# Cardiogenetics and Congenital Cardiopathies

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*"To love and be loved is to feel
the sun from both sides".
-- DAVID VISCOTT, M.D.*

**INTRODUCTION**

Hello dear colleagues. I was designated to tell you about the future of Cardiology and how Genetics is a milestone for all branches of medicine. In fact, to talk about pediatric cardiology is to talk basically about congenital heart diseases (CHD), and to talk about CHDs now is to talk about Molecular Cardiology, Embryology, Development and Genes.

In short … cardiogenetics.

Let me introduce you to the fascinating world of genes, their function and how they can produce cardiovascular abnormalities.

Cardiogenetics is a branch of molecular cardiology in which molecular and cellular biology is applied to search for disarray of cellular function during development, for the genes involved and the mutations and polymorphisms inside these genes that can explain this disarray.

Based on these findings will establish a comparison of the patient's phenotypical and genotypical characteristics and try to find a connection between them.

Congenital anomalies are now the leading cause of death during the first five years of life. Until 14 years of age they are among the three leading causes. Until 24 years of age they remain among the 10 leading causes (1). Specifically, the prevalence of congenital heart disease has been remarkably constant throughout the world and over time (2). There is no reason to suspect any change to occur in the near future. Not only is the incidence of congenital heart disease relatively predictable, but the relative frequency of the various congenital cardiac defects varies little (3).

Worldwide, heart disease in children continues to be a major public health problem. In some European countries and in Canada, USA, Australia, New Zealand and Japan, congenital heart disease accounts for almost all heart disease in children (4). An estimated of 1 million infants are born each year with heart defects worldwide (5,6) . In the USA, congenital heart defects are recognized in approximately 0.5% to 1.25% of live births, which means 40,000 new cases each year (5-7).

However, chromosomal aberrations and mutations in single genes known to be related to congenital heart diseases amount to approximately 6 per cent (8).. Because of the lack of a basic mechanistic understanding, the pathogenesis of most congenital malformations, as well as most diagnostic and therapeutic approaches, is based on empirical observation rather than on the precise cellular and molecular dysfunction underlying the disease. This means that there is still a vast ground to study in this field (9).

We need to pinpoint the exact role of each gene involved in the development of the cardiovascular system in order to establish its specific function, and find the mutations that produce dysfunction in these genes. Continued research

on the genetic cause of congenital heart disease (CHD) promises to augment our understanding of the mechanisms underlying the normal and abnormal development of the cardiac structures (10). Cardiogenetics also can confirm the diagnosis of CHD, establish a presymptomatic diagnosis, predict the severity of the condition, determine the risk status of the patients' relatives and establish the basis to augment our ability to counsel families on the recurrence risk with greater accuracy. In the future, it will allow the physician to modify his or her clinical management based on the genetic cause (10,11).

Identifying the cause and understanding the disease mechanism allows for early intervention that may modify the degree of cardiac maldevelopment or prevent cardiac malformation altogether (10).

> "Nothing is so embarrasing as watching someone do something that you say couldn't be done".
> -- SAM EWING

## BACKGROUND

The basic unit of life is the cell. Cells are microfactories in which raw materials (amino acids, simple carbohydrates, lipids, and trace elements) are received, new substances (proteins, complex lipids, carbohydrates, and nucleic acids) are produced, and wastes are removed. Each cell has the ability to self-replicate using the deoxyribonucleic acid (DNA) code as the blueprint, raw materials as building blocks, and enzymes as catalysts.

Within eukaryotic cells most of the DNA is nuclear; only a minor extranuclear quantity is found in the mitochondria and plant plastids.

All of the genetic information required to produce a human is stored in the nucleus of each cell in the form of deoxyribonucleic acid (DNA). In eukaryotes, the nuclear DNA is divided into chromosomes. The human karyotype consists of 22 matched pairs of autosomes and two sex chromosomes; the female is designated XX and the male XY (Fig.1). One from each pair, plus a sex chromosome, is derived from each parent at the time of conception. The chromosomes in all body (somatic) cells are in the diploid state, with two sets of chromosomes per cell; gametes (egg or sperm) have only the haploid, or single, set of chromosomes (12).
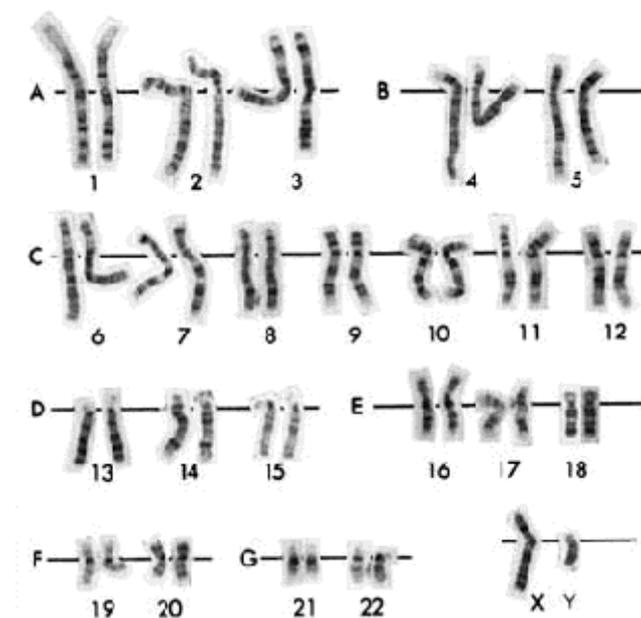


Fig. 1: Set of Chromosomes the human being possess.

Pairs of chromosomes are termed *homologous* because they have a common evolutionary origin (a common ancestral chromosome). A copy of each gene resides at the same position (locus) on each chromosome of the homologous pair. Chromosomes have two parts using the centromere as a point of division. The short arm is p and

the long arm is q. Each part can be divided into different regions that are further divided into smaller bands. It is in these mapped regions where genes are located, having DNA as their substance (13,14).

According to the Watson-Crick model, the DNA chain is a long, unbranched polymer composed of a double helix with the complementary strands linked by hydrogen bonds. These bonds are readily broken by heating for a few minutes at 95°C to 100°C and they reform at about 65°C. The basic unit of the DNA molecule is the deoxyribonucleotide containing the bases adenine (A), cytosine (C), guanine (G) and thymine (T). The nucleotides are linked together by covalent phosphodiester bonds that join the 5´ carbon of one deoxyribose group to the 3´ carbon of the next. The four subunits are attached to this repetitive sugar-phosphate chain following the **complementary base pairs** (also called Watson-Crick model) in which nucleotide A will pair only with nucleotide T and nucleotide C only with G (Fig. 2).
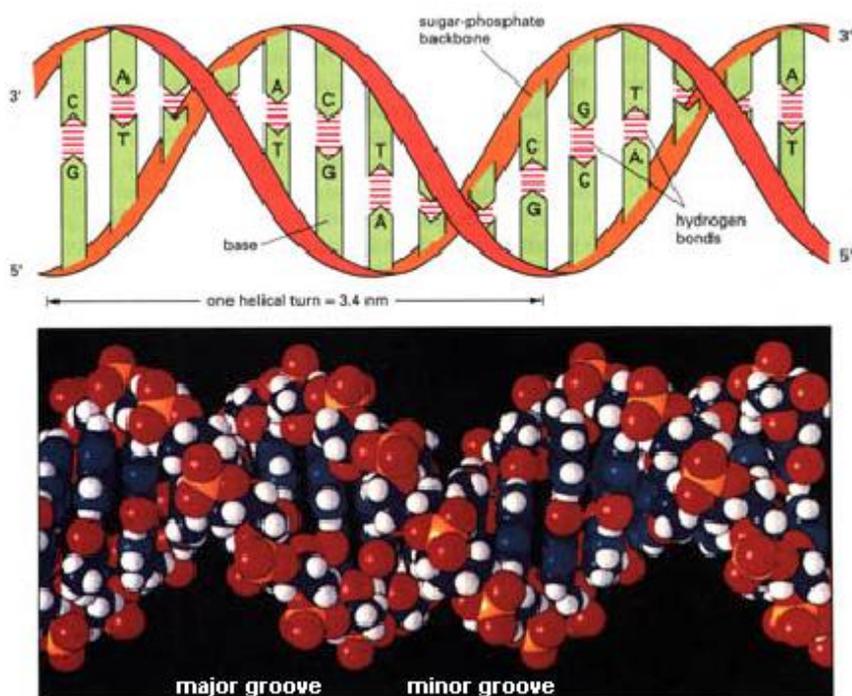


Fig. 2: DNA double helix. A schematic representation (top) and a space-filling model (bottom) are illustrated here.

A gene carries biological information in a form that must be precisely copied and transmitted from each cell to all of its progeny. The "replication machine" begins with the separation of the two strands of the DNA double helix. Each strand then acts as a template for the formation of a new DNA molecule by the sequential addition of deoxyribonucleoside triphosphate. The genetic information is duplicated completely, so that two complete DNA double helices are formed, each identical in nucleotide sequence to the parental DNA helix that served as the template.

Genes must be copied exactly from generation to generation because their main function is to synthesize proteins; proteins that will be helpful to cells and the development of tissues and organs to work well. For example, in moderate to severe cases of hypertension oxygen radicals are involved in the development of tissue injury. Genes immediately produce antioxidant proteins such as glutathione to avoid oxidative stress (15). The elevated levels of such antioxidant activity will be normalized by other proteins triggered by $Ca^{2+}$ antagonists (16).

The transfer of information from DNA to protein proceeds by means of an intermediate, called messenger RNA (mRNA). After a process of *transcription* a large RNA is transcribed (coding sequences called exons plus noncoding sequences called introns); but, before this RNA leaves the nucleus, a complex of RNA-processing enzymes removes all of the intron sequences, thereby producing a much shorter RNA molecule. This step is called **RNA splicing**. The RNA then moves to the cytoplasm and directs the synthesis of a particular protein (13).

The sequence of nucleotides in the mRNA molecule that acts as an intermediate was found to be read in serial order in groups of three. Each triplet of nucleotides, called a **codon**, specifies one amino acid. Since RNA is a linear polymer of four different nucleotides, there are $4^3 = 64$ possible codon triplets. However, the so-called **genetic code** is problematic; only 20 different amino acids are commonly found in proteins, so that most amino acids are specified by several codons.

However, sometimes this perfect procedure fails. The replication machinery skips or adds a few nucleotides, or puts a T where it should have put a C, or an A instead of a G. Any change of this kind in the DNA sequence constitutes a genetic mistake, called a **mutation**, which will be copied in all future cell generations since "wrong" DNA sequences are copied as faithfully as correct ones. The consequence of such an error can be enormous, as even a single nucleotide change can have important effects on the cell, depending on the location of the mutation (17).This is how congenital cardiopathies are produced.

The real work begins when we need to elucidate which gene or genes are involved in the CHDs and how many mutations are present. But the work is not only to find the gene, but also to elucidate the mechanism of how the gene produces the disease.

Genes probably start to function after cleavage during the first week of development, but the cardiovascular system originates from the mesoderm that begins to form from the third week of development, so the key time for cardiovascular research is late during the second week until the end of the third week of development (18). But even during this time there are some special points in time that are helpful more than others because at these points most of the genes begin to work. One is when gastrulation just begins, usually between days 14 and 15 (Fig. 3) at which time epiblasts coming from the ectoderm go into the primitive streak and replace the hypoblast to form definitive endoderm. Another important period is from day 16, when epiblasts keep entering across the primitive streak, but by this time the endoderm is already formed. Therefore, these cells come between the ectoderm and endoderm forming the mesoderm layer. There are several paths of migration, and all come from the primitive streak and primitive node (19).
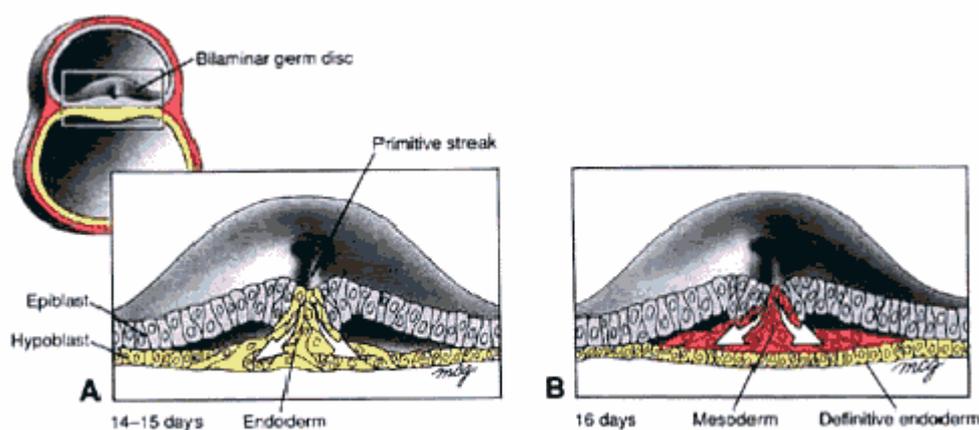


Fig. 3: Germ discs sectioned through the region of the primitive streak, showing gastrulation. (A) On days 14 and 15, the ingressing epiblast cells replace the hypoblast to form the definitive endoderm. (B) The epiblast that ingresses on day 16 migrates between the endoderm and epiblast layers to form the intraembryonic mesoderm.

Other important period for gene development is during day 17, when formation of the notochordal process and prechordal plate mesoderm takes place.

The notochord is the common factor of all species of the phylum Chordata. It is the center of development of the axial skeleton. This is also another important period: the process by which the hollow notochordal process is transformed into a solid notochord, usually between days 17 to 22 (Fig. 4).

Fig. 4: The process by which the hollow notochordal process is transformed into a solid notochord between days 16 and 22. (A,B) First, the ventral wall of the notochordal process has begun to fuse with the endoderm to form the notochordal plate. As shown in Fig. B, this process commences at the caudal end of the notochordal process and proceeds cranially (the dotted line marks the level of section. A) An open neurenteric canal is briefly created between the amniotic cavity and the yolk sac cavity. C) The stages by which the notochordal process becomes the notochordal plate and then the notochord.

Once we detect how a gene functions, we need to understand the mechanisms of the genes around it, because genes never work alone; there are interactions between them (Fig. 5)(20).
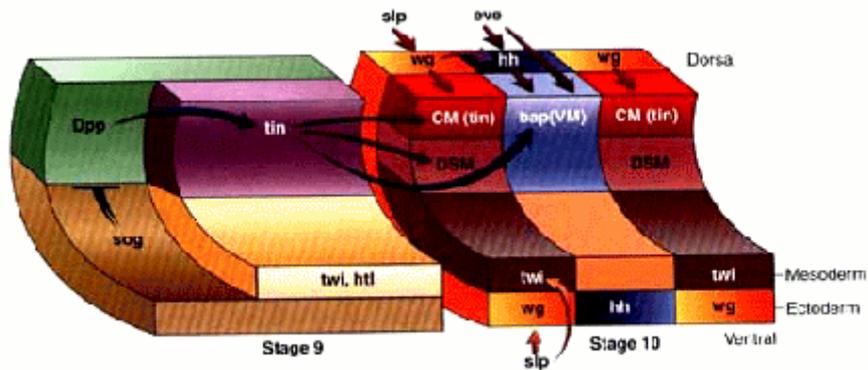


Fig. 5: Summary of genetic interactionsduring early mesoderm patterning.

"My father always told me, 'Find a job you love and you'll never have to work a day in your life'."
-- JIM FOX

## MATERIALS

Although every cell in the body has a complete genome in its nucleus, medical researchers prefer to extract blood cells from patients or control subjects because we can isolate lymphocytes from these cells. These mononuclear, nonphagocytic leukocytes are used to create cell lines using the Ebstein-Barr virus. Lymphocytes reproduce by mitosis in the way cancer cells do, increasing the number of cells and therefore, the amount of DNA. Usually this procedure is performed using special techniques and an uncontaminated environment (Fig. 6).

Fig. 6: Equipment usually used for culture of cell lines, DNA recombination, targeting vectors, gene cloning, etc.

However, we usually need to elucidate the exact mechanisms by which the genes work, and be sure of their real function.

One attempt is to start with an interesting phenotype, then proceed to identify the gene involved (like identifying a face without knowing the name). Recently, genome researchers have "invented" a new territory, called functional genomics, in an effort to understand the roles of the genes, in which the genome is sequenced without regard to function (first name, then face). One can simply infer function from a homologue (often safe, but by no means certain), or one can mutate known genes individually (21).

That is when animal models begin to play important roles in cardiogenetics.

One of the models is an insect. Its name: *Drosophila melanogaster*, a fruit fly (Fig. 7).


Fig. 7: Drosophila melanogaster, one of the most used animal models in genetics

The invertebrate *Drosophila melanogaster* is well known for its tractability in genetic studies. In general, insect models offer many advantages, such as ease of maintenance, availability of pure genetic lines and well-defined mutants, short life span, and the existence of tremendous biological variability. In particular, *Drosophila* offers the advantages of a substantial literature detailing its genetic, biochemical, physiological and behavioral characteristics. However, as an invertebrate, *Drosophila* lacks entire genetic programs unique to vertebrates (22).

Another model is an African frog: *Xenopus laevis*, the amphibian most widely used today.

The advantages of *Xenopus* as an experimental animal include its robustness and ease of husbandry, the fact that it is a vertebrate, the accessibility of embryonic material from the earliest stage onward, the ready availability of oocytes and eggs in large quantities. The comparatively large size of the egg and embryo facilitates physical manipulation from fertilization onward, and many of the early patterning processes have proven generalizable to all vetebrates (23). These advantages allow for many embryological experiments that are difficult or not possible to perform in other model organisms (Fig. 8), and in spite of the limitation of the almost total inapplicability of classical genetics, have stimulated much research on Xenopus over the past three decades. Specially in two aspects: I. The role of localized cytoplasmic information and of inductive interactions in the establishment of the polarity and initial tissue differentiation in the embryo, and on the nature and molecular basis of embryonic induction; II. The study of genes for RNA components of the rybosome and the control of their expression (24).
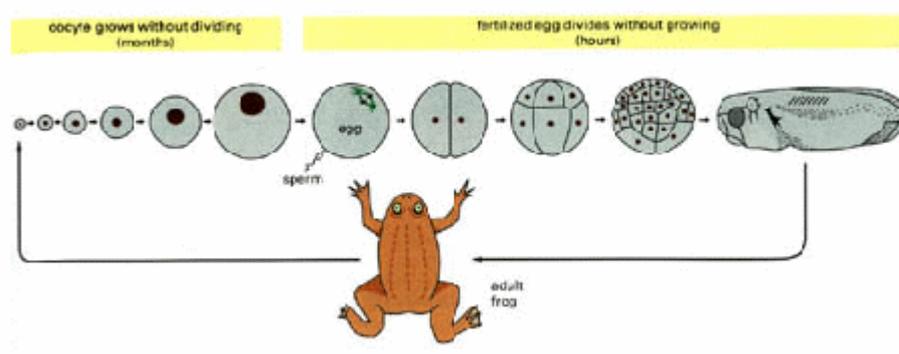


Fig. 8: Xenopus laevis. One of the best models for cardiogenetics, because its ready availability of oocytes and eggs in large quantities, the large size of the egg and embryo and the fact is a vertebrate.

In fact, the expression of transgenes is not a private property of mice anymore since it is possible now to establish transgenic lines of Xenopus laevis (25).

Another interesting model is the tropical freshwater fish, the cyprinid teleost Danio renio, the zebrafish, that has been considered as a candidate organism for wide-ranging genetic studies (Fig. 9). There are many advantages for using zebrafish: they are small and inexpensive to house; their natural method of mating is entirely extracorporeal and hence easily controlled in the laboratory setting; embryos are optically transparent throughout development, and pigmentation can be suppressed for longer periods of time using simple treatments; and development is rapid with most processes corresponding to mammalian embryology completed within 48 to 72 hours of fertilization. Several attributes of zebrafish make them particularly useful for genetic studies. The genetic advantages include the following: (1) high female fecundity generates many meioses rapidly (a female can lay 100 eggs twice per week at her reproductive peak, all of which are potentially viable); (2) methods exist for generating haploid embryos that are viable for up to 5 days postfertilization; and (3) adult-viable gynogenetic diploid embryos can be generated. Advantages for assaying cardiovascular function in the embryo: their embryos are transparent and can be remonitored without being removed from their natural environment. Heart rate, oxygen consumption, and blood pressure all have been assayed in developing wild-type embryos. In the zebrafish, the cardiovasculature is functional at 24 hrs. postfertilization , but it is not essential for survival of the early embryo, which obtains adequate oxygen by simple diffusion. Thus zebrafish mutants that completely lack a circulation still develop relatively normally for a day or

two. This distinguishes fish embryos from mammalian embryos, whose survival depends on an intact circulation, so that mutations with cardiovascular effects are inevitably complicated by secondary degeneration or death (22,26).



Fig. 9: The zebrafish model Danio renio, a candidate organism for wide-ranging genetic studies. A female can lay 100 eggs twice per week at her reproductive peak, all of which are potentially viable.

In the future, the mutants affecting cardiac development will especially stand out, and should provide useful models for human congenital heart diseases. The zebrafish screens cannot identify all the functionally interesting mutants, but this is neither surprising nor disappointing. Even in *Drosophila*, new interesting developmental mutants are still being discovered. There is, in any event, more than enough that comes out of the fish screens to drive good research for many years (21,27). Lately, researchers are succeeding in creating loss-of-function genes in zebrafish models using morpholino-modified oligonucleotides (28).

In Japan, an alternative fish species used as a model for vertebrate developmental genetics is the Oryzias latipes, the Japanese medaka (29). In the medaka, more than 80 spontaneous visible mutants including about 30 morphological mutants have become available for experimental work and are currently mantained at Nagoya University (30).

For at least 50 years, the mouse has been the classic animal model for experimental cardiologists. It has the advantage of being a mammal like humans and the murine genome also conserves mammalian molecular idiosyncrasies. This close genetic relationship results in many proteins retaining cross-species activity, making them useful in biochemical and biological experiments (Fig. 10). The mouse has the longest history in genetic studies, and is fairly well understood at the anatomical and physiological levels. So there would be a great advantage in having a large collection of mouse mutants (29). The long period of murine experimental cell biology has resulted in the variability of many validated reagents for studying the phenotype of mutant mice. Since the syntenic relationships between the two mammalian genomes are well characterized, progress in sequencing and understanding the structure of the human genome can facilitate gene mapping projects in mutant mice. Furthermore, the sequencing of the murine genome itself is not far from completion (22).

Fig. 10: The classic animal model for experimental cardiologists: the mouse.

One approach to the investigation of function might be to selectively eliminate genes in mice, one by one. However, such grand-scale targeted mutagenesis programs would be inordinately expensive. In addition, mouse embryos with severe cardiac malfunction die very early, making functional analysis difficult, if not impossible (26).

Finally, there are more animal models like avians, primates, but cardiovascular research is one of the most expensive as the screening programs are very costly. Therefore, mammals are not suitable for such mutagenetic programs. Again, that is why cardiovascular research programs are using zebrafish, medaka, or Xenopus frogs and some invertebrates.

"The truth of the matter is that
you always know the right thing
to do. The hard part is doing it."
--GEN. H. NORMAN SCHWARZKOPF

**METHODS**

Cardiogenetics tries to describe cardiovascular, physiological and pathological processes at the cellular, molecular and genetic levels, but still have its limitations and remains an elusive goal for most cardiovascular processes (15). Genes are not separate fragments of DNA inside chromosomes, they are joined as a long piece of rope in the loci. We need to elucidate the limits of the selected gene, separating exons from introns, and identifying the promoter and the polyadenylation signal.

Usually for mutagenesis programs cardiogenetic researchers use two ways: one is coming from pure lymphocytes where we can extract DNA directly for the polymerase chain reaction (PCR). The second one is coming from Ebstein Barr virus lymphocytes, which are reproduced as cancer cells, and the main advantage is we can use as much DNA as we want. However, the second method is not so accurate as the first, since viruses sometimes produce mutations on their own and change the real sequence of the gene (Fig. 11).

Fig. 11: Methodology for mutagenetic screening program.

The polymerase chain reaction will be used to copy DNA fragments, and reverse transcriptase PCR (RT-PCR) for the production of more mRNA fragments. Southern and Northern blot analyses will be used for hybridization. To search for polymorphisms we will use single-strand conformation polymorphism analysis (SSCP) and restriction length fragment polymorphism (RLFP) analysis using restriction enzymes (Fig. 12). The mutations found will be confirmed by direct sequencing using radioisotopes.



Fig. 12: RLFP of the ACVR2B gene

We also must follow other family members, ancestors, trying to get the whole family history of the patient. We call this study a **pedigree** of a patient, key point in the analysis of inheritance (Fig. 13).
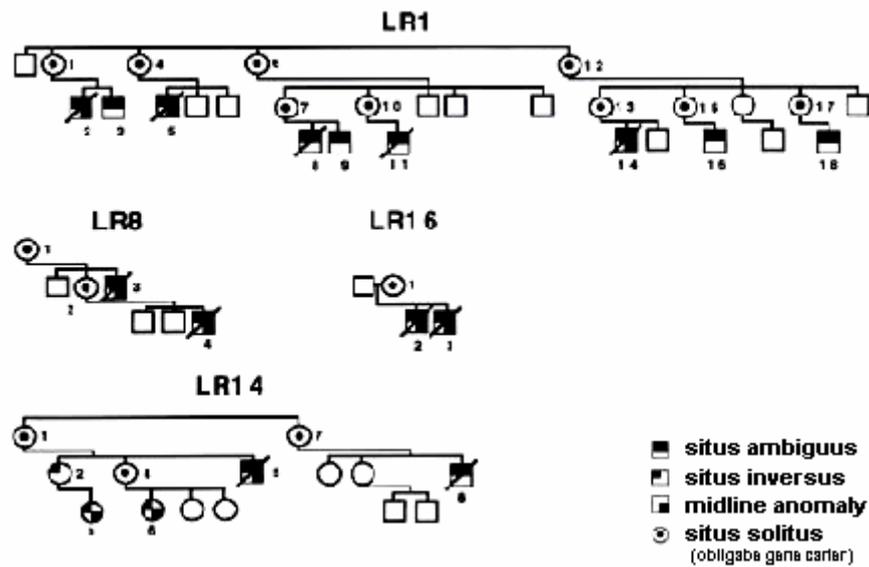
Fig. 13: Example of the pedigree of a familial case of heterotaxy syndrome

Once we get some mutations on a desired sequence of the gene, we need to determine which chromosome that gene is located on. For this it is necessary to prepare short sequences of single-stranded DNA of the gene, called probes, which are complementary to a particular region of a chromosome. The **locus-specific probes** hybridize to one chromosome that will be the chromosome for our gene. This technique is called fluorescence in situ hybridization (FISH) and is very useful in cardiogenetics (31) (Fig. 14).
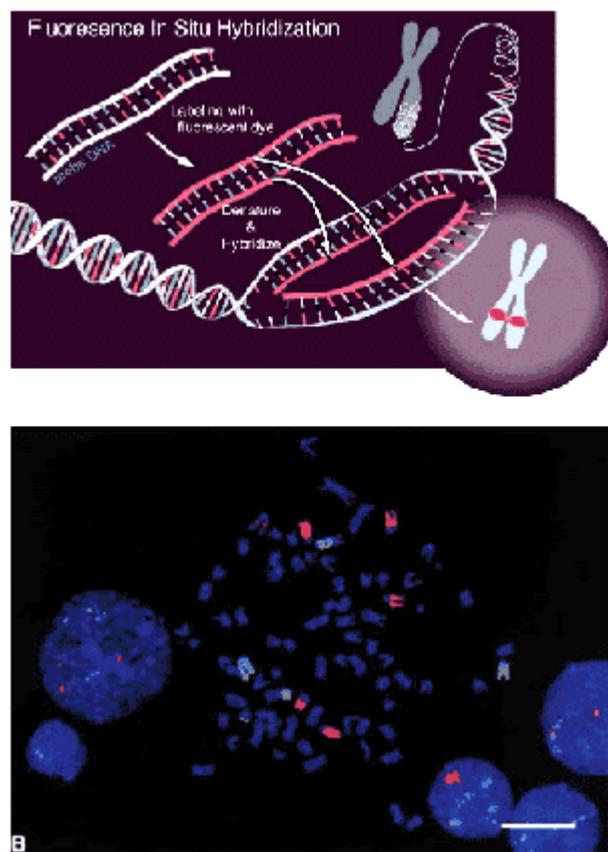


Fig. 14: Fluorescence In Situ Hybridization method and example of how to visualize the image (below).

To characterize the function of a gene, a genomic vector with the desired null mutant gene fragment will be obtained from a genomic library and introduced into ES cells. Only ES cells with recombinant DNA sequences will be injected into an early mouse embryo to produce heterozygous male and female animals. Once we mate these mice, one-fourth of the progeny will be homozygous for the altered gene. We will study these homozygotes to see the function of the altered gene. This work is slow, and in mouse, sometimes frustratingly unrewarding for the work involved.

Also, applying the $F_2$ screen for *Drosophila* and zebrafish . The $F_2$ screen was developed by Nusslein-Volhard and Wieschaus to identify new genes in Drosophila, and it has now been used in zebrafish (29). The screen was designed to reveal mutations that have a zygotic effect in a range of developmental events.

The use of transgenic mice, frog or zebrafish animal models have brought a revolutionary approach to cardiogenetics. Functional Genomics is an event that has saved many years of research. Thanks to this, we can understand the interactions, functions and mechanisms of many genes that play important roles in the development of cardiovascular system.

<div align="right">
"Experience is the name every-<br>
one gives his mistakes."<br>
--ELBERT HUBBAR
</div>

## RESULTS

Congenital heart disease (CHD) is a cardinal feature of multiple genetic syndromes associated with known chromosomal alterations. Specific chromosomal alterations are associated with specific types of CHD, implying that the genetic alteration has a specific rather than a global affect on cardiac development. The best known example is Down syndrome (trisomy 21) where approximately 40% of patients have CHD, typically, atrial septal defect (ASD), ventricular septal defect (VSD), patent ductus arteriosus (PDA) and common atrio-ventricular canal defects (CAVC). There are other syndromes, such as Edward's syndrome (trisomy 18) and Patau's syndrome (trisomy 13), which both have VSD, ASD and PDA (11,32).

However our interest is focused on genetic syndromes, i.e., on a CHD for which a consistent chromosomal or genetic anomaly may not yet be recognized. There are already many loci recognized to be related or to origin CHDs. However, they are only the tip of the iceberg since there are now numerous syndromes reported in CHD, as follows: **Holt-Oram Syndrome**, which is a highly penetrant, autosomal-dominant disorder with ASD and VSD (33). Recent studies suggest that there is a genotype-phenotype correlation between the clinical manifestations of Holt-Oram syndrome and TBX5 mutations (34,35). Specific mutations within TBX5 are associated with either more significant cardiac or skeletal anomalies, whereas null alleles result in severe abnormalities in both the heart and limbs (36).

**22q11.2 deletion or CATCH 22**, is a common genetic disease shared by 4 syndromes: DiGeorge syndrome (DGS), velocardiofacial syndrome (VCFS), conotruncal anomaly face syndrome (CTAFS) and Opitz G/BBB syndrome (37).These syndromes not only affect the cardiovascular system but also show other symptoms. The clinical presentation is highly variable (38). Because a large proportion of 22q11 deletion patients have CHD, several studies have asked the complementary question and assessed the frequency with which a patient with a conotruncal cardiac defect has 22q11 deletion. For example, tetralogy of Fallot is present in 16% of all patients with CATCH 22 (39,40). Conotruncal anomaly face syndrome shares many features with DGS and VCFS, not only cardiac defects, but also those related to characteristic facial appearance. The finding in CTAFS patients of deletions of the region of 22q11 deleted in DGS and VCFS confirmed that CTFAS and VCFS are the same entity(41). With respect to Opitz syndrome, additional studies are required to determine if the autosomal form of Opitz syndrome results from a mutation in a gene which maps within the deleted region or whether the Opitz G/BBB locus maps outside the DGS/VCFS chromosomal region (37).

**Williams syndrome** is a genetic disorder characterized by mild mental retardation, distinctive craniofacial dysmorphology, hypertension, an associated outgoing personality, elfin facies, short stature, infantil hypercalcemia and various congenital heart defects, especially supravalvular aortic stenosis (SVAS), VSD, pure pulmonary stenosis (PPS), pulmonary stenosis (PS) and mitral valve prolapse (MVP)(42). In this syndrome there is a deletion of the

region of 7q11.23, and the neurological symptoms of WS may be related to the hemizygous deletion of HPC-1/STX1A and LIMK1 *loci* (43).

Heterotaxy syndrome, also called asplenia-polysplenia syndromes or situs ambiguous, is the anomalous placement or transposition of viscera or parts, and is characterized by single ventricle (SV), single atrium (SA), total anomalous pulmonary venous connection (TAPVC), endocardial cushion defect (ECD) and VSD. Although mammalian experiments have identified a number of genes involved in establishing asymmetry in the developing embryo, only in one human disease-related gene, a novel zinc-finger transcription factor ZIC3, have been found mutations in familial and sporadic cases of situs ambiguous (44). Studies to find more genes implicated in this syndrome have been performed, but mutations were rarely found (45,46). In the ACVR2B gene a mutation at position +7 of intron 2 was identified (Fig. 15), and also in the WNT11 gene a mutation at position 1209 of a non-coding region was identified (Fig. 16) (47).
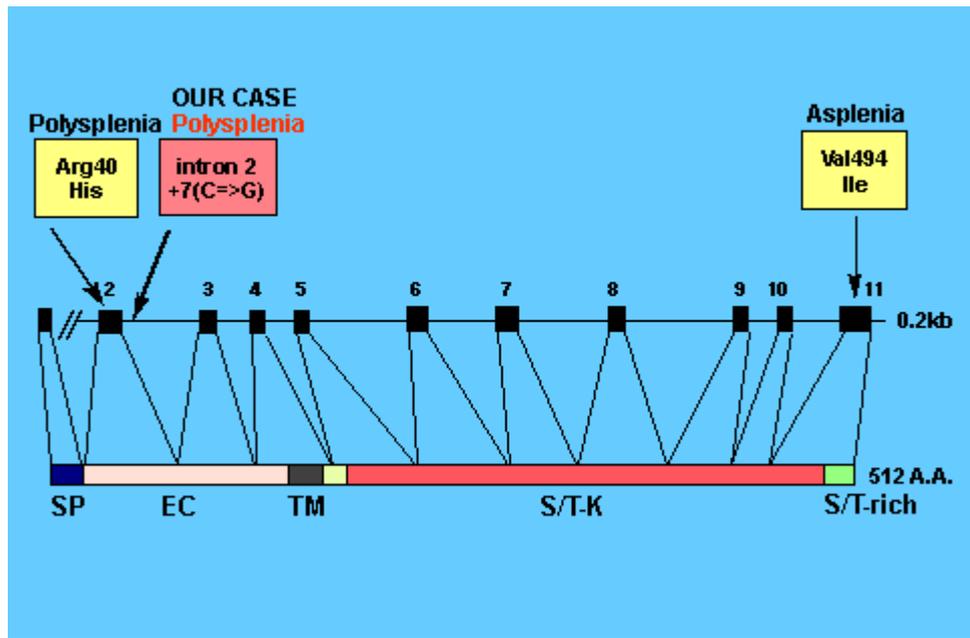


Fig. 15: Mutations in the gene ACVR2B. Genomic organization of human ACVR2B. Three mutations are illustrated: two of them come from polysplenia patients, one from asplenia. Our finding is in red. Lines show locations of amino acids encoded by each exon within the final protein product. SP signal peptide; EC, extracellular domain; TM, transmembrane domain; S/T -K, serine threonine kinase domain; S/T -rich, serine-threonine-rich domain.
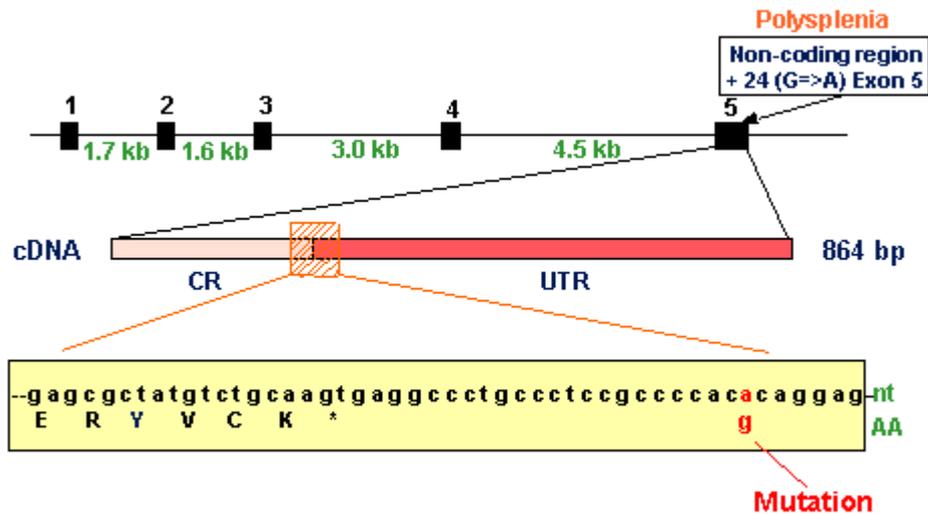
Fig.16: Mutations in the gene WNT11. WNT11 gene organization. The figure shows the complete sequence of the gene in black (Intron' s length shown in green). Inside exon 5 we describe the cDNA, showing in yellow the sequence in which the mutation (red) takes place. CR, coding region; UTR, untranslated region; nt, nucleotide; AA, aminoacid.

**Single gene defects**, which are usually atrial septal defects (ASD) and nonlethal forms of CHD. Multiple familial cases have been reported, some of which are associated with conduction disturbances. Reports have demonstrated that the genetic cause of familial ASD is heterogeneous (48). Using linkage analysis followed by evaluation of a candidate gene, the first disease-related gene for nonsyndromic ASDs has been identified (49). In a subsequent study, NKX2.5 mutations were identified in additional patients with a variety of lesions, including ASDs, isolated atrioventricular conduction disturbances, TOF, double outlet right ventricle, Ebstein's anomaly, and muscular ventricular septal defects (50). NKX2.5 seems to be a disease-gene for a wide range of CHD, the scope of which remains to be defined.

The first major breakthrough in the genetics of familial cardiomyopathy was reported ten years ago, with the discovery of mutations in a group of sarcomere proteins in patients with hypertrophic cardiomyopathy (51). Progress in unravelling the genetics of familial dilated cardiomyopathy has been slower, in part because the disease is less common, more heterogeneous, and harder to diagnose.

Recent insights into the molecular causes of sudden death, frequently due to cardiac arrhythmia, have been no less remarkable. Six genes responsible for long QT syndrome have been identified (52), including HERG, which codes for the cardiac potassium channel. While almost 100 mutations have been documented in this channel alone, drug-induced cases of arrhythmia are also associated with blockage of HERG, which appears to be susceptible because of an unusually spacious inner cavity.

**Alagille syndrome**, is an autosomal-dominant disorder characterized by bile duct paucity in conjunction with cardiac disease (particularly right-sided defects); skeletal and ocular abnormalities and a characteristic face. JAG1, a gene coding for a cell surface protein known to function as a ligand for the Notch transmembrane receptor, was recently identified as the Alagille syndrome disease gene (53,54).

Finally, **Char syndrome**, with PDA as a main characteristic. Char was the first to describe a syndrome with a variable phenotype that included a short philtrum, duck-bill lips, ptosis, low-set ears, and PDA , which was later referred to as Char syndrome. A disease gene for Char syndrome was recently identified (55). It remains to be seen whether this gene is the cause of PDA only in the context of Char syndrome, or whether it is the cause for other, nonsyndromic cases of PDA.

"Success is not the result of spon-
taneous combustion. You must
first set yourself on fire."
-- FRED SHERO

## CONCLUSIONS

After this lengthy explanation about cardiogenetics and its tools for studying genes and mutations now we are able to understand this fascinating world. However, still there is a lot more work to do.

We are here entering the post-genomic era, and we expect to obtain more and more findings and to identify gene more easily than before. The Human Genome Project was essentially completed almost five years ahead of the initial predictions, and few people could imagine its real significance in terms of gathering and analyzing information when the Human Genome Project officially began, in 1991.

It is true that cardiogenetic researchers are still resigned to spend many tedious years identifying genes. But, from now on, the work will be easier than before since we have an invaluable tool in our hands: the human genome sequence.

Just to understand this idea, identification of the gene for cartilage-hair hypoplasia took eight years (56). Now, the

raw sequence deposited nightly by the public genome consortium over the past couple of years has facilitated the identification of more than 30 disease genes, including genes for deafness and epilepsy (57).

Nevertheless, is necessary to understand that the true challenge begins now in interpreting and using this bounty of genetic data (58). As everyone involved in cardiogenetic research says: "the sequence is just the beginning..."

We are living in a time of historic discoveries in the world of research and cardiogenetics, and pediatric cardiologists, cardiologists and medical practitioners in general should be acknowledged for.

We are entering an exciting era for molecular cardiology in which studies of a variety of organisms are contributing to a detailed molecular understanding of normal and abnormal cardiac development. Genetic screening and counseling will become increasingly important for individuals with CHD, particularly as improvements in medical care allow a growing number of these individuals to reach reproductive age (59).

Gene therapy for children with CHD remains a distant prospect. However, patients with acquired heart disease may benefit much earlier from the studies of cardiac development discussed here. For example, determination of the genes involved in proliferation, differentiation, and growth of embryonic cardiac myocytes will greatly facilitate efforts to regenerate heart muscle in patients with myocardial cell loss. Potential modes of such therapy include direct delivery of genes into the myocardium or introduction of genetically altered cells into the heart.

The combined advances in molecular biology, genetics, and gene therapy will undoubtedly lead to improvements in both our understanding and our therapeutic interventions for cardiovascular disease (60).

Finally, I would like to say that research in molecular cardiology, is not easy. However, the effort is not hard enough compared to the satisfaction in making this world a better world, to help our children grow healthy, without congenital heart diseases (Fig. 17).



Fig. 17: Our goal, to guarantee or children a normal growth and development. Free of congenital heart diseases. They deserve it.

pediatric cardiologist, researcher and father of two children. To my friends Drs. Susumu Minamisawa, Mariko Tatsuguchi and Taichi Kato, thanks for helping me when I asked for it. And Michiko Furutani, Misa Kimura, Kumiko Ikeda and Yoshiuki Furutani for their continual support and for making me feel good working with them. Thanks for your example. Now I know what the word "hard-worker" really means.

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# REFERENCES

1. Behrman RE et al. Nelson Textbook of Pediatrics. 15th ed. Philadelphia, Pa: WB Saunders Co. 1996; Chapter 1:1-3.

2. Grabitz RG, Joffres MR, Collins-Nakai RL: Congenital heart disease: incidence in the first year of life: The Alberta Heritage Pediatric Cardiology Program. AM J Epidemiol 1998; 128: 381-388.

3. Larsen HB: Some epidemiologic aspects of congenital heart disease in Denmark. Acta Paediatri Scand 1980; 69: 619-624.

4. Fyler DC, Buckley LP, Hellenbrand WE et al: Report of the New England Regional Infant Cardiac Program. Pediatrics 1980; 65 (Suppl):376-460.

5. Lefkowitz RJ, Willerson JT. Prospects for Cardiovascular Research. JAMA, 2001; 285(5): 581-587.

6. Lefkowitz RJ, Willerson JT. Incidence of Congenital Heart Disease. JAMA, 2001; 285(20): 2579-2580.

7. Hoffmann JIE. Incidence, prevalence and inheritance of congenital heart disease. In: Moller JH, Hoffmann JIE, eds. Pediatric Cardiovascular Disease. New York, NY: Churchill Livingstone, 2000: 257-262.

8. Nora JJ, Nora AH: Genetic Epidemiology of congenital heart disease. Prog Med Genet 1983; 5: 91-137.

9. Nadal-Ginard B, Mahdavi V. A Cellular and Molecular approach to Pediatric Cardiology. Nada's Pediatric Cardiology, Donald Fyler Ed. 1992; chapter 55: 747-759.

10. Braunwald E. Cardiovascular Medicine at the turn of the millennium: triumphs, concerns, and opportunities. The New England Journal of Medicine Nov. 1997; 337 (19): 1360-1369.

11. Goldmuntz E. The Epidemiology and genetics of congenital heart disease. Clinics in Perinatology. March 2001; 28(1): 1-10.

12. Kirby, LT. DNA Fingerprinting: An introduction. 2nd. Edition. Stockton press Co. 1993; Chapter 2: Genetic Principles:7-15.

13. Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson J. Molecular Biology of the Cell. Third Edition. Garland Publishing Inc. 1994; Chapter 3: Macromolecules: Structure, Shape, and Information: 89-102.

14. Lewin B. Genes V. Oxford University Press Inc., New York. 1994; Part 1: DNA as a store of information: 57-127.

15. Díaz C.M.,Gotoh S.,Urata Y.,Niwa M.,Kondo T.,Tsuji Y. Glutathione-related Enzyme activities in Spontaneous Hypertensive Rat Hearts. The Acta Medica Nagasakiensia. June 1998; Vol.43, Numbers 1-2: 23-28.

16. Díaz M. Carlos, Goto S, Urata Y, Iida T, Cho S., Niwa M, Tsuji Y, Kondo T. Nicardipine normalizes elevated levels of antioxidant activity in response to xanthine oxidase-induced oxidative stress in hypertensive rat hearts. Free Rad. Res. 1998; 29: 143-150.

17. Braunwald E. Heart Disease: A Textbook of Cardiovascular Medicine, Fifth Edition. W.B. Saunders Company. 1996; Vol. 2, Part IV, chapters 50 and 51: 1602-1655.

18. Larsen W. Human Embriology. 2nd. Edition. Churchill Livingstone ed. 2000; chapter 3: 49-60.

19. Moore K, Persaud TVN. The Developing Human: Clinically oriented embryology. 6th edition. 1998; Chapter 4: 63-79.

20. Sadler TW. Langman's Medical Embryology. Eighth Edition. Lippincott Williams & Wilkins, 2000; Chapters 3 and 4: Second and Third weeks of Development: 49-82.

21. Felsenfeld AL. Defining the boundaries of Zebrafish developmental genetics. Nature Genetics. November 1996; 14: 258-263.

22. Lieschke GJ. Zebrafish - An emerging Genetic Model for the study of Cytokines and Hematopoiesis in the Era of Functional Genomics. International Journal of Hematology. 2001; 73:23-31.

23. Kroll KL, Kirschner MW. Easy passage: Germline transgenesis in frogs. Proceedings of the National Academy of Sciences of the United States of North America. December 7, 1999; 96 (25): 14189-14190.

24. Dawid I, Sargent T. Xenopus laevis in Developmental and Molecular Biology. Science, June 1988; 240:1443-1448.

25. Marsh-Armstrong N, Huang H, Berry D, Brown D. Germ-line transmission of transgenes in Xenopus laevis. Proceedings of the National Academy of Sciences of the United States of North America (PNAS), December 1999; 96 (25): 14389-14393.

26. Warren KS, Fishman MC. "Physiological genomics": mutant screens in zebrafish. Am. J. Physiol. 1998; 275 (Heart Circ. Physiol. 44): H1-H7.

27. Fishman Mark C. Zebrafish genetics: The enigma of arrival. Proceedings of the National Academy of Sciences of the United States of America. September 1999; 96: 10554-10556.

28. Nasevicius A, Ekker S. Effective targeted gene "knockdown" in zebrafish. October 2000; 26: 216-220.

29. Holder N, McMahon A. Genes from zebrafish screens. Nature. December 1996; 384: 515- 516.

30. Ishikawa Y, Hyodo-Taguchi Y, Tatsumi K. Medaka fish for mutant screens. Nature. March 1997; 386: 234.

31. Phillips RB, Reed KM. Localization in repetitive DNAs to zebrafish (Danio rerio) chromosomes by fluorescence in situ hybridization (FISH). Chromosome Research. 2000; 8 (1): 27-35.

32. Matsuoka R, Matsuyama S, Yamamoto Y, et al. Trisomy 18q. A case report and review of karyotype-phenotype correlations. Hum Genet 1981; 57: 78-82.

33. ?????. Holt-Oram ???. ??????IV. ???? 1996; 15: 267-70.

34. Basson C, Huang T, Bachinsky DR, et al. Mutations in human TBX5 cause limb and cardiac malformation in Holt-Oram syndrome. Nat Genet 1997; 15: 30-35.

35. Li QY,Newbury-Ecob RA, Terrett JA, et al. Holt-Oram Syndrome is caused by mutations in TBX5, a member of the brachyury (T) gene family. Nat Genet 1997;15:21-29.

36. Basson C, Huang T, Lin RC, et al. . Different TBX5 interactions in heart and limb defined by Holt-Oram syndrome mutations. Proc Natl Acad Sci USA 1999; 96: 2919-2924.

37. Emanuel B, Budarf M, Scambler P. The genetic basis of conotruncal cardiac defects: The chromosome 22q11.2 deletion. Heart Development. Richard Harvey & Nadia Rosenthal Ed. 1999; Chapter 26: 463-478.

38. Matsuoka R, Kimura M, Scambler PJ., et al. Molecular and clinical study of 183 patients with conotruncal anomaly face syndrome . Hum Genet 1998; 103: 70-80

39. Momma K, Kondo C, Ando M, et al. Tetralogy of Fallot associated with chromosome 22q11 deletion. Am J Cardiol 1995; 76: 618-621.

40. Momma K, Kondo C, Matsuoka R. Tetralogy of Fallot with pulmonary atresia associated with chromosome 22q11 deletion. J Am Coll Cardiol 1996; 27: 198-202.

41. Burn J, Takao A, Wilson D, et al. Conotruncal anomaly face syndrome is associated with a deletion within chromosome 22q11. J Med Genet 1993; 30:822-824,.

42. Nakayama T, Matsuoka R, Kimura M, et al. Hemizygous deletion of the HPC-1/syntaxin1A gene (STX1A) in patients with Williams syndrome. Cytogenet Cell Genet 1998; 82: 42-51.

43. Kimura M.,Hirota H.,Nishikawa T.,Ishiyama S.,Imamura S.,Koremberg J.R., Mizuno K.,Nakayama T.,Akagawa K.,Shimizu N.,Joh-o K.,Díaz C.M.,Tatsuguchi M., Komatsu K.,Ando M.,Takao A., Monma K.,Matsuoka R.Chromosomal deletion and phenotype correlation in patients with Williams Syndrome. Etiology and Morphogenesis of Congenital Heart Disease: Twenty Years of Progress in Genetic and Developmental Biology. Edited by Edward B. Clark, Futura Publishing Company, Inc. Armonk NY. October 2000. Section VII: 381-384.

44. Gebbia M, Ferrero GB, Pilia G, et al. X-linked situs abnormalities result from mutation in ZIC3. Nat Genet 1997; 17: 305-308.

45. Kosaki R, Gebbia M, Kosaki K, et al. Left-right axis malformations associated with mutations in ACVR2B, the gene for human activin receptor type IIB. Am J Med Genet 1999; 82: 70-6.

46. Kosaki K, Bassi MT, Kosaki R, et al. Characterization and mutation analysis of human LEFTY A and LEFTY B, homologues of murine genes implicated in left-right axis development. Am J Hum Genet 1999; 64: 712-21.

47. Diaz CM, Furutani M, Iida A, Imamura S, Takao A, Momma K, Matsuoka R. Assessment of novel mutations in five candidate genes in twenty-three patients with heterotaxia. Pediatric Cardiology, 2001, In press.

48. Benson DW, Sharkey A, Fatkin D, et al. Reduced penetrance, variable expressivity, and genetic heterogeneity of familial atrial septal defects. Circulation, 1998; 97: 2043-2048.

49. Schott JJ, Benson DW, Basson CT, et al: Congenital heart disease caused by mutations in the transcription factor NKX2.5. Science, 1998; 281:108-111.

50. Benson DW, Silverbach GM, Kavanaugh-McHugh A, et al. Mutations in the cardiac transcription factor NKX2.5 affect diverse cardiac developmental pathways. J Clin Invest, 1999; 104: 1567-1573.

51. Seidman JG, Seidman C. The genetic basis for cardiomyopathy: from mutation identification to mechanistic paradigms. Cell , February 23, 2001; 104: 557-567.

52. Keating MT, Sanguinetti MC. Molecular and Cellular mechanisms of cardiac arrhythmias. Cell, February 23, 2001; 104: 569-580.

53. Li L, Krantz ID, Deng Y, et al. Alagille syndrome is caused by mutations in human Jagged 1, which encodes a ligand for Notch 1. Nat Genet, 1997; 16: 243-251.

54. Oda T, Elkahloun AG, Pike BL, et al. Mutations in the human Jagged1 gene are responsible for Alagille syndrome. Nat Genet, 1997; 16: 235-242.

55. Satoda M, Zhao F, Diaz GA, et al. Mutations in TFAP2B cause Char syndrome, a familial form of patent ductus arteriosus. Nat Genet 2000; 25: 42-46.

56. Ridanpää M, van Eenennaam H, Pelin K, Chadwick R, Johnson C, Yuan B, vanVenrooji W, Pruijn G, et al. Mutations in the RNA component of Rnase MRP cause a pleiotropic human disease, cartilage-hair hypoplasia. Cell, 2001; 104: 195-203.

57. International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome. Nature 2001; 409: 860-921.

58. Davies K. After the genome: DNA and Human Disease. Cell. February 23, 2001; 104: 465- 467.

59. Yamagishi H, Garg V, Matsuoka R, et al. A molecular pathway revealing a genetic basis for human cardiac and congenital defects. Science, 1999; 283: 1158-61.

60. Olson E, Srivastava D. Molecular pathways controlling heart development. Science. May 3, 1996; 272: 671-676.

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