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Identification of maternal serum microRNAs as novel non-invasive biomarkers for prenatal detection of fetal congenital heart defects

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ABSTRACT

Background: Congenital heart defects (CHD) are the most common form of malformation during embryonic development. Circulating miRNAs have recently been shown to serve as diagnostic/prognostic biomarkers in patients with cancers. Our current study focused on the altered expression of maternal serum miRNAs and their correlation with fetal CHD.

Methodology/principle findings: We systematically performed SOLiD sequencing followed by individual quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays to identify and validate the expression of maternal serum miRNAs at 18–22 weeks of gestation. Four miRNAs (miR-19b, miR-22, miR-29c and miR-375) were found to be significantly up-regulated in pregnant women with fetal CHD, with an area under the receiver operating characteristic (ROC) curve (AUC) of 0.79, 0.671, 0.767 and 0.693, respectively. Furthermore, the combination of the four miRNAs using multiple logistic regression analysis showed a larger AUC (0.813) that was more efficient for the early detection of fetal CHD.

Conclusions/significance: We identified and validated a class of four maternal serum miRNAs which could act as novel non-invasive biomarkers for the prenatal detection of fetal CHD.

1. Introduction

Congenital heart defects (CHD) are the most common major congenital malformation, comprising numerous structural and functional abnormalities of the heart and great vessels, with a prevalence of approximately 8 in every 1000 newborn infants [1]. Although details of the underlying causes of CHD have been well studied [2,3], such defects remain a serious problem, accounting for approximately 40% of perinatal deaths and more than one fifth of deaths in the first month of life [4]. Multiple previous studies have reported that in the fetal deaths, the incidence of CHD is associated with the gestational age of fetal loss. The main causes of the earliest deaths are the presence of complex CHDs [5]. Furthermore, it is proved that the earlier the diagnosis of CHD, the better the prognosis [6]. Thus, the prenatal detection of fetal CHD is a key to the decrease of the mortality and the improvement of the prognosis of individuals with CHD.

Even with the advent of fetal echocardiography as a screening tool for CHD, cardiac abnormalities are still overlooked during routine prenatal care, with disappointing detection rates ranging from 6% to 35% [7–9]. In addition, results of ultrasound examinations vary from center to center due to a lack of standardization [10]. Several studies have reported that many factors influence the accuracy of prenatal sonographic investigations to detect CHD, such as the experience of operators, the quality of the ultrasound equipment, the lesion type, different departmental policies and guidelines [11–15]. Recently, biomarkers have been found to correlate with CHD in utero, including elevated levels of nuchal translucency (NT), free beta-human chorionic gonadotropin (β-hCG) and lowered levels of pregnancy-associated plasma protein-A (PAPP-A) in the first trimester[16–21]. However, differences in these CHD biomarkers are not specific enough to be used as biomarkers for fetal CHD screening [22].

MicroRNAs (miRNAs) are a class of small non-coding RNAs which are 19–23 nt in size. To date, more than 800 miRNAs have been identified in animal cells and have been reported to be involved in various biological processes, including cell growth, the modulation on differentiation, cell proliferation and apoptosis [23–25]. An increasing number of studies have shown that circulating miRNA levels could have great potential as novel prognostic and predictive biomarkers for many cancers or to assess treatment outcomes. They have unique merits for these roles, including their abundance, stability, ease of

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detection and disease-specific nature [26–29]. A relationship between miRNAs and cardiogenesis has been identified [30,31]. Moreover, specific miRNAs that relate to fetal CHD can be found in placental tissues from fetuses with CHD, and it has been shown that miRNAs of placental origin can also be detected in the peripheral blood of pregnant women [32,33].

Herein, we hypothesized that maternal serum miRNA could act as candidate biomarkers for the prenatal detection of fetal CHD in relatively early pregnancy. This present study applied SOLiD sequencing to the screening of maternal serum miRNAs systematically and performed individual quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays to validate them.

2. Materials and methods

2.1. Study design and patient samples

This multistage nested case-control study was designed to identify and confirm that maternal serum miRNA levels could detect fetal CHD in the second trimester. 98 participants in this study were recruited at the Nanjing Maternity and Child Health Care Hospital of Nanjing Medical University between July 2011 and June 2012. Women with a history of heart disease, a family history of cardiovascular disease, pregnancy complications and multiple pregnancies were excluded (35 cases). In addition, if the affected fetuses have a diagnosis that may have caused the CHD (e.g., Down's, 22q11DS, CHARGE, etc), the pregnant women were also subtracted (3 cases). At last, a total number of 60 pregnant women were selected as the subjects of the study. Thirty pregnant women bearing a single fetus with a CHD were chosen as cases, while the other thirty women bearing a single normal fetus were defined as controls. All the thirty cases were fetuses with ventricular septal defect (VSD), atrial septal defect (ASD) and tetralogy of Fallot (TOF). The diagnosis of fetal CHD was confirmed with fetal echocardiography. In order to reduce heterogeneity, controls were matched with cases on the basis of gestational age and maternal age. The study protocol was approved by Nanjing Medical University and Nanjing Maternity and Child Health Care Hospital of Nanjing Medical University, and was conducted according to the tenets of the Declaration of Helsinki. Clinical information for each subject was collected from their obstetric medical records, and written informed consent was also obtained from all the participants before their enrollment in this research.

The study was divided into two stepwise phases. In phase I (biomarker discovery), we randomly pooled the maternal serum of three pregnant women with fetal CHD and three matched controls, respectively, to identify the differential miRNAs profile by SOLiD sequencing between these two groups. The three cases represented the three specific CHDs. By comparing the relative expression level of maternal serum miRNAs, significantly up- or down-regulated miRNAs were preliminarily selected for further analysis in the next phase. In phase II, we subsequently conducted quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on the rest 27 women who were pregnant with a child with fetal CHD and 27 women with healthy fetuses to validate the miRNAs which was initial screened. Based on the results of this phase, receiver operating characteristic (ROC) curve-based risk assessment analysis was conducted to assess the sensitivity and specificity of maternal serum for predicting fetal CHD.

2.2. Serum preparation and RNA extraction

A venous blood sample of up to 5 ml from each participant in the case and control groups was collected at the routine 22–28 week obstetric examination. Each sample was aliquoted into a procoagulant drying tube. The whole blood was allowed to stand for 30–50 min at room temperature before centrifugation at 4000 rpm for 10 min to separate them into serum and cellular fractions. The supernatant serum was aliquoted into 1.5-ml Eppendorf tubes and stored at −80 °C until the further analysis.

Total RNA, including miRNAs, was purified from 400 μl serum by using the mirVana PARIS Kit (Ambion, Foster City, USA), according to the manufacturer's protocol with some minor modifications [34]. As there is currently no consensus on sample normalization for the qRT-PCR analysis, we spiked in a synthetic *Caenorhabditis elegans* miR-39, (cel-miR-39; Invitrogen, CA) at a final concentration of 10^{-4} pmol/μl for each serum sample after the addition of 2X Denaturing Solution, which was provided in the kit. Owing to the absence of homologous sequences in humans, cel-miR-39 could control sample-to-sample variations in the RNA extraction and/or purification procedures as a housekeeping miRNA [35,36]. Then, the aqueous phase was pipetted onto the Filter Cartridge after organic isolation. The RNA pellet was washed and dried by spinning the assembly for 1 min according to the manufacturer's instructions. Then, we dissolved the RNA in 20 μl of preheated (95 °C) Elution Solution. RNA was measured using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE) to assess its quantity and quality, and stored at −80 °C. In general, we simultaneously performed RNA extraction and cDNA transcription for all subjects. Furthermore, the serum must be stored under the same conditions and added in equal volumes during every experiment step to reduce any potential bias.

2.3. SOLid sequencing

In the discovery stage, we screened differentially expressed miRNAs with the use of the SOLiD version 2 sequencing system (Applied Biosystems). Briefly, we took equal mass of total RNA from each sample to hybridize and ligate overnight with 5′ and 3′ adaptors, reverse-transcribed, RNase H-treated and PCR amplified. Subsequently, we cleaned up the PCR products and selected them on agrose gels by size of 105–150 bp. Template bead preparation, emulsion PCR, and deposition were conducted in order. After the completed sequencing run, mapping of SOLiD reads were analyzed by SOLiD System Small RNA Analysis Pipeline Tool (RNA2MAP, version 0.5.0). We decoded the barcodes. If the reads matched a barcode uniquely, they were used for mapping. Following the calculation of length distribution and mapping to other small RNAs reference of rRNA, snRNA, snoRNA and tRNA, reads were compared with miRBase (release 15.0 at http://microRNA.sanger.ac.uk/). Finally, we mapped reads to the database of human genome. Since low copy number was less liable, only the miRNAs with more than 10 copies were picked up. Fold changes were calculated based on the expression profiles which were normalized to the total counts of 1,000,000. Based on the SOLiD sequencing results, 11 significantly varied miRNAs were initially selected for the validation stage.

2.4. Reverse transcription (RT) and quantitative PCR (qPCR)

The total RNA was reverse-transcribed to cDNA with the TaqMan MicroRNA reverse transcription kit and miRNA-specific stem-loop primers (Applied Biosystems Inc.). The 15-μl reaction mix consisted of 0.15-μl of 100 mM dNTP mix, 1 μl of Multiscribe RT enzyme (5 U/μl), 1.5 μl of 10 × RT Buffer, 0.19 μl of RNase Inhibitor (20 U/μl), 7.16 μl of nuclease-free water, 3 μl of TaqMan RT primer and 2 μl total RNA. Reverse transcription was initiated at 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, and held at 4 °C. Subsequently, real-time PCR was performed in triplicate for each maternal serum sample and no-template negative controls included. For the final volume of 20 μl reaction, 1 μl synthesized cDNA was mixed with 8 μl diethylpyrocarbonate (DEPC)-treated water, 10 μl TaqMan Gene Expression Master Mix and 1 μl TaqMan MicroRNA Assay (Applied Biosystems). The MicroRNA Assay IDs was shown in Table 3. The mixture was incubated at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and