The role of microRNAs in congenital heart disease

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ABSTRACT

Congenital heart diseases (CHDs) are the leading inherited cause of perinatal and infant mortality. CHD refers to structural anomalies of the heart and blood vessels that arise during cardiac development and represents a broad spectrum of malformations, including septal and valve defects, lesions affecting the outflow tract and ventricules. Advanced treatment strategies have greatly improved life expectancy and led to expanded population of adult patients with CHD. Thus, a better understanding of the pathogenesis and molecular mechanisms underlying CHDs is essential to improve the diagnosis and prognosis of patients. The etiology of CHD is largely unknown, genetic and environmental factors may contribute to the disease. In addition to the mutations affecting genomic DNA, epigenetic changes are being increasingly acknowledged as key factors in the development and progression of CHDs. The posttranscriptional regulation of gene expression by microRNAs (miRs) controls the highly complex multi-cell lineage process of cardiac tissue formation. In recent years, multiplex experimental models have provided evidence

that changes in expression levels of miRs are associated with human cardiovascular disease, including CHD. The newly described correlations between miRs and heart development suggest the potential importance of miRs as diagnostic markers in human cardiovascular diseases. In the future, more intensive research is likely to be carried out to clarify their contribution to personalized management and treatment of CHD patients. In this paper, we discuss the current knowledge on the causative role of miRs in cardiac development and CHDs.

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INTRODUCTION

CHD defines functional and structural disorders of the heart and blood vessels that arise during embryogenesis. CHD is the most common cause of perinatal and infant mortality. The reported birth prevalence varies widely across countries and continents, the best estimation generally accepted is 8 per 1,000 live births (1). CHDs are the leading cause of infant morbidity and mortality and account for approximately 40% of prenatal deaths and 20% of mortality in the first year of life (2, 3). CHDs encompass a wide spectrum of phenotypes including atrial septal defect (ASD), ventricular septal defect (VSD), patent ductus arteriosus (PDA), transposition of the great arteries (TGA), pulmonary valve atresia (PVA), coarctation of the aorta (CoA), tricuspid atresia (TA), tetralogy of Fallot (TOF), hypoplastic left heart syndrome (HLHS) and univentricular heart (UH). The severity of the different forms of CHD varies extensively from simple mild lesions with follow-up for decades without any treatment to complex cyanotic malformations requiring urgent surgical intervention (4). Recent advances in pre- and postnatal diagnosis as well as surgical treatment approaches have increased life expectancy of patients with CHD. Nowadays more than 75% of

CHD patients live to adulthood, increasing the pediatric CHD cohort and requiring lifelong specialized cardiac care. As a result, an increased number of patients will reach the reproductive age and can transmit the disease with a high risk (5, 6). Therefore, a better understanding of the etiology is essential in order to give early diagnosis, allow timely interventions, effective patient management and proper genetic counseling. To date, approximately 20% of CHD cases have known causes, such as teratogen effects or genetic alteration. Half of the genetic abnormalities are chromosomal aneuploidy, submicroscopic copy number variation or nucleotide sequence mutations. These abnormalities may cause syndromes (with extracardiac symptoms) or isolated heart defects. Clarification of genetic background is complicated due to low penetrance, high phenotypic variability and variable expression of symptoms (7, 8). Very little is known about the etiology of the remaining 80% of cases, most of them follow multifactorial inheritance including genetic and environmental factors in disease development (7). Recently, epigenetics is being increasingly acknowledged as a key mechanism in the pathophysiology of cardiovascular diseases, including CHDs. Epigenetics refers to a set of mechanisms that regulate gene expression without changing the underlying nucleotide sequence. Beside DNA methylation and histone modification as two basic elements of epigenetic machinery, noncoding RNA (ncRNA) molecules can also induce epigenetic modifications by post-transcriptional regulation of gene expression. The best studied group of noncoding RNAs are miRs, which are small, evolutionarily conserved, single-stranded RNA molecules, approximately 22 nucleotides in length at mature stage. They suppress expression of target genes by inhibiting translation or encouraging degradation of their mRNA by complementary base pairing at 3'UTR. The degree and nature of complementary sites

between the miR and target mRNA appear to determine the gene silencing mechanism. Since the first description in the early 1990s, more than 1000 miRs have been identified in mammals regulating more than 30% of genes. A single miR can regulate multiple target mRNAs, and individual mRNA can be targeted by many miRs (2, 9, 10).

The aim of this paper is to review the current knowledge of the potential role of miRs in abnormal cardiac development and consequently in CHD.

THE ROLE OF MICRORNAS
IN HEART DEVELOPMENT

Various roles of miRs were identified in the pathogenesis, progression, and remodeling of cardiovascular diseases. The process of cardiac tissue formation and cardiac gene expression is so highly complex that it requires formation of diverse cell types, including cardiomyocytes, valvular and endothelial cells, conduction system, smooth muscle cells, in a tightly regulated, spatiotemporal manner. MiRs increase this complexity even further by adding another layer of regulation at the post-transcriptional level (11, 12). The significance of miRs in cardiogenesis was first revealed in gene deletion experiments in animal models, using mice and zebrafish.

Dicer is an essential endonuclease in the miR biogenesis pathway, disruption of its function removes all mature miRs. Dicer deletion in mice and mutation in zebrafish caused lethality by arresting gastrulation, providing convincing evidence on the essential role of miRs in early animal development. To better understand the function of miRs in cardiac tissues, studies on tissue specific Dicer deletion were carried out on mouse heart at different developmental stages. Deletion of cardiac specific Dicer prenatally resulted in defective heart morphogenesis and embryonic lethality. However, heart

specific Dicer deletion after birth led to abnormal expression of contractile proteins, and remarkable sarcomere disarray coupled with reduced cardiac function and rapid progression to dilated cardiomyopathy and heart failure. This cardiac phenotype closely resembled human dilated cardiomyopathy and heart failure (13-15). To understand the contribution of individual miRs to cardiac development, much research has been undertaken with gain- and loss-of-function studies of specific miRs (16-18). The outcomes of these studies clearly indicated that single miRs have crucial roles in cardiac development and function.

MiR-1 (miR-1-1, miR-1-2, miR-206) and miR-133 (miR-133a-1, miR-133a-2, miR-133b) families are highly conservative. They are abundantly, but not exclusively expressed in the heart. These miRs are produced from the same polycistronic transcript, encoded by two separate genes in the mouse and human genomes, therefore they have identical sequences and consequently target the same mRNAs.

Last decades of intensive research using developmental models of the heart have revealed that these miRs control fundamental cardiac transcription factors, such as *GATA4*, *TBX1*, *MEF2C*, *SRF* and *MHC* genes. MiR-1/-133 have a crucial role in regulation of cardiomyocyte proliferation. They are also known to induce mesoderm formation and differentiation of embryonic stem cells. In the developing mouse heart, excessive expression of miR-1 inhibited ventricular myocyte proliferation (17).

In contrast, targeted deletion of miR-1-2 was found to be lethal at nearly 50% of mouse embryos at weaning age, and some animals showed ventricular septal or conduction system defects in later stage (18). Deletion of either miR-133a-1 or miR-133a-2 did not affect seriously the cardiogenesis, but resulted in VSD and chamber dilatation leading to late embryonic or

early postnatal death (19). Both miR-1 and miR-133 seemed to regulate growth-related cardiac genes as growth suppressors and had an opposite role in cardiomyocyte apoptosis (20, 21). Furthermore, miR-1 was linked to NOTCH1 receptor, whose ligand is essential in normal asymmetric division (22). To understand the exact molecular mechanism underlying the function of miRs, their target genes are required to be identified. In case of miR-1, one important validated target is *Hand2* cardiac transcription factor. Deletion of *Hand2* gene in mouse models leads to similar failure in ventricular myocytes as miR-1 overexpression, which also reduces expression of *Hand2* (17).

The miR-15 family includes six miRNAs: miR-15a, miR-15b, miR-16-1, miR-16-2, miR-195, miR-497. By inhibiting these miRs, mitosis of myocardial cells increases and the suppression of the most important target gene, *CHEK1* is eliminated. Of particular importance is the miR-195, whose overexpression was detected during cardiac hypertrophy in human and mouse hearts, and it was sufficient alone to induce hypertrophic growth of cultured rat cardiomyocytes (23).

MiRs belonging to the miR-208 family (miR-208a, miR-208b, miR-499) are commonly referred to as MyomiRs, expressed specifically in the heart. MiR-208-null animals do not show gross developmental defects although their overexpression induces cardiac hypertrophy and conduction defects (24). Among their target genes, the *THRAP1* is involved in the negative regulation of myocardial growth and hypertrophy. MiR-499 is responsible for the expression of myosin genes in the heart muscle of mice (24-26).

The 17-92 miRs are clustered, and initially they were known as oncogenes (oncomiR). A few of these miRs (miR-17, miR-18a, miR-19a, miR-19b-1, miR-20a, miR-92a-1) play an important role in cardiogenesis, participating in the differentiation of progenitor cells in the heart muscle. Their elevated level causes CHDs due to inhibition of the expression of the main progenitor genes (*ISL1*, *TBX1*) (12).

The miR-143 family expressed in endocardial, myocardial and cardiac progenitor cells is involved in the regulation of the *ADD3* target gene, which encodes the F-actin capping protein. This pathway is involved in the proper design and function of the heart chambers. By knocking out these miRs, outflow tract or ventricular disorders can develop in the heart (27, 28).

Evolutionarily conserved miR-218a-1, miR-218a-2 and miR-218b together with *SLIT2* and *ROBO* genes provide the adequate development of the heart tube. Together with the transcription factor *TBX5*, they participate in the morphogenesis of the heart. Downregulation of miR-218 causes overexpression of *TBX5*, resulting in heart looping and chamber disorders (29).

ALTERED MICRORNA EXPRESSIONS IN CONGENITAL HEART DISEASES AND THEIR USE AS BIOMARKERS

During the past decade, findings of miR expression profiling experiments have demonstrated that significant changes of specific miR signatures occur in various forms of cardiovascular diseases, including CHDs (22). The first studies documented deregulated miR expression patterns in humans mostly in the myocardial tissues, such as ventricular myocardium or outflow tract tissues (2, 10, 30). Subsequently, it became clear that some miRs released from their cells of origin can also be detected in different bodily fluids (31-33). Numerous studies started to investigate tissue- and disease-specific miR profiles in bodily fluids in different human pathological conditions (34-36). Extracellular miRs became attractive candidates as noninvasive

biomarkers for early diagnosis or prognosis of CHDs (34, 37, 38) (Table 1).

However, extracellular miR profiling as a valid diagnostic or prognostic tool is still in the descriptive phase. The highly variable miR assessments from blood are derived from the different extraction methods, analysis platforms applied and the fact that the extracellular miRs are either associated with proteins or contained in cellular fragments (e.g. microparticles, exosomes). An excellent review summarizes the currently used methodological approaches in utilizing miRs as circulating biomarkers.

The authors highlighted optimal miR isolation protocols, the advantages and disadvantages of various expression methods and provided a workflow in designing miR profiling from plasma or serum samples (39).

Table 1	MicroRNA profiling methods and expression changes in CHD*						

Sample	CHD	Control	Methods		MicroRNAs			Reference
			Screening	Confirmatory	Candidate	Confirmed	Expression	
Cardiac tissue	TOF (n=16)	n=8 normally developing hearts	Microarray	RT-qPCR	miRs (n=61)	miR-27b miR-421 miR-1275	Upregulated	(10)
						miR-122 miR-1201	Downregulated	
Cardiac tissue	TOF (n=21)	n=6 normal controls	Microarray	RT-qPCR	miRs (n=41)	miR-19a miR-130b miR-146b-5p miR-154 miR-155 miR-181c miR-181d miR-192 miR-222 miR-337-5p miR-363 miR-424 miR-424* miR-660 miR-708	Upregulated	(30)
						miR-29c miR-181a* miR-720	Downregulated	

Cardiac tissue	VSD (n=28)	n=9 normal heart tissues	Microarray	RT-qPCR	miRs (n=25)	miR-181c	Upregulated	(41)
						miR-1-1	Downregulated	
Cardiac tissue	TOF (n=16)	n=8 normally developing hearts	Microarray	RT-qPCR	miRs (n=61)	miR-421	Upregulated	(44)
Cardiac tissue	TOF (n=26)	n=15 healthy individuals	Microarray	RT-qPCR	miRs (n=75)	miR-940	Downregulated	(46)
Cardiac tissue	HLHS (n=15)	n=6 non-failing control hearts	Microarray	RT-qPCR	miRs (n=93)	miR-204	Upregulated	(49)
						miR-99a miR-100 miR-137-3p miR-145a	Downregulated	
Cardiac tissue	CHD (n=30)	n=30 normal samples	Gene expression microarray	RT-qPCR	miR-145 miR-182	miR-145	Downregulated	(52)
Cardiac tissue	CHD (TOF, VSD, PA) (n=10)	n=11 acyanotic cardiac defects	-	RT-qPCR	-	miR-138	Upregulated	(45)
Cardiac tissue	TOF (n=30)	n=10 normal samples	Gene expression microarray	RT-qPCR	miR-1 miR-19a miR-30a miR-30c miR-30d miR-30e miR-130a miR-130b miR-144 miR-206	miR-1 miR-206	Downregulated	(36)
Cardiac tissue	Cyanotic CHD (n=10)	n=10 acyanotic cardiac defects	-	RT-qPCR	-	miR-184	Downregulated	(53)

Serum	TGA (n=26)	n=20 healthy subjects	Microarray	RT-qPCR	miRs (n=23)	hsa-let-7e miR-16 miR-18a miR-25 miR-93 miR-106a miR-144* miR-451 miR-486-3p miR-486-5p miR-505*	Upregulated	(47)
Serum	CHD (n=61)	n=20 normal individuals	-	RT-qPCR	-	miR-873	Upregulated	(54)
Serum	Cyanotic CHD (n=32)	n=20 acyanotic CHD individuals	-	RT-qPCR	-	miR-182	Downregulated	(55)
Plasma	VSD (n=20)	n=15 VSD-free participants	Microarray	RT-qPCR	miRs (n=36)	miR-498	Upregulated	(40)
						hsa-let-7e-5p miR-155-5p miR-222-3p miR-379-5p miR-409-3p miR-433 miR-487b	Downregulated	
Plasma	CHD (ASD, VSD, AVSD) (n=26)	n=27 healthy children	miRNA PCR Array	RT-qPCR	miRs (n=84)	hsa-let-7a hsa-let-7b miR-486	Upregulated	(37)
Maternal serum	CHD (ASD, VSD, TOF) (n=30)	n=30 normal fetuses	SOLiD sequencing	RT-qPCR	miRs (n=11)	miR-19b miR-22 miR-29c miR-375	Upregulated	(42)

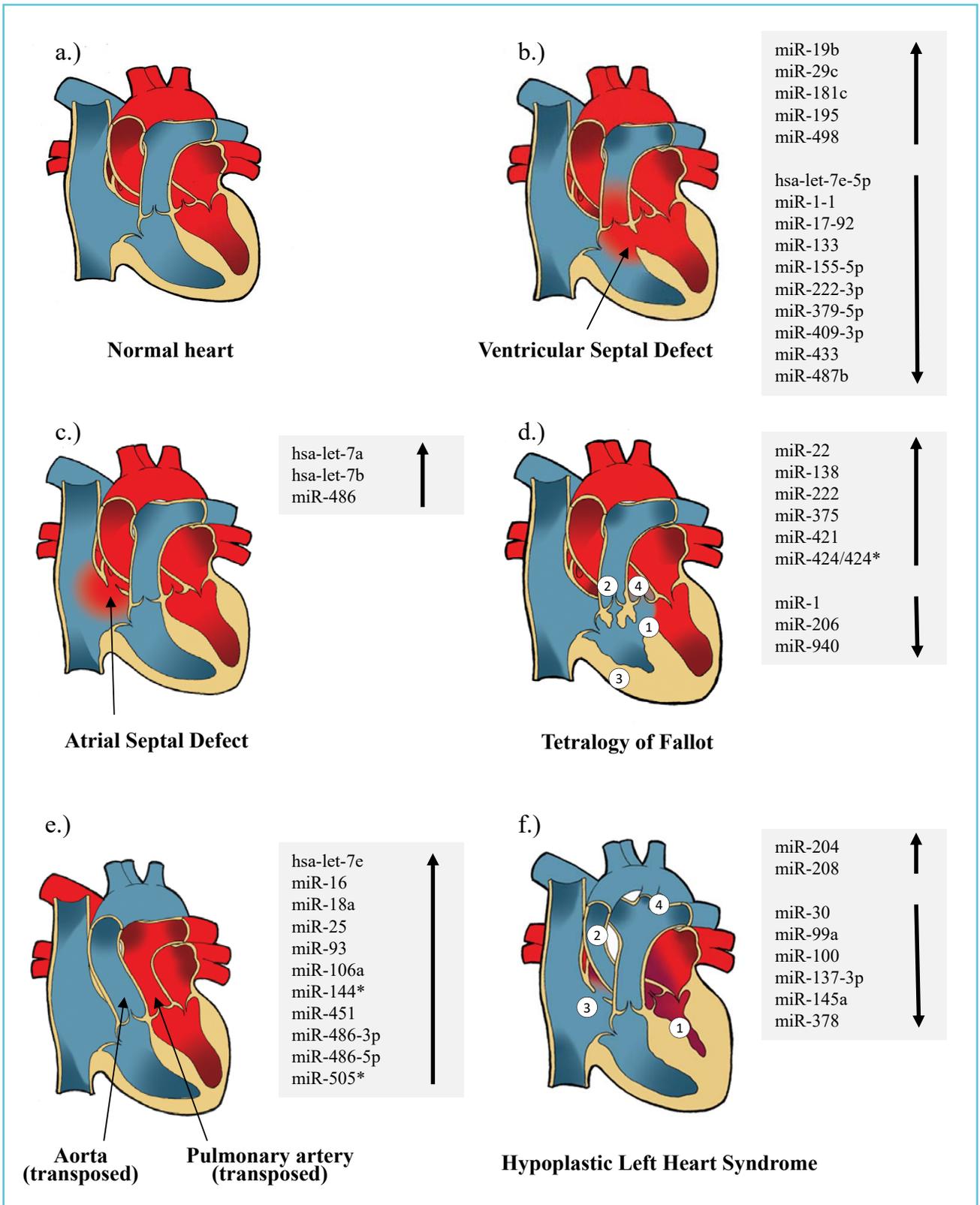| Venous blood	TOF (n=37)	n=15 healthy controls	Microarray	RT-qPCR	miRs (n=49)	miR-183-5p miR-421 miR-625-5p miR-1233-3p	Upregulated	(11)
						miR-140-3p miR-142-5p miR-181d-5p miR-206 miR-339-5p	Downregulated	

*ASD: atrial septal defect, AVSD: atrioventricular septal defect, CHD: congenital heart disease, HLHS: hypoplastic left heart syndrome, miRs: microRNAs, PA: pulmonary atresia, RT-qPCR: reverse transcription-quantitative polymerase chain reaction, TGA: transposition of the great arteries, TOF: tetralogy of Fallot, VSD: ventricular septal defect.

To date, especially the common and/or severe CHDs have been studied extensively by miR expression profiling. VSD, as a discontinuation in the septal wall between left and right ventricles, accounts for approximately 20-40% of CHDs (Fig. 1b) (40). Large defects lead to severe heart failure requiring urgent surgical intervention, while smaller ones may be asymptomatic and close spontaneously. The more severe forms can result in left-to-right shunt with consecutive left ventricle overload and pulmonary hypertension. Although its embryology and pathology have been clarified, its etiology remains unknown. After initial microarray screening, Li *et al* (41) compared the expression of a set of 25 candidate miRs in heart tissues of patients with VSD and healthy controls by qRT-PCR. They found that miR-1-1 expression was decreased in patient samples and associated with the increased level of its target genes, *GJA1* and *SOX9*. The miR-181c overexpression, linked with the downregulation of *BMPR2* gene, was identified as well. Later on, another research group reported for the first time circulating miR profiles for patients with VSD with upregulation of miR-498 and downregulation of hsa-let-7e-5p, miR-155-5p, miR-222-3p, miR-379-5p, miR-409-3p, miR-433 and miR-487b in plasma samples of patients (40). Zhu *et al* hypothetize that miRs in maternal serum can be used as biomarkers for the prenatal detection of fetal CHD in early pregnancy. They analyzed miR profiles in serum samples from pregnant women, who had fetuses with ASD, VSD or TOF and from women with normal pregnancies. They identified significantly higher expression of miR-19b and miR-29c in case of VSD and upregulation of miR-22 and miR-375 in TOF. Results of this study raised the possibility of using miRs in the maternal serum for early diagnosis of fetal cardiac disorders as noninvasive biomarkers (42). However, because of the limitations of the study, further research is required to confirm the usefulness of miRs in the clinical practice for prenatal diagnosis of CHD.

There are much less data on the expression profile of miRs in congenital ASD (Fig. 1c). In a more recent study Song and colleagues (37) identified significant upregulation of hsa-let-7a, hsa-let-7b and miR-486 in children with ASD, VSD and AVSD. The hsa-let-7a and hsa-let-7b were specifically overexpressed in ASD cases supporting their hypothesis that specific miRs are associated with specific types of CHD. Similar expression profiles of hsa-let-7a and hsa-let-7b were detected in mothers of ASD children. Based on their results, not only do these miRs have diagnostic but also predicting roles for CHD risk in offspring.

Figure 1   Altered expression of miRs in CHDs*

a.)

Normal heart

b.)

miR-19b
miR-29c
miR-181c
miR-195
miR-498

hsa-let-7e-5p
miR-1-1
miR-17-92
miR-133
miR-155-5p
miR-222-3p
miR-379-5p
miR-409-3p
miR-433
miR-487b

Ventricular Septal Defect

c.)

hsa-let-7a
hsa-let-7b
miR-486

Atrial Septal Defect

d.)

miR-22
miR-138
miR-222
miR-375
miR-421
miR-424/424*

miR-1
miR-206
miR-940

Tetralogy of Fallot

e.)

hsa-let-7e
miR-16
miR-18a
miR-25
miR-93
miR-106a
miR-144*
miR-451
miR-486-3p
miR-486-5p
miR-505*

**Aorta
(transposed)**   **Pulmonary artery
(transposed)**

f.)

miR-204
miR-208

miR-30
miR-99a
miR-100
miR-137-3p
miR-145a
miR-378

Hypoplastic Left Heart Syndrome

TOF (Fig. 1d) is the most common form of cyanotic CHD that represents 5-8% of all CHDs (43). It is characterized by: 1) pulmonary outflow tract obstruction; 2) VSD; 3) overriding aortic root; and 4) right ventricular hypertrophy. Without surgery, patients have poor prognosis and follow-up studies show that even after repair of TOF they have a higher risk for heart failure. Despite its prevalence and clinical significance, the underlying mechanism is poorly understood.

More recently, miRs have been investigated as etiological factors in the pathogenesis of TOF. O'Brien and colleagues (10) examined expression patterns of miRs in right ventricular myocardium from infants with nonsyndromic TOF compared to infants with normally developing heart. Microarray analysis revealed that the levels of 61 miRs significantly changed, especially miR-1275, miR-27b, miR-421, miR-1201 and miR-122, moreover the levels of expressions were similar to those in the fetal myocardium. Potential targets of miRs with altered expression were concentrated in gene networks critical to cardiac development. Subsequently, this group performed a follow-up study focusing on miR-421 by under- and overexpressing of miR-421 on primary cells from ventricular myocardium of TOF heart.

The authors found an inverse correlation between expression of miR-421 and *SOX4* that is a key regulator of the Notch signaling pathway, which suggests the association of miR-421 with TOF (44). He *et al* (45) studied miR-138 expression in myocardial samples from patients with cyanotic TOF and identified a two-fold increase compared to patients with acyanotic CHD. Based on studies from Liang and colleagues (46) miR-940 is the most downregulated miR in myocardium from patients with TOF among the identified 75 dysregulated miRs. Zhang *et al* (30) applied microarray analysis to identify deregulation of miRs in right ventricular outflow tract tissue in infants with TOF. They found 18 miRs with significantly altered expression, among which miR-424/424* and miR-222 were shown to affect cardiomyocyte proliferation and differentiation. MiR target gene network analysis showed that 16 of the 18 miRs targeted 97 genes involved in heart development.

Abnormal expression of connexin 43 (*Cx43*) has been documented in conotruncal anomalies although the underlying mechanism is unknown. Wu and colleagues (36) investigated the potential role of miRs in altered expression of *Cx43* in myocardium samples of patients with TOF. They showed that miR-1 and miR-206 were significantly decreased in the TOF patients as compared with controls, which may cause upregulation of *Cx43* protein synthesis and suggest the role of these miRs in the pathogenesis of TOF.

Little is known about the expression profile of miRs in CHD with systemic right ventricle. In patients with TGA (Fig. 1e) after atrial switch

operation, late systemic right ventricular dysfunction develops. Lai *et al* (47) tested the circulating miR expression profile in these patients. They found that 23 miRs were upregulated, 11 were validated to be increased in patients compared with controls. MiR-18a and miR-486-5p expression signature correlated negatively with systemic ventricular contractility.

HLHS (Fig. 1f) represents 2% to 9% of CHDs and accounts for 23% of neonatal deaths. Characteristics of HLHS include varying degrees of hypoplasia of the left ventricle, mitral valve and aortic valve atresia/stenosis, and hypoplasia of the ascending aorta. This is one of the most serious cardiac malformation and leads to death in the newborn period without treatment. Nowadays as a result of surgical palliative techniques a five-year survival can be reached in more than half the cases (48). Sucharov *et al* (49) investigated the miR expression in the right ventricle myocardium of HLHS patients. They found that pediatric HLHS population has a unique miR profile, and some miRs (miR-100, miR-145a, miR-99a, miR-137-3p, miR-204) are modulated by changes in volume loading of the right ventricle. Biostatistical analysis revealed that target genes of these miRs are important for cardiac development and disease.

The most common genetic defect leading to CHD is the trisomy 21 in Down syndrome. Five miRs are overexpressed in Down syndrome, miR-99a, hsa-let-7c, miR-125-b2, miR-155 and miR-802, all of them are located on chromosome 21 (50).

Patients with DiGeorge syndrome, which is the most common microdeletion syndrome, have heterogeneous phenotype including CHD, immunodeficiency and hypocalcemia. The genetic cause is the deletion of 22q11.2 region including *TBX1*, which is essential in normal cardiac development. The *DGCR8* gene encodes a crucial component of the RNA induced silencing complex (RISC), necessary for miR biogenesis.

Haploinsufficiency of this complex leads to impaired miR expression and development of CHD (51).

CONCLUSION

CHDs account for a significant part of cardiovascular diseases, perinatal morbidity and mortality. Improved survival of children with CHD has led to high number of adult patients facing multiple challenges throughout their lifetime. Despite our increasing knowledge on the genetic basis and signaling pathways involved in cardiac development, there are still huge gaps that require further examination.

In the cardiovascular research field, numerous studies demonstrated that miRs are required for proper heart development and function. Characteristic expression profiles of miRs have specific and generalized effects on cell signaling pathways associated with CHD.

MiRs are attractive diagnostic and prognostic biomarkers as they remain stable in bodily fluids and avoid RNA degrading enzymes and hereby may provide an additional diagnostic tool to assess heart disease.

According to recent reports miRs can serve as noninvasive biomarkers for the extent of myocardial damage and the postoperative clinical course of pediatric patients with CHDs following surgery.

Despite the results having been reported in the past decade, the exact role of miRs in CHDs is still unclear. Large-scale studies are needed to provide a better understanding of the molecular interactions causing CHD.

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