

Elevated hsa-miR-99a levels in maternal plasma may indicate congenital heart defects

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Abstract. The current standard for prenatal screening is mostly based on biochemical marker tests and the use of ultrasonography. There is no secure stand-alone screening marker for congenital heart defects (CHDs). MicroRNAs (miRNAs) that are associated with cardiogenesis enter the maternal peripheral bloodstream during pregnancy and allow non-invasive prenatal testing (NIPT). The present study investigated the plasma expression profile of fetal hsa-miR-99a in maternal blood. Peripheral blood samples were collected from 39 pregnant patients, comprising 22 with CHD-positive fetuses and 17 with CHD-free controls. miRNAs were isolated from the maternal serum and reverse transcription-quantitative polymerase chain reaction was carried out to determine the expression of hsa-miR-99a. While the miRNA concentrations were almost identical among the affected and control groups (5.54 vs. 6.40 ng/ μ l), significantly upregulated hsa-miR-99a levels were identified in the affected group ($1.78 \times 10^{-2} \pm 3.53 \times 10^{-2}$ vs. $1.09 \times 10^{-3} \pm 3.55 \times 10^{-3}$ ng/ μ l, $P=0.038$). In conclusion, according to the present study, hsa-miR-99a is involved in cardiac malformation and may serve as a biomarker during fetal development, and therefore presents as a candidate for monitoring cardiomyogenesis and potential use as a NIPT-biomarker for fetal CHD.

Introduction

Congenital heart defects (CHDs) represent the most common form of major birth defects currently affecting ~1% of live births (1). The introduction of reliable markers for early screening and identification of CHDs is likely to reduce the morbidity and mortality of the affected neonates, the economic burden placed on the National Health Service (NHS) and the stress placed on families, including proactive medical

treatment and parental counseling regarding options during the pregnancy following detection.

The present detection methods of screening CHDs comprise fetal echocardiography and various biomarkers, such as β -human-chorionic-gonadotropin (β -hCG) and pregnancy-associated plasma protein A (PAPP-A); however, none of them establish specific indices without high incidences of false-positive results. Recent studies have identified specific microRNAs (miRNAs) associated with cardiogenesis, linked positively to placental miRNA expression. Detection of these in maternal peripheral blood during pregnancy allows for novel biomarkers used for screening and validating fetal CHDs.

While the reported birth prevalence differs worldwide, the estimate of 8/1,000 live births is generally accepted as the best approximation (1). Rosano *et al* (2) linked ~20% of infant fatalities to anomalies present at birth; CHDs account for 28% of mortalities during the neonatal period and 50% in the first 2-12 months of life (2,3).

Studies have reported 25-50% of cases with CHD as severe, requiring invasive diagnostics, and one or more surgical procedures within the neonatal period or during infancy (4,5). Frequently, the affected children struggle with more than heart disease due to the associated defects in other organ systems and substantial neurodevelopmental problems (6). In a statement of the American Heart Association, Marino *et al* (7) reported moderate to severe neurodevelopmental disabilities in >50% of neonates born with severe cardiac defects and in 25% of those with less severe malformations.

Prenatal ultrasonography (US) constitutes one of several standard-screening tools in current obstetric care. Routinely performed at gestational weeks 18-20, fetal echocardiography indicates CHDs by examination of a four-chamber view and additional visualization of the outflow tracts of great vessels and the aortic arch.

While experienced US operators approach sensitivities close to 60-100% for diagnosing major CHDs in the second and third trimester, general screening specificity and sensitivity differ significantly (8). Although high in tertiary care, such as university-associated hospitals, detection rates drop to $\leq 39\%$ when performed in community-based centers or by less experienced operators (9,10).

Among the various biomarkers currently available, the assessment of nuchal translucency prevails as one of the most popular screening markers for cardiac malformations (11). Regularly performed between gestational weeks 11-14,

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measurement of the hypoechogenic space between skin and subcutaneous tissue, covering the cervical spine of the fetus, quantifies the risk for possible cardiac malformations. However, the sensitivity of this screening method depends profoundly on the percentile used as the cut-off point and the experience of the US operator conducting the examination (12,13).

A different approach utilizes maternal serum markers, including elevated levels of free β -hCG and lowered levels of PAPP-A, commonly used for screening Down's syndrome (14). Although highly useful in predicting chromosomal abnormalities when combined, these biomarkers fall short in establishing stand-alone indicators for CHDs (15).

In recent years, research of the fetal products that are able to pass the placental barrier and enter the maternal circulation has increased. Numerous studies linked specific miRNAs associated with cardiogenesis to placental miRNA expression (16,17). Similar to cell-free fetal DNA, these miRNAs enter the maternal peripheral bloodstream during pregnancy and allow for non-invasive prenatal testing, thus introducing novel biomarkers for screening and validating congenital heart malformations (18).

miRNAs belong to a recently discovered family of small endogenous non-coding RNAs and constitute an additional level of control in gene expression. Acting as RNA silencers and at the post-transcriptional level, these molecules modify the expression of 30-60% of human protein-encoding genes (19,20). Approximately 22 nucleotides long, these short, single-stranded RNA molecules interact with their target coding mRNAs to reduce translation by either reducing mRNA stability or inhibiting translation without target degradation (21).

The various locations of the miRNA-encoding genes on the genome allow differentiation to be separated into 3 groups: Intergenic, intronic and exonic miRNAs. While intergenic miRNA genes reside between 2 protein-coding genes with their own promoters and regulatory units, intronic and exonic miRNA genes rely on co-transcription together with their host genes in intron and exon regions, respectively (22).

Deviation of an miRNA-related pathway may lead to serious malformations and/or early death of the affected mammal as shown by Bernstein *et al.* (23) who examined their importance for correct development of mouse embryonic stem cells (ESC) through disruption of the Dicer1 gene. In particular, the changing miRNA expression profile during ESC differentiation and the inability of Dicer-deficient ESC to undergo any type thereof, suggest the involvement of miRNAs in the regulation of transcription factors that are essential for cellular differentiation and survival (24).

Therefore, the present study explores hsa-miR-99a as a marker, examined the correlation between hsa-miR-99a and congenital heart malformations, and therefore, established an evidence-based foundation for future use in diagnostics.

Materials and methods

Patients. A total of 39 pregnant patients with an average age of 31.59 ± 5.47 years and an average gestational age of 22.22 ± 5.20 weeks were included in the study. The participants were divided on grounds of fetal health and assigned to a control group, containing 17 women with healthy fetuses and a

patient group, consisting of 22 women, satisfying the following criteria led to patient group assignment: Positive US indicating one or more congenital heart defects. The Ethical Scientific Board approved the study, patients were informed and they provided signed consent.

Blood sample collection and plasma preparation. Venous blood samples were drawn into 2x9 ml ethylenediaminetetraacetic acid tubes, which were centrifuged at 800 x g at first and subsequently the obtained supernatant was centrifuged at 4,800 x g for an interval of 10 min at 4°C, respectively, using an Eppendorf Centrifuge 5810 R (Eppendorf AG, Hamburg, Germany). Following the second centrifugation, the supernatant maternal plasma was stored in 1.5 ml Eppendorf tubes at -80°C for later miRNA extraction and analysis.

miRNA extraction. miRNA was isolated from 300 μ l maternal plasma using the NucleoSpin® miRNA Plasma kit (Macherey-Nagel GmbH and Co., KG, Düren, Germany). The concentration of total miRNA was measured on a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The purity of each sample was determined by the ratio absorbance (optical density value = A260/A280).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT of 4 μ l total miRNA was performed by applying cDNA-miScript II RT (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions, allowing further amplification. RT-qPCR was performed in a LightCycler II (Roche Diagnostics GmbH, Mannheim, Germany), with the miRCURY LNA™ Universal RT microRNA PCR kit (Exiqon A/S, Vedbaek, Denmark) in combination with the Roche Master mix (Roche Diagnostics GmbH), and preceded the application of hsa-miR-99a-specific primers and the microRNA LNA™ PCR primer set, (Exiqon A/S) targeting the sequence: 5'-AACCCGUAGAUCCGAUCUUGUG-3'.

The results were normalized using the U6-snRNA miRNA, and the relative expression of hsa-miR-99a in the retrieved plasma samples was analyzed with the $2^{-\Delta\Delta Ct}$ method. Sample concentrations of hsa-miR-99a and U6 were calculated in proportion to the globin reference gene used in 4 different quantities (15, 1.5, 0.15 and 0.015 ng/ μ l).

Statistical analysis. All the data are expressed as mean \pm standard deviation non-invasive prenatal testing, and analyzed using Microsoft Excel 2011 version 14.4.6 (Microsoft Corp., Redmond, WA, USA). The statistical significance of differentially expressed miRNAs was computed using Student's two-tailed t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Concentration of miRNA. The 39 samples had an average miRNA concentration of 5.92 ± 1.89 ng/ μ l with a variance of 3.57 ng/ μ l, a median concentration of 5.90 ng/ μ l and $P = 0.15$. The 22 samples collected from the patient group showed an average miRNA concentration of 5.54 ± 1.99 ng/ μ l with a variance of 3.94 ng/ μ l and median concentration of 5.50 ng/ μ l, while the control group containing 17 samples

Table I. Concentration of miRNA.

	Patient group (n=22), ng/ μ l	Control group (n=17), ng/ μ l
Total miRNA concentration		
Average \pm SD	5.54 \pm 1.99	6.40 \pm 1.69
Median	5.50	5.90

SD, standard deviation.

Table II. Concentration of miR-99a.

miR-99a concentration	Patient group (n=22), ng/ μ l	Control group (n=17), ng/ μ l
Average \pm SD	1.78 $\times 10^{-2}$ \pm 3.53 $\times 10^{-2}$	1.09 $\times 10^{-3}$ \pm 3.55 $\times 10^{-3}$
Median	6.45 $\times 10^{-3}$	<0.001 $\times 10^{-3}$

SD, standard deviation.

exhibited an average miRNA concentration of 6.40 \pm 1.69 ng/ μ l with a variance of 2.86 ng/ μ l and median concentration of 5.90 ng/ μ l (Table I).

hsa-miR-99a concentrations. Based upon the normalized U6-snRNA data, the hsa-miR-99a expression profile was normalized as follows: The patient group had an average hsa-miR-99a concentration of 1.78 $\times 10^{-2}$ \pm 3.53 $\times 10^{-2}$ ng/ μ l with a variance of 1.25 $\times 10^{-2}$ ng/ μ l and a median concentration of 6.45 $\times 10^{-3}$ ng/ μ l, whereas the control group showed an average hsa-miR-99a concentration of 1.09 $\times 10^{-3}$ \pm 3.55 $\times 10^{-3}$ ng/ μ l with a variance of 1.26 $\times 10^{-5}$ ng/ μ l and a median concentration of <0.001 $\times 10^{-3}$ ng/ μ l. The difference between the 2 groups was significant with P=0.038 (Table II).

The measured and normalized data presented no significant difference for the average concentration of miRNA, however the hsa-miR-99a concentration showed a statistically significant difference in the expression.

Discussion

In the present study, the plasma expression profile of fetal hsa-miR-99a was investigated in maternal peripheral blood of 22 pregnant patients with CHD-positive fetuses and 17 CHD-free controls by RT-qPCR and differentially expressed miRNAs were identified in affected patients with significantly upregulated miR-99a levels. While there was no significant difference between the isolated miRNA concentrations among the affected and control groups (P=0.15), significant differences were identified in normalized hsa-miR-99a concentrations between the 2 groups (P=0.038).

Similarly, studying the role of the miRNAs associated with fetal heart development, Lazar *et al* (25) analyzed maternal peripheral blood samples and reported increased hsa-let-7c expression levels in cases of cardiac malformations, which is in line with the present observations. Similarly, it is located on the let-7c-cluster on 21q21.1.

Thus far, numerous studies investigated the crucial role of miRNAs in the regulation of cell proliferation and differentiation, formation of complex organ systems, such as the heart, and throughout pregnancy. While the majority of placental miRNAs expressed during the first trimester exhibit angiogenic features, miRNAs promoting cell differentiation are commonly expressed during the third trimester, suggesting that aberrant expression of miRNAs is likely associated with compromised pregnancies.

Trisomy 21 (Down's syndrome) is caused by the presence of a third copy of the human chromosome 21 (hsa21), which leads to altered expression of its products, causing cardiac malformations, mental deficits and phenotypic deviation as described in recent literature. Located on chromosome 21, the let-7c cluster serves a distinguished regulatory purpose in cardiac and fetal development, with its products targeting mRNAs and thereby precisely modifying organ formation. The present results are in accordance with the published data of Coppola *et al* (26), in which miR-99a was also overexpressed in CHD samples. hsa-miR-99a is one of its products and therefore a noteworthy candidate for monitoring cardiomyogenesis.

The estimated average medical costs (AMCs) of CHD-affected children <3 years of age excess the AMC for non-affected children of the same age by 10-20 times, with the greatest cost difference observed in the care of infants (27). Placing a significant economic burden on the NHS, Connor *et al* (28) investigated the cost burden of CHD, examined the associations of the social impact on families and reported 'high levels of stress in terms of finances, emotional drain and family member burden.'

These findings emphasize the fundamental importance of early screening on the identification for neonates with moderate or severe CHD, affected families and society in general. Early awareness permits further testing for associated defects in other organ systems, allocation of additional resources for proactive treatment or pregnancy termination after counseling.

Furthermore, studies of the obesity rate among pregnant women indicate a large-scale body mass index increase in the western hemisphere during the last decades, impeding detection further. According to Fuchs *et al* (29), estimates of maternal obesity range from 10% in France to >28% in the United States of America (29,30). The report concludes that US scans of obese women are feasible, however, they are accompanied by a significant decline in image quality and global anatomical scores, complicating identification of cardiac malformations further. Concisely, the success of fetal echocardiography depends significantly upon maternal obesity, equipment quality and operator proficiency.

Thus, it is reasonable to assume that identification of supplemental methods prior and in addition to second trimester US examination benefit early, accurate and operator-independent assessment of cardiac anomalies significantly. The early awareness and accurate diagnosis of CHDs allow for informed decisions on treatment options, including termination, while appropriate follow-up and delivery in tertiary care centers should improve overall perinatal survival in those pregnancies that continued.

In early embryonic development, following gastrulation and subsequent formation of the 3 germ layers, organ development begins with the formation of the human heart (31). As

morphogenesis, growth and integrated function are essential for survival and require precise cardiac gene expression, deviations in heart development may result in congenital heart disease, the most frequent form of major birth defects in humans (32).

These pathological alterations present at birth, classified as CHDs, affect the structure and function of the heart. Based on size, location and other associated defects, these malformations differ in severity and determine survival (33).

The crucial role of miRNAs in animal development is best explained by the results of their absence, such as due to the prior mentioned disruption of the *Dicer1* gene, significantly altering the miRNA formation. *Dicer1*-deficient mice suffer depletion of functional mature miRNAs and exhibit developmental arrest during gastrulation, causing lethality early in development (23). In particular, cardiac-specific knockout of *Dicer* later in development results in severe dilated cardiomyopathy, heart failure and postnatal lethality (32).

Organisms contain a large set of different miRNA families within their tissues, with ≥ 1 miRNA specifically expressed per tissue, such as in muscle or brain (33). While the miRNA-133 family embodies the most abundant miRNA group expressed in muscle tissue, other families, such as the miRNA-1 family, were also positively identified as muscle-specific miRNAs. However, miRNA-208 is the only known miRNA exclusively expressed in cardiac tissue thus far (34,35).

Cardiac and muscle-specific expression of the highly conserved miRNA-1 (miRNA-1-1, miRNA-1-2 and miRNA-206) and miRNA-133 (miRNA-133a, -133a-2 and miRNA-133b) families follow a strict spatiotemporal pattern in vertebrates (35). In a gain-of-function study by Chen *et al* (35), overexpression of miRNA-1 in transgenic *Xenopus laevis* led to thin-walled ventricles due to premature myocyte differentiation and lethality in early embryonic development. Concordantly, overexpression of miRNA-1 in developing *Xenopus laevis* resulted in accelerated myogenic differentiation and decreased cardiac tissue proliferation, and while the exact target structures differ, misexpression of miRNA-133 also led to pathological tissue formation, frequently including disorganized looping and chamber formation (36).

The cardiac-specific miRNA-208 family (miRNA-208a, miRNA-208b and miRNA-499) induces similar pathological conditions in hosts following overexpression, such as gain-of-function studies in mice with miRNA-208a misexpression induced cardiac hypertrophy and conduction deficits (36).

While current research on miR-99a is rare or generally focuses on the oncological role, there are only a few studies regarding the role of miR-99a in heart development and function. Of those, the majority focus on its role in recovering from myocardial infarction when miR-99a is overexpressed. The present study shows the overexpression of miR-99a in the maternal plasma of CHD fetuses. More extensive investigations are required to identify reliable miRNA-based biomarkers for the prenatal detection of CHDs.

In conclusion, in order to evaluate the role of miRNAs in the development of CHDs, the present study found differentially expressed levels of hsa-miR-99a from pregnant patients positively screened for fetal CHD and pregnant women without any signs of fetal malformation. miR-99a is a prospective

novel biomarker that provides valuable information for further research of predictive markers and possible therapeutic target structures of cardiac malformation at a molecular level.

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