Rise in antifibrotic and decrease in profibrotic microRNA protect the heart against fibrosis during pregnancy: A preliminary study

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Conflict of interest

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Abstract

Background. Physiological pregnancy is associated with volume overload. Unlike cardiac pathologies linked with volume overload, such as mitral or aortic regurgitation, pregnancy is thought to be unrelated to fibrosis of the heart. However, changes in the cardiac extracellular matrix during pregnancy remain poorly understood.

Objectives. The aim of the study was to examine the expression of 11 microRNAs associated with cardiac fibrosis (miR-21, miR-26a, miR-26b-5p, miR-29b-3p, miR-29c-3p, miR-101a, miR-146a, miR-208a, miR-223 and miR-328) during pregnancy and to compare them with a healthy control group.

Material and methods. Six women in singleton pregnancy (30–36 weeks) and 6 non-pregnant women as a control group were included in the study. Each woman underwent an echocardiographic examination, and had blood pressure on both arms measured and a blood sample taken. MicroRNAs expression was analyzed using Custom TaqMan® Array MicroRNA Cards (Applied Biosystems, Foster City, USA).

Results. Median age of the pregnant women was 34 years (range 25–39 years) and of the control group 32 years (range 29–43 years). Median week of pregnancy was 34 years (range 31–36 years). Most of the examined microRNAs had a lower expression in the pregnancy group (fold change 1.0).

Conclusions. In the 3rd trimester of physiological pregnancy, there is a 244% increase in expression of miR-101a and a decrease by 73% in expression of miR-328. Both of these changes can protect against fibrosis during volume overload occurring in physiological pregnancy.

Key words: pregnancy, microRNA, cardiac remodeling, cardiac fibrosis, volume overload

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Introduction

Extracellular matrix (ECM) plays a pivotal role in the proper systolic and diastolic function of the heart as well as in transduction of electrical and chemical signals. It provides structural support for the cardiomyocytes, allowing them to perform their mechanical function. It also ensures communication between cardiomyocytes and fibroblasts. Collagen types I and III are the main types of fibrillar collagens in the cardiac muscle. They are produced by cardiac fibroblasts. The secretion profile of cardiac fibroblasts is influenced by several factors, such as neurohormonal activation, inflammation, age, injury and overload.^{1,2} In reaction to different stress stimuli, the action of cardiac fibroblasts changes in order to maintain ECM homeostasis.

Volume overload results in chamber dilation, eccentric cardiac myocyte hypertrophy and changes in ECM remodeling. Increased preload causes augmentation in mechanical stress and rise in neurohormonal activation. Mitral and aortic valve regurgitation are the primary pathological causes of volume overload.³ This remodeling is different from the reaction to pressure overload. Volume overload is characterized by thinning of the wall and chamber dilatation, which changes the ratio of left ventricle end-diastolic diameter to wall thickness and increases mechanical stress on the wall of the heart. This may be due to expression of anti-fibrotic factors by cardiac myocytes in response to acute volume overload, leading to changes in the proportion between cardiac-specific metalloproteinases and tissue inhibitors of metalloproteinase. As a result, degradation of ECM appears and a structure of changed geometry and shifted proportions between collagen I and III is produced. During end-stage heart failure, which develops as a consequence of prolonged volume overload, an increase of collagen is noted.³

Pregnancy is a state which results in progressive continuous volume overload of the heart due to a rise in blood volume with a decrease of peripheral vascular resistance.⁴ In this situation, volume overload causes mild eccentric hypertrophy to develop, thought to be physiological. Crucially, the increase in chamber dimension coexists with a proportional increase in wall thickness as well as a proportional increase in myocyte length and width. The mass of the left and right ventricle enlarges.⁵ The hemodynamic changes develop throughout the pregnancy and reach their peak in the 3rd trimester, around 30–34 weeks of the pregnancy. It is thought that pregnancy-related cardiac remodeling is not related to fibrosis, but changes in the ECM remain poorly understood.

The aim of this study was to examine the expression of microRNAs associated with fibrosis in pregnant women in the 3rd trimester of pregnancy in order to identify the potential mechanism of ECM remodeling during pregnancy. The hypothesis was that, in pregnant women, an increase in activity of antifibrotic microRNAs or a decrease

in profibrotic microRNAs will be observed. This could be a protective mechanism to prevent fibrosis in a state of cardiac overload.

Material and methods

Study population

Six women in singleton pregnancy and 6 non-pregnant women as a control group were included in the study. The recruitment took place in the Cardiology Department of the Institute of Mother and Child in Warszawa, Poland, from October 2013 to October 2014. The study was approved by the Bioethics Committee of the Institute, in accordance with the principles of Good Clinical Practice and the Declaration of Helsinki. All patients gave written informed consent. Inclusion criteria were age >18 years old and singleton pregnancy between 30 and 36 weeks of gestation. Exclusion criteria included known prior cardiovascular disease such as congenital heart disease, myocardial infarction, heart failure, prior myocarditis, clinically relevant arrhythmia, hypertension, and hypertension associated with pregnancy. Each woman underwent an echocardiographic examination, and had blood pressure on both arms measured and a blood sample taken.

Sample preparation and RNA isolation

Blood was collected and subjected to centrifugation. Obtained serum samples were stored in -80°C until the extraction of RNA. Total RNA containing a fraction of miRNA was isolated from 350 μL of each serum sample with a mirVana PARIS Kit (Ambion: Thermo Fisher Scientific, Waltham, USA), according to the manufacturer's protocol.

MicroRNA measurement

Expression of microRNA was analyzed using Custom TaqMan[®] Array MicroRNA Cards (Applied Biosystems, Foster City, USA). The selected miRNAs were reverse-transcribed using a TaqMan[®] MicroRNA Reverse Transcription Kit (Applied Biosystems Foster City, USA), according to the manufacturer's instruction. Briefly, 3 μL of sample containing 18 ng of total RNA was added to a prepared reverse-transcription (RT) reaction mix consisting of 6 μL of custom RT primer pool, 0.3 μL of deoxynucleotides (dNTPs) with deoxythymidine triphosphate (dTTP) (100 mM), 3 μL of MultiScribe Reverse Transcriptase (50 U/ μL), 1.5 μL of 10 $\times$ RT buffer, 0.19 μL of RNase inhibitor (20 U/ μL) and 1.01 μL of nuclease-free water (Ambion; Thermo Fisher Scientific, Waltham, USA). A reverse transcription reaction was carried out under the following thermal conditions: 16 $^{\circ}\text{C}$ for 30 min, 42 $^{\circ}\text{C}$ for 30 min, 85 $^{\circ}\text{C}$ for 5 min and then the RT mixture was held at 4 $^{\circ}\text{C}$. Next, the preamplification reaction was

performed using TaqMan® PreAmp Master Mix, ×2 and Custom PreAmp Primer pool (Applied Biosystems, Foster City, USA), according to the protocol of the manufacturer. In brief, 22.5 µL of the preamplification reaction mix was combined with 2.5 µL of each RT product and the reaction was run with thermal-cycling conditions specified by the manufacturer. The preamplification reaction products were diluted 8-fold with ×0.1 TE buffer, pH 8.0 (Ambion; Thermo Fisher Scientific, Waltham, USA) to a final volume of 200 µL. To prepare the quantitative polymerase chain reaction (qPCR), 1.13 µL of diluted PreAmp product was combined with 56.25 µL of TaqMan® Universal Master Mix II, No AmpErase UNG (×2) (Applied Biosystems, Foster City, USA) and 55.12 µL of nuclease-free water (Ambion; Thermo Fisher Scientific, Waltham, USA), and then mixed. Finally, 100 µL of the qPCR reaction mix was dispensed into each port of the TaqMan® MicroRNA Array Card (Applied Biosystems, Foster City, USA), subjected to centrifugation (2 × 1 min, 1200 rpm) and mechanically sealed. Quantitative reverse-transcription polymerase chain reactions (qRT-PCRs) were performed in triplicate for each sample and carried out on ViiA7 Real-Time PCR System (Applied Biosystems, Foster City, USA) with thermal cycling conditions recommended by the manufacturer. Automatic baseline and manual cycle threshold (Ct) were set to 0.2 for all samples. The relative expression level of each miRNA was calculated using the delta delta cycle threshold ($\Delta\Delta Ct$) method. For analysis, we selected 11 microRNAs associated with fibrosis: miR-21 (Assay ID 000397), miR-26a (Assay ID 000405), miR-26b-5p (Assay ID 000407), miR-29a-3p (Assay ID 002112), miR-29b-3p (Assay ID 000413), miR-29c-3p (Assay ID 000587), miR-101a (Assay ID 002253), miR-146a (Assay ID 000468), miR-208a (Assay ID 000511), miR-223 (Assay ID 002295) and miR-328 (Assay ID 000543). Five small RNAs (HY3 (Assay ID 001214), U6 (Assay ID 001973), RNU44 (Assay ID 001094), RNU48 (Assay ID 001006) and miR-103 (Assay ID 000439) were chosen and tested as candidates for endogenous controls. Finally, miRNAs levels were normalized to a U6 snRNA molecule.

Statistical analysis

Due to the small number of participants, variables were not tested for a normal distribution and in all statistical analyses nonparametric tests were used. Continuous variables are represented as median and range of collected values. Categorical variables are presented as number (percentage). In comparisons of nonparametric variables between 2 studied groups, the Mann-Whitney U test was used. To compare nominal variables, a χ^2 test was performed. ΔCt and $\Delta\Delta Ct$ were calculated. Descriptions regarding microRNA findings were presented in 2 forms – as frequency of expression, defined as the number of subjects in whom microRNA expression was found/

sample size, and as fold change in microRNA expression between the pregnancy group and the control group. Fold change was defined as the amount of times microRNA expression was changed between the control group and pregnancy. A difference in fold change between the groups larger than 2 or less than 0.5 was considered relevant; p-value <0.05 was regarded as statistically significant. SPSS Statistics v. 21 (IBM Corp., Armonk, USA) was used for statistical analysis.

Results

Study group characteristics

The median age of the pregnant women was 34 years (range 25–39 years) and of the control group was 32 years (29–43 years). The median week of pregnancy was 34 (range 31–36 weeks). Upon echocardiographic examination, we did not find any abnormalities to exclude any participant from further analysis. The median left ventricle ejection fraction in the pregnancy group was 60.5% (range 57–62%) and in the control group was 64.5% (range 59–72%) (Mann-Whitney U test = 23.5; p = 0.394). The walls of the heart were thicker in the pregnancy group: for the intraventricular septum the median thickness was 9.5 mm in the pregnancy group (8–11 mm) and 8 mm for the control group (6–11 mm) in diastole (Mann-Whitney U test = 6.5; p = 0.065). For the posterior wall, the median thickness was 8 mm in the pregnancy group (7–10 mm) and 7.5 mm for the control group (7–8 mm) in diastole (Mann-Whitney U test = 9; p = 0.18). The median systolic left ventricle diameter was 32 mm (25–40 mm) in the pregnancy group and 30 mm in the control group (28–37 mm) (Mann-Whitney U test = 16; p = 0.818) and for diastolic the left ventricle diameter was 44.5 mm (39–50 mm) in the pregnancy group and 49 in the control group (40–51 mm) (Mann-Whitney U test = 25; p = 0.310). Upon examination of mitral inflow, the E/A ratio was lower in the pregnancy group (1.19 vs 1.35; Mann-Whitney U test = 25; p = 0.310). A higher dimension of the left atrium (34.5 vs 30.5 mm; Mann-Whitney U test = 8; p = 0.132) and right ventricle (23.5 vs 20.5 mm; Mann-Whitney U test = 7.5; p = 0.093) were observed in the pregnancy group.

Frequency of expression of selected microRNAs

Firstly, we assessed whether the selected microRNAs were expressed in participants from both study groups by calculating expression frequency. Full results are shown in Table 1. In brief, most of the selected microRNAs (miR-21, miR-26a, miR-29a-3p, miR-146a and miR-223) were found in all of the participants, regardless of the study group. There was no presence

Table 1. Frequency of expression of selected microRNAs – number of subjects in whom microRNA expression was found/sample size

Group	miR-21	miR-26a	miR-26b-5p	miR-29a-3p	miR-29b-3p	miR-29c-3p	miR-101a	miR-146a	miR-208a	miR-223	miR-328
Control	6/6	6/6	6/6	6/6	0/6	5/6	3/6	6/6	0/6	6/6	6/6
Pregnancy	6/6	6/6	5/6	6/6	0/6	6/6	4/6	6/6	0/6	6/6	5/6

Table 2. Fold change and interquartile range (IQR) for selected microRNAs in pregnant women in comparison to healthy controls. Difference in fold change between the group larger than 2 or less than 0.5 is considered relevant

Pregnancy	miR-21	miR-26a	miR-26b-5p	miR-29a-3p	miR-29c-3p	miR-101a	miR-146a	miR-223	miR-328
Fold change	0.57	0.60	0.66	1.30	0.95	2.44	0.72	0.91	0.27
IQR (25–75%)	0.23–2.64	0.40–1.25	0.43–2.22	0.72–1.86	0.61–1.96	0–6.42	0.56–1.41	0.72–1.65	0.11–1.29

of miR-29b-3p or miR-208a detected in any of the serum samples. Both miR-26b-5p and miR-328 were found in all of the participants from the control group and 5 in the pregnancy group (83.3%). Expression of miR-29c-3p was detected in 5 samples from the control group and 6 samples from the pregnancy group. As for expression of miR-101a, it was identified in 3 subjects from the control group and 4 from the pregnancy group.

Fold change for selected microRNAs

Further analysis to establish fold change for selected microRNAs in pregnant women in comparison to healthy controls was performed (Table 2). Most of the examined microRNAs had a lower expression in the pregnancy group expressed by a fold change <1.0. The lowest fold change was observed for miR-328, miR-21 and miR-26a. Fold change for miR-29a-3p and miR-101a was >1.0. For miR-101a, fold change between pregnant participants and control group was 2.44 and for miR-29a-3p it was 1.30.

Discussion

Our results show that expression of miR-101a and miR-328 are changed in pregnancy compared to non-pregnant women. Expression of miR-101a increases and miR-328 decreases.

MicroRNA-101a

The data regarding the role of miR-101a indicates that it might play a pivotal role in protecting against fibrosis modulated by transforming growth factor beta signaling (TGFβ). In an animal model of myocardial infarction, it was observed that administration of miR-101a significantly suppressed the expression of TGFβ receptor type 1 (*TGFβRI*), cardiac fibroblast differentiation and collagen content of cardiac ECM. Moreover, overexpression of miR-101a lowered the migration and proliferation of cardiac fibroblasts in response to hypoxia.⁶ It was also observed that miR-101a inhibited the hypoxia-induced

up-regulation of *TGFβRI* but not TGFβ itself in cardiac fibroblasts. Additionally, it reversed the hypoxia-induced proapoptotic intracellular changes and reduced calcium overload.⁶ These indicate the possible role of miR-101a in protecting against hypoxia-induced apoptosis.

MicroRNA-328

Among many others, microRNA miR-328 was tested as a marker of myocardial infarction. He et al. proved that miR-328 and miR-134 are elevated in patients with myocardial infarction.⁷ It was also proved that miR-328 was a predictor of mortality and development of heart failure in a 6-month observation. This remained significant after including values of troponin, NT-proBNP, age, gender and smoking status into the multivariable analysis model. It was also proven to be a good biomarker for differentiation of heart failure with reduced or preserved ejection fraction.⁸ Interestingly, an up-regulation of miR-328 suppresses matrix metalloproteinase-2 in human osteosarcoma, influencing negatively the metastatic ability of the cancerous cells.⁹ This shows its role on the extracellular matrix in different organs and pathological states. High levels of miR-328 seem to have a profibrotic influence on cardiac tissue, resulting in worse prognosis in patients after myocardial infarction. A possible mechanism might be connected to the suppression of metalloproteinases, as in osteosarcoma cells.

Other microRNAs

In our study, there were no differences between the groups in fold change of miR-21, miR-26a, miR-26b-5p, miR-29a-3p, miR-29c-3p, miR-146a and miR-223.

Both miR-146a and miR-21 were assessed in ischemic heart disease. The data suggests that those miRs are related to left ventricle remodeling after myocardial infarction.¹⁰ It is suggested that miR-21 is an important factor of cardiac fibrosis mediated by angiotensin II, through the osteopontin pathway and by increasing fibroblast survival.¹¹ Other studies suggest, however, that angiotensin II does not influence the level of miR-21 in cardiac fibroblasts.¹²

On the other hand, miR-26a seems to be an antifibrotic agent whose expression level depends on the activity of NF- κ B. A rise in the level of miR-26a attenuates collagen I and connective tissue growth factor gene expression in the presence of angiotensin II.¹³ Moreover, miR-26 has been linked with stopping cardiac hypertrophy through attenuating glycogen synthase kinase 3 β expression.¹⁴

Other highly examined microRNAs in the context of fibrosis are those of the miR-29 family. They seem to be involved in fibrosis processes in many different organs.¹⁵ The miR-29 family suppresses the expression of several collagens and ECM proteins. Also, miR-29b is downregulated after myocardial infarction, probably influencing scar formation.¹⁶ In vitro studies, pre-miR-29b reduced collagen and matrix metalloproteinase-2 secretion by cardiac fibroblasts. MiR-29b seems to reduce the profibrotic influence of transforming growth factor beta.¹⁷ Others point to its involvement in apoptosis and angiogenesis after myocardial infarction.¹⁸

Most mechanisms of action of the miRs described above, in which we did not notice any differences between the 2 examined groups, are related to angiotensin II and its negative influence on cardiac muscle. Very little data is available on the renin–angiotensin–aldosterone system in physiological pregnancy. The data on its comparison between pregnant and non-pregnant women is also scarce. However, this problem has been studied in the context of pregnancy-induced hypertension and preeclampsia. Most available data compares the activity of angiotensin II and angiotensin-converting enzyme between normal pregnancy and the preeclampsia state. Some observations show that, from the renin–angiotensin–aldosterone system, only plasma levels of angiotensin II are elevated in preeclampsia compared to healthy pregnant women. Serum levels of angiotensin 1–7, known for its hypotensive role, were reduced in preeclampsia when compared to normal pregnancy.^{19,20} According to other researchers, the levels of angiotensin II is comparable between normal pregnancy and severe preeclampsia in the last weeks of pregnancy.²¹ It is suggested that although angiotensin II levels are comparable between normal pregnancy and preeclampsia, there is a lower response to the vasoconstrictive action of angiotensin II. There seems to be an increase of angiotensin II inactivation by angiotensinase in the serum and placenta with advancing gestation in normal pregnancy.^{19,20,22} Therefore, the data on the function and role of the angiotensin–aldosterone system is conflicting and is based on a small amount of data. These observations should be a topic for further studies in order to establish whether the heart of a pregnant woman in a normal pregnancy remains immune to the negative influence of the angiotensin–aldosterone system and what are the possible underlying mechanisms.

It is worthy of note that the data regarding microRNA associated with fibrosis mainly concentrates on patients with myocardial infarction. Data concerning microRNA expression in volume overload is available mostly for animal models and on a small number of subjects.^{23,24}

One study compares microRNA expression in patients with bicuspid aortic valve with either stenosis or insufficiency requiring surgical treatment. In this study, microarrays containing 1421 miRNAs were used. The authors describe a 65% reduction in miR-26a, 59% reduction in miR-195 and a 62% reduction in miR-30b in patients with aortic stenosis in comparison with aortic insufficiency. The authors comment that, additionally in vitro testing, these miRNAs may modulate the genes responsible for the calcification process.²⁵

Conclusions

The results of our study show that in physiological pregnancy in the 3rd trimester, there is a 244% increase in the level of miR-101a and a decrease by 73% in the levels of miR-328. Both of these changes can be protective of fibrosis during the volume overload occurring in physiological pregnancy.

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