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Research Article

Dysregulation of micro-RNA contributes to the risk of unexplained recurrent pregnancy loss

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ABSTRACT

Although there are plenty of evidence that dysregulation of microRNA (miRNA) level is involved in many human diseases, it is still unknown whether abnormal levels of specific miRNAs are associated with recurrent pregnancy loss (RPL). We believe that such an association do exist as this study confirmed significant differences in the level of specific miRNAs between RPL cases and healthy controls. The study was conducted on 100 Palestinian women: 60 patients with at least two unexplained consecutive pregnancy losses half of them were pregnant at the first trimester and the rest were non-pregnant and 40 healthy controls with at least two live births and no history of pregnancy loss; half of them were at their first trimester of pregnancy and the rest were non-pregnant. We investigated the relative expression of miR-21, miR-126, miR-155, miR-182, miR-222 and miR-517* using quantitative real-time polymerase chain reaction and Ct method experiments. Differential expression was evaluated using Student t-test and fold change analyses. The expression difference of miR-21, miR-126 and miR-182 between patients and controls in the pregnant subjects showed statistically significant difference (p-value $\leq$ 0.05) with fold decrease of 1.5, 1.6 and 5.6, respectively. In the non-pregnant group miR-21, miR-126, miR-222 and miR-517* expressions were significantly different with fold decrease of 2.4, 2.9, 2.7 and 11.8, respectively. In conclusion, at least miR-21 and miR-126 could serve as potential markers for idiopathic RPL as their levels were significantly lower in patients before being pregnant and during pregnancy. Moreover, restoration of the normal level of those miRNAs might be a novel intervention strategy in unexplained RPL.

Keywords: miRNAs, Recurrent pregnancy loss

INTRODUCTION

Micro-RNAs are small, evolutionarily conserved, single stranded, non-coding RNA molecules that bind target mRNA to prevent protein production by one of two distinct mechanisms: translation inhibition or degradation of mRNA. Mature miRNA is generated through two-step cleavage of a precursor miRNA (pre-miRNA), where RNA-induced silencing complex (RISC) is involved in the process (Hutzinger and Izaurralde, 2011).

The miRNA functions as a guide by base-pairing with target mRNA to negatively regulate its expression. The level of complementarity between the guide and mRNA target determines which silencing mechanism will be employed; cleavage of target mRNA with subsequent degradation or translation inhibition (Hutzinger and Izaurralde, 2011).

Earlier studies have revealed that miRNAs have key roles in diverse regulatory pathways, including control of developmental timing, hematopoietic cell differentiation, apoptosis, cell proliferation and organ development (Kim, 2005).

miRNAs have also been implicated in various multifactorial human diseases such as cancers (Calin and Croce, 2006), cardiovascular disease (Thum et al, 2008),

primary muscular disorders (Eisenberg et al., 2007) and diabetes (Joglekar et al., 2011).

Studies on miRNA expression across several organs have revealed that miRNA expression is tissue-specific, and that some miRNAs are also expressed abundantly in placenta (Kotlabova et al., 2011). Therefore, miRNAs produced predominantly in the placenta are probably involved in placental differentiation and maintenance of pregnancy.

Cell-free placental DNA and/or RNA in maternal plasma are possible molecular markers for noninvasive prenatal monitoring or early detection of pregnancy-associated adverse outcomes (Miura et al., 2010).

RPL has been historically defined as three or more consecutive pregnancy losses before 20 weeks of gestation. An estimated 1% of couples attempting pregnancy suffer three or more consecutive losses, and as many as 5% have two or more consecutive losses (Coulam, 1991). Causes of RPL can be categorized as genetic abnormalities, hormonal and metabolic disorders, uterine anatomic abnormalities, infectious causes, autoimmune disorders, thrombophilic disorders, alloimmune causes, and idiopathic. This latter group accounts for over 50% of RPL cases (Lee and Silver, 2000).

As the normal pregnancy is controlled and maintained by the action of several maternal and fetal genes and the simultaneous regulation of genes' expression is possible by miRNAs, the study of some of these miRNAs and their expression in normal pregnancy and unexplained RPL is both plausible and justified.

METHODS

After having the subjects' written informed consent and the approval of the local ethics committee, samples of peripheral blood (4 mL) were collected into EDTA tubes from a 100 women: 60 patients with at least two unexplained consecutive pregnancy losses half of them were pregnant at the first trimester and the rest were non-pregnant and 40 healthy controls with at least two live births and no history of pregnancy loss; half of them were at their first trimester of pregnancy and the rest were non-pregnant. Both groups were recruited from Gaza strip private Ob/Gyn clinics, IVF centers and hospitals. All subjects were in the age 18-35 years and their husbands were not their family relatives.

To harvest cell-free plasma, whole blood samples were centrifuged twice at 1200×g for 10 min at room temperature. RNA was extracted immediately after sample collection using miRNeasy RNA isolation kit (Qiagen, USA), according to the manufacturer protocol.

For this study six miRNAs were selected (miR-21, -126, -155, -182, -222 and -517*). This set of miRNAs was analyzed using the real-time RT–PCR scheme for miRNA quantification according to the protocol of Applied Biosystems (P/N: 4364031); this two-steps protocol consists of reverse transcription with a miRNA-specific primer, followed by real-time PCR with TaqMan probes. The TaqMan miRNA assay kits used were also purchased from Applied Biosystems. In brief, for each RT–PCR 10 ng RNA was reverse transcribed to cDNA using 3 µl specific looped RT primers (Applied Biosystems, USA). The 15 µl reactions were incubated in a BioRad thermocycler for 30 min at 16 ºC, 30 min at 42 ºC, 5 min at 85 ºC and then kept at 4 ºC. Real-time PCR was performed in duplicate using a standard protocol on the Applied Biosystems 7500 real time PCR System. The reactions were incubated in a 96-well plate at 95 ºC for 10 min, followed by 40 cycles at 95 ºC for 15 s and 60 ºC for 1 min. In each sample the relative amount of miRNA was calculated using the comparative threshold method using miR-223 as the endogenous control with ΔCt= Ct (miRNA) – Ct (miR-223). Relative quantification of miRNA expression was calculated $2^{-\Delta Ct}$ method which was calculated using the following formula

$$2^{-\Delta Ct} = (2^{-(Ct\ selected\ miRNA\ -\ Ct\ endogenous\ control)}).$$

The data was analyzed by SPSS software (version 14). The independent sample t-test was used for mean comparisons.

RESULTS

The results indicated that the expression difference of miR-21, miR-126 and miR-182 between the pregnant patients and controls is statistically significant with fold decrease of 1.5, 1.6 and 5.6, respectively. Table 1 illustrates the fold change comparison of the six investigated miRNAs in patients and controls in the pregnant group. As shown in the table the fold decrease of miR-155 and miR-222 was not significant. In contrast to other miRNAs, miR-517* level was elevated in the patient group and it was not statistically significant.

Table 1: The fold change in expression of the selected miRNAs in the pregnant group.

miRNA	Fold change	Trend	p-value
miR-21	1.5	Decreased	**0.049**
miR-126	1.6	Decreased	**0.012**
miR-155	2.3	Decreased	0.488
miR-182	5.6	Decreased	**0.046**
miR-222	2.4	Decreased	0.114
miR-517*	0.52	Increased	0.347

In the non-pregnant patient group miR-21, miR-126, miR-222 and miR-517*expressions were significantly different with fold decrease of 2.4, 2.9, 2.7 and 11.8,

respectively. miR-155 and miR-182 were also lower in the patients but the decrease in their levels did not reach significance (Table 2).

Table 2: The fold change in expression of the selected miRNAs in the non-pregnant group.

miRNA	Fold change	Trend	p-value
miR-21	2.4	Decreased	**0.011**
miR-126	2.9	Decreased	**0.006**
miR-155	12.5	Decreased	0.054
miR-182	1.06	Decreased	0.953
miR-222	2.7	Decreased	**0.001**
miR-517*	11.8	Decreased	**0.037**

DISCUSSION

Pregnancy is an intricate process facilitated by multiple molecular and cellular events that, in turn, are controlled by several proteins, enzymes, angiogenic and immunologic factors which are differentially expressed and coordinated. Regulation of these gene-associated activities could be achieved through the participation of particular miRNAs especially that one miRNA can regulate the expression of many genes simultaneously.

Moreover, accumulating evidence from recent reports have shown that various miRNAs are associated with one or more aspects of pregnancy and pregnancy outcomes. For instance, Renthal et al (2010) have shown that miR-200 family regulates uterine quiescence and contractility during pregnancy and labor (Renthal et al., 2010). Mice knockout studies of miRNA biogenesis proteins (e.g., Dicer1 and Ago2) confirmed that miRNAs are crucial for reproduction (Hong et al., 2008; Morita et al., 2007). Chakrabarty et al. (2007) have shown that a number of miRNAs are specifically expressed during the peri-implantation and pre-implantation periods in mice (Chakrabarty et al., 2007).

Interestingly, miRNAs seem to cross the placenta and appear in the maternal circulation and their concentration and patterns in plasma may be utilized for the detection of adverse pregnancy outcomes (Fu et al., 2013).

Absence of known causes in around 50% of RPL cases and scarcity of studies on the role of miRNAs in RPL prompted us to investigate the expression level of certain miRNAs in the plasma of pregnant and non-pregnant RPL patients and healthy subjects. The miRNAs investigated in the current study were selected because earlier studies have shown that they are involved in cellular proliferation, invasion and migration abilities, differentiation, adhesion, apoptosis and angiogenesis (Malumbers et al., 2003; Segura et al., 2009; Kong et al., 2012), events that are essential for pregnancy.

In the present study miR-21 was found to be significantly associated with increased risk of RPL, as its level was highly decreased in pregnant and non-pregnant RPL subjects when compared to corresponding healthy women. A result that points to a potential role of miR-21 and its target gene(s) in maintaining a healthy pregnancy. Consistent with our results, Maccani et al. (2011) in their analysis of term human placentas observed that the expression of miR-16 and miR-21 were markedly reduced in infants with the lowest birth weights (Maccani et al., 2011).

Various genes have been reported to be the targets of miR-21 but we believe that *PTEN* gene is the candidate that might be involved in the etiology of RPL as it has been implicated in cellular proliferation, invasion and migration abilities (Meng et al., 2007; Lou et al., 2010). Interestingly, Tokyol et al. (2008) have shown that altered patterns of *PTEN* expression may be associated with abortion (Tokyol et al., 2008).

Regarding miR-126, our results showed decreased level of this miRNA in idiopathic RPL cases compared to their corresponding healthy control before and during pregnancy with fold decrease of 2.9 (p=0.001) and 1.6 (p=0.006), respectively. One of the important functions of miR-126 is its involvement in angiogenesis by enhancing expression of VEGF (Nikolic et al., 2010).

Abnormalities of placental vasculature may result in several gestational complications, including pregnancy loss, intrauterine fetal death, intrauterine growth restriction and preeclampsia. VEGF plays an essential role in fetal and placental angiogenic development and diminished placental trophoblastic VEGF has been described in the decidual endothelium of spontaneous miscarriages (Su et al., 2011). Our results are consistent with Dai et al. (2011) findings who showed that collapsed blood vessels and cranial hemorrhages occurred in zebrafish with reduced miR-126 abundance, and mice deficient in miR-126 exhibited delayed angiogenic sprouting, widespread hemorrhaging, and partial embryonic lethality (Dai et al., 2011).

The decreased level of miR-126 in idiopathic RPL patients in both groups indicates its importance in the initiation and maintenance of healthy pregnancy.

miR-182 was found to be 5.6 fold deceased in the pregnant RPL patients (p=0.046) but its decline in the non-pregnant group was not significant. miR-182 regulates a plenty of genes most of which function in cell invasion, cell migration and cell cycle regulation which are essential for a healthy pregnancy (Zhang et al., 2011; Hirata et al., 2013; Bin et al. 2012). This miRNA has been shown to be among a group of placental miRNAs that are up-regulated in pre-eclampsia (Fu et al, 2013).

Thus, dysregulation of miR-182 might also be responsible for increasing the risk of RPL.

Although the level of miR-155 was also decreased in the RPL patients it did not, however, reach statistical significance. The fold decrease was 2.3 (p= 0.488) in the pregnant group and 12.5 (p= 0.054) in the non-pregnant group. Several targets and activities pertinent to pregnancy have been associated with this miRNA including: placental development and vascular integrity through regulation of *CYR61(CCN1)* gene (Zhou et al., 2005; Mo et al., 2002; Zhang et al., 2010), regulating endothelium-dependent vasorelaxation by targeting endothelial nitric oxide synthase gene (Sun et al., 2012), remodeling of human-trophoblast-derived HTR-8/SVneo cells (Dai et al., 2011) and pathogenesis of severe pre-eclampsia (Cheng et al., 2011).

In the present study it was observed that miR-222 was significantly decreased in the non-pregnant patients with fold decrease of 2.7 (p=0.001). Its level was also decreased in the pregnant RPL cases, however, this decline did not reach the statistical significance level (p=0.114). This miRNA has been shown by Qian et al (2009) to participate in endometrial stem cell differentiation, a step critical to decidualization and implantation (Qian et al., 2009). Moreover, Sang et al (2013) have reported that miR-222 participates in regulating estradiol concentration (Sang et al., 2013). Therefore, this miRNA seems important in the very early stages of pregnancy. Interestingly, Zhao et al. (2011) have shown that miR-222 is significantly decreased in gestational diabetes mellitus women (Zhao et al., 2011).

miR-517* is recognized as pregnancy-associated and has been shown to be highly expressed in the maternal circulation especially at the first trimester and to escape from maternal circulation after delivery (Kotlabova et al., 2011). Its level was higher, though not significant, in the pregnant patients when compared to healthy control group. However, it was decreased 11.8 fold in the non-pregnant RPL patients when compared to healthy controls (p=0.037). Hromadnikova et al (2012) have concluded that screening of a collection of placental miRNAs including miR-517* early in gestation can differentiate between women with normally progressing pregnancies and those who may later develop placental insufficiency-related complications (Hromadnikova et al., 2012).

In light of the obtained results we can conclude that proper levels of maternal plasma microRNAs, particularly miR-21, miR-126 and miR-182 are important for pregnancy and could serve as possible targets for diagnosis and intervention of RPL cases. As more than one of the investigated miRNAs were concurrently downregulated in both pregnant and non-pregnant subjects we believe that the underlying defect could be somewhere in the pathway of miRNAs biogenesis.

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