA transcripts are found in the adult murine aortic valve. These findings suggest that Notch1 signaling is important for aortic valve formation and potentially in adult valve diseases.

53.3 Notch1 Signaling and Aortic Valve Calcification

With the increased longevity of human population, calcific valvular disease is becoming more prevalent. Calcific aortic stenosis affects an estimated 2–3 % of the population by 65 years of age [12]. Calcification of the normally thin aortic valve cusps leads to valvular thickening with resultant stenosis/regurgitation that ultimately requires surgical replacement. Examination of calcified human valves has demonstrated increased expression of osteogenic markers such as Runx2 [12]. The process of valvular calcification was traditionally proposed to be a degenerative process that occurred with aging, but increasing evidence suggests that molecular pathways underlie this complex disease [12]. In addition to clinical risk factors such as hypertension and hypercholesterolemia, BAV is a major risk factor for CAVD.

The role of the Notch signaling pathway in the development of CAVD has becoming increasingly recognized. Our initial studies demonstrated that Notch1 repressed the activity of Runx2, a transcriptional regulator of osteoblast cell fate [6]. Subsequently our studies focused on the molecular changes that occur with inhibition of Notch signaling in the aortic valve [11]. Consistent with this hypothesis, diseased human aortic valves have decreased expression of NOTCH1 in areas

of calcium deposition. To identify downstream mediators of Notch1 during valve calcification, the gene expression changes that occur with chemical inhibition of Notch signaling in rat aortic valve interstitial cells (AVICs) were studied. Downregulation of Sox9 along with several cartilage-specific genes that were direct targets of this transcription factor was identified. Loss of Sox9 has been published to be associated with aortic valve calcification in mouse models [13]. Utilizing an in vitro porcine aortic valve calcification model system, inhibition of Notch activity resulted in accelerated calcification, while stimulation of Notch signaling attenuated the calcific process. Overexpression of Sox9 was able to prevent the calcification of porcine AVICs that occurs with Notch inhibition. These studies demonstrated that loss of Notch signaling contributes to aortic valve calcification via a Sox9-dependent mechanism. Additional work by other investigators has supported these conclusions and have also demonstrated a role for Bmp2 as a downstream target of Notch1 signaling in this process and found that *Notch1* haploinsufficient mice develop aortic valve calcification with aging [14, 15].

Dysfunction of the valvular endothelium is thought to initiate calcification of neighboring AVICs leading to CAVD. The molecular mechanism by which endothelial cells communicate with AVICs and cause disease is not well understood. Using a coculture and transwell assays, it was shown that a secreted signal from endothelial cells inhibits calcification of porcine AVICs [16]. Nitric oxide (NO), which is secreted by endothelial cells, is critical for numerous physiologic and pathologic processes and had been implicated in the process of aortic valve calcification. In addition, mice lacking *Nos3*, which encodes for endothelial nitric oxide synthase, display partially penetrant BAV and making NO a potential candidate for this secreted signal. NO prevents calcification of AVICs in vitro, similar to the presence of endothelial cells, while the absence of NO increases calcification. Overexpression of a constitutively active Notch1 in AVICs prevented calcification that occurs with NO inhibition linking NO and Notch signaling in this process. Consistent with this, endothelial-derived NO signaling increases the expression of a Notch signaling target genes in AVICs and inhibition of NO decreased nuclear localization of NICD in AVICs. Conversely, increased nuclear localization of NICD was noted with the addition of NO donor. Lastly, the NOS3 and Notch1 signaling pathways genetically interact in vivo as *NOS3*;*Notch1* compound mutant mice display a highly penetrant aortic valve disease [16]. These mice have highly penetrant BAV and develop hemodynamically significant aortic valve stenosis and regurgitation. These studies suggest that NO signaling in valve endothelial cells regulates Notch1, Sox9, and Bmp2 in the neighboring AVICs and this pathway may be critical in the pathogenesis of adult-onset aortic valve calcification (Fig. 53.1).

53.4 Future Directions and Clinical Implications

While mutations in *NOTCH1* were identified in a family with a common cardiac valve malformation, subsequent work has demonstrated a role for Notch1 in aortic valve calcification. The development of a highly penetrant mouse model of BAV

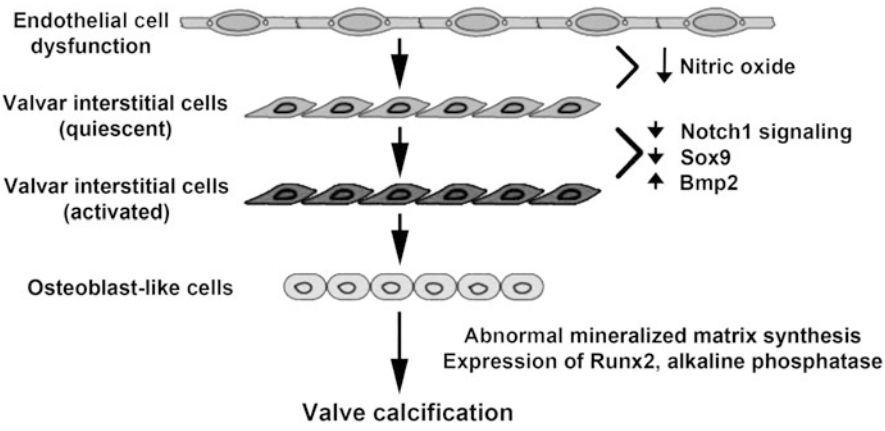

Fig. 53.1 Role of Notch1 signaling in aortic valve calcification. Endothelial cell dysfunction results in decreased nitric oxide production which decreases Notch1 signaling in aortic valve interstitial cells and leads to calcification by Sox9-, Bmp2-, and Runx2-dependent mechanisms

will assist in the dissection of the molecular pathways that lead to the development of this common cardiac malformation. Interestingly, mice deficient for *Gata5* display partially penetrant BAV and have reduced expression of Nos3 and Notch signaling [17]. These mice also offer an opportunity to study the development of BAV-associated ascending aortic aneurysms. Future investigations into the downstream targets of Notch1 signaling may lead to novel therapies for BAV-associated diseases such as CAVD.

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To Detect and Explore Mechanism of CITED2 Mutation and Methylation in Children with Congenital Heart Disease

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Abstract

In this study we found four CITED2 coding region mutations (c.550G>A, c.574A>G, c.573–578del6) which led to alterations of amino acid sequence (p.Gly184Ser, p.Ser192Gly, p.Ser192fs) in 120 children with congenital heart disease. The CITED2 mutation associated with the dysregulation of HIF-1α, TFAP2c, and CITED2 methylation accompanied with its decrease in mRNA expression might be involved in the pathological process of congenital heart disease.

Keywords

CITED2 • Mutation • Methylation • Congenital heart disease

CITED2 mutation and methylation may be the cause of CHD. The purpose of this study was (1) to identify CITED2 mutation in children with CHD in China, (2) to analyze the mechanism of CITED2 mutation in cellular level if CITED2 gene mutation affects expression of HIF-1α and TFAP2c, and (3) to examine if CITED2 CpG island methylation exists in children with congenital heart disease.

1. Four CITED2 coding region mutations (c.550G>A one case, c.574A>G one case, c.573–578del6 two cases) exist in 120 children with congenital heart disease (Fig. 54.1) [1].
2. CITED2 mutation can inhibit TFAP2c expression. Our study also demonstrated that CITED2 has negative inhibition for HIF-1α. But this negative mechanism

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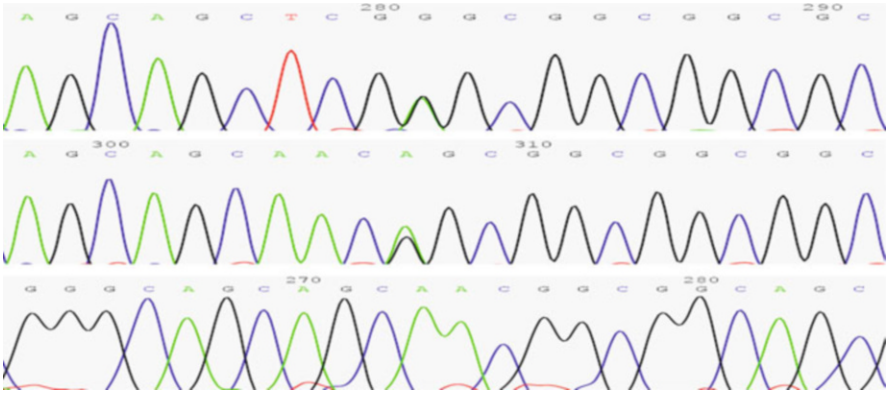

Fig. 54.1 The sequence of CITED2 mutation

will be weakened owing to CITED2 mutation in congenital heart disease; HIF-1α expression was elevated in CITED2 mutant group.

3. The CITED2 methylation is another mechanism of promoting congenital heart disease. CITED2 abnormal methylation was found in 26 of 31 congenital heart diseases. The abnormal methylation leads to decreased CITED2 mRNA expression [2].

CITED2 mutation and methylation may play an important role for the development of congenital heart disease.

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