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Association of Micro-RNAs (320-b, 101-3p) and Parvovirus B19 with Recurrent Miscarriage

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Abstract

Miscarriage is the spontaneous end of pregnancy before 20 weeks of gestation. miRNAs serve as important regulators of target genes to control different pathophysiological events. Parvovirus B19 is a human pathogenic virus. Non-immune pregnant women are at risk for fetal infection with greater complications if transmission occurs in the first or second trimester. Here we try to study the expression of micro RNAs (320-b and 101-3p) and b) to detect the incidence of Parvovirus B19 antibodies among pregnant women with recurrent miscarriage. A prospective study comprised 80 pregnant women from the Prenatal Diagnosis and Fetal Medicine Department, National Research Centre, Egypt between January 2022 and March 2024 divided into the case group including 40 pregnant women with 2 or more histories of recurrent miscarriages and presenting with threatened abortion at gestational age 6 – 14 weeks. The control group consisted of 40 pregnant women with no history of miscarriages and at least one living child. micro-RNA (320-b and 101-3p) expression was done using Real-time PCR. Quantification of parvovirus B19 antibodies IgG and IgM using Enzyme-linked Immunosorbent Assay. Results revealed that miR 320-b was upregulated with a highly significant difference between the control group and the case group ($p<0.05$), while miR 101-3p was downregulated with a highly significant difference between the control group and the case group ($p<0.05$). Anti-B19 IgM antibodies (recent infection) tested negative in all subjects, while anti-B19 IgG antibodies (prior infection) were detected in 28.75 % (23/80). In conclusion, miR 320b and miR 101-3p can serve as biomarkers for early diagnosis of recurrent miscarriages. Acute Parvovirus B19 infection was not directly related to miscarriage incidences.

Keywords: Egypt; Miscarriage; miR 320-b; miR 101-3p; Parvovirus B19; Pregnancy.

1. Introduction

Miscarriage is known as the spontaneous end of pregnancy before 20 weeks of gestation (1). There is no specific reason for pregnancy loss in more than 50 % of women having recurrent miscarriages (2-3). There are different causes for recurrent miscarriage including anatomic and environmental factors, endocrine dysfunctions, antiphospholipid syndrome, genetic factors, autoimmune diseases and infections (4-5). Many ultrasound markers have been established to be potential predictors for early miscarriage, such as fetal crown-rump length, mean gestational sac diameter, and mean uterine artery pulsatility index (6). Many biochemical markers related to placental function have also been demonstrated for the prediction of subsequent early miscarriage, including glycodelin-A (7), kisspeptin (8), and angiogenesis-related factors such as placental growth factor (9).

Small non-coding RNAs are a group of short non-coding RNAs that have an important role in several biological processes and diseases (6). Those small non-coding RNAs include; micro-RNA (miRNA). miRNA is a small endogenous single-stranded RNA that controls the expression of different target genes after transcription. The nature of mRNA controls target gene expression and does not produce proteins themselves. So, it was supposed that miRNAs have a vital role in regulating genes controlling many physiological or pathophysiological conditions (7-8). miR-320b is an important member of the miR-320 family. It has been shown to regulate many biological processes. Qin et al reported that the higher expression level of circulating miRNA-320b could be used as a biomarker for unexplained recurrent spontaneous abortion (9). miR-101-3p greatly affects Phosphoinositide 3-kinases, phosphatase and tensin homolog, and protein kinase B, enhancing apoptosis and inhibiting cell proliferation of granulosa cells (10).

Parvovirus B19 (B19V) is a single-stranded DNA virus belonging to the Parvo-viridae family and is pathogenic in humans (11). Parvovirus B19 is transmitted through respiratory secretions, blood transfusion, and transplacental transmission (12). The incidence of infection with B19V may affect 5% of pregnant women, increasing

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up to 20% during the epidemic (13). It was estimated that the incidence of human parvovirus B19 was 31.11% in pregnant women with a history of miscarriage (14). The fetus may be infected through vertical transmission from the mother to the fetus. Most infected infants are asymptomatic. However, B19 infection during pregnancy may lead to many complications such as fetal anemia, spontaneous abortion, non-immune hydrops fetalis, and intrauterine fetal death (15).

2. Material and Methods

2.1. participant

This Case-control prospective study was conducted at the Prenatal Diagnosis and Fetal Medicine Department, National Research Centre, Egypt between January 2022 and March 2024. The study was conducted after the approval of the Medical Research Ethics Committee of the National Research Centre, Egypt; approval number (19045).

This study included 80 pregnant women who attended routine first-trimester screening between 6-14 weeks of gestation. They were divided into 2 groups; The Case group included 40 pregnant women aged 26.9 ± 2.47 years with 2 or more histories of recurrent miscarriage and presenting with threatened abortion. The control group included 40 pregnant women aged 26.5 years ± 2.26 years with no history of miscarriages and at least one living child. Women with multiple pregnancies, chronic infections, diabetes, hypertension, induced abortion, and inherited or acquired thrombophilia were excluded from the study. Extended personal obstetric, family histories, demographic and clinical data such as age, number of living births, miscarriages and gestational age were taken from all participants. All subjects were examined and followed up with ultrasound.

2.2. Sampling

5 ml of blood from all women was withdrawn and placed in a tube containing ethylenediamine tetraacetic acid (EDTA). Plasma was centrifuged at 3000 xg for 15 min., divided into aliquots, and stored at (-20°C) till use.

2.3. Extraction of RNA and microRNA quantification

An aliquot of plasma was used to extract RNA from maternal plasma samples using the miRNeasy® Serum/Plasma kit (QIAGEN, Germantown MD, USA). The extracted RNA's quantity, quality, and integrity were determined using a NanoDrop® ND1000 spectrophotometer (NanoDrop Technologies, LLC, Wilmington, DE, USA). cDNA was done using reagents from the Taqman® MicroRNA Reverse Transcription Kit, followed by quantification using Real-time quantitative (RT-PCR) (Thermo-Fisher Scientific, Waltham, MA, USA) according to manufacturer protocol as shown in **(table 1)**. Amplification was performed using the following thermal cycling program: an initial denaturation step at 95°C for 30 seconds, 40 cycles at 94°C for 5 seconds, and 60°C for one minute. The $2^{-\Delta\Delta CT}$ technique has been used as a relative quantification method for data analysis of quantitative real-time polymerase chain reaction (qPCR) (16). This technique calculates relative gene expression in target and reference samples using the threshold cycle (CT) information from a qPCR system and the reference gene U6 used as the normalizer **Fig 1.**

Table (1): RT-PCR protocol

Component	Volume per reaction
TaqMan™ Small RNA Assay (20X)	1.00 µL
PCR Master Mix	10.00 µL
Nuclease–free water	7.67 µL
Total PCR Reaction Mix volume	17.67 µL

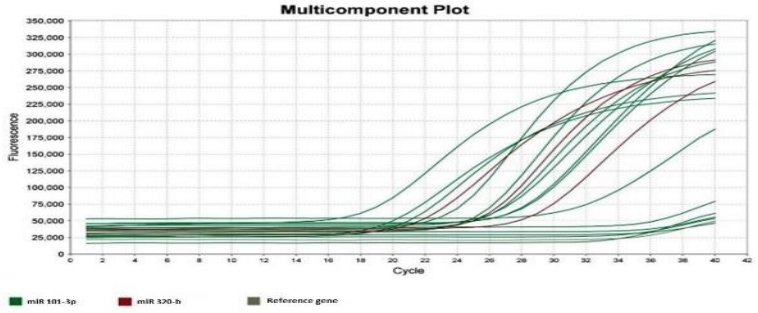

Fig. (1): Expression of miRNA 320b and miRNA 101-3p

2.4. Detection of parvovirus b19 antibodies

Parvovirus B19 specific Immunoglobulin (Ig) IgM and IgG antibodies were detected by parvovirus Enzyme-linked Immunosorbent Assay (ELISA) test (RIDASCREEN®, R-Biopharm, Hesse, Germany) as instructed by manufacturer guidelines. According to the results of parvovirus B19 IgG and IgM antibodies by ELISA, patients were categorized into: No infection if both IgM & IgG were negative; prior infection if IgG +ve and IgM -ve; recent infection if IgM +ve and IgG -ve or +ve.

2.5. Statistical analysis

The data was analyzed using IBM SPSS statistics (Statistical Package for Social Sciences) software version 18.0, IBM Corp., Chicago, USA, 2009. Descriptive statistics were used for quantitative data as minimum & maximum of the range and mean ± SD (standard deviation) for quantitative normally distributed data. The median was used for qualitative data as a percentage and number. Quantitative variables were analyzed using the Shapiro-Wilk test for normality testing, and independent t-tests in cases of two independent groups with normally distributed data analyses for independent variables were done using the Chi-square test to differentiate between ratios and Fisher's accurate test for variables with small predictable numbers. The ROC curve was used to estimate the accuracy of different tests and to test the differences between definite groups. The significance level taken at P value < 0.05 is significant. The sample size was determined using an online calculator https://riskcalc.org/samplesize/ /, which had a confidence interval of 95% and a type I error of 0.05.

3. Results

3.1. Clinicopathological data of study participants

There was no significant difference in the means of maternal age, hemoglobin level, and gestational age between the case and control groups. The mean maternal age of the participant in the **control group** was (26.5 years ± 2.26) in comparison to the **case group** which was (26.9 ± 2.47); P, (CI) = 0.425, (-1.48-0.63). The mean hemoglobin level in the **control group** was (11.21 mg/dl ± 1.47) in comparison to the **case group which was** (11.18 mg/dl ± 1.39); P, (CI) = 0.913, (-0.60- 0.67). The mean gestational age in the **control group** was (9.25 ± 1.7), while in the **case group,** the gestational age was (9.02 ± 1) P, (CI) = 0.471, (-0.41- 0.83) respectively.

3.2. micro-RNA expression levels:

The mean expression levels for miR 320-b and miR 101-3p among the control compared to the case group were statistically highly significant. Figure 2 showed a significant upregulation in expression levels of circulating miR 320-b in the plasma samples from the healthy controls (0.75 ± 0.49) compared with patients (1.13 ± 0.12) with a 95% confidence interval (95% CI) of (0.69-0.07) p<0.05. Figure 3 showed significant downregulation in expression levels of circulating miR 101-3p in the plasma samples from the healthy controls (0.98 ± 0.08) compared with patients (0.65 ± 0.52) with a 95% CI of (0.05 - 0.58) p<0.05 (Table 2).

Table (2): Comparison of miRNA expression in different studied groups

	Control	Case	Std . Error		P Value	95% CI
miR 320-b	0.75 ±0.495	1.13 ±0.757	0.097		0.017*	(0.69-0.07)
miR 101-3p	0.98 ±0.455	0.65 ± 0.527	0.089		0.018*	(0.05 - 0.58)

miR 320-b: micro RNA 320-b, miR 101-3p: micro RNA 101 -3p, Std: standard, CI: Confidence Interval, miR-: MicroRNA.

* p< 0.05: significant

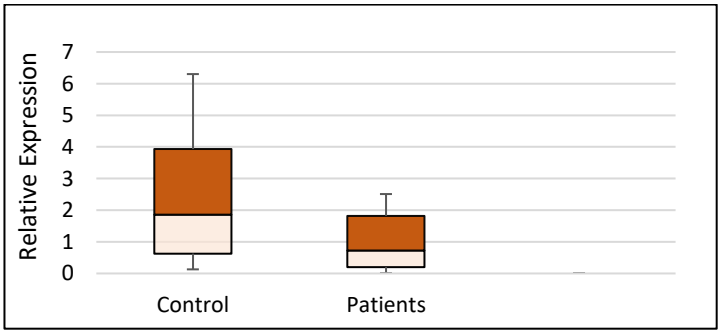

Fig. (2): miR-320-b expression in different studied groups.

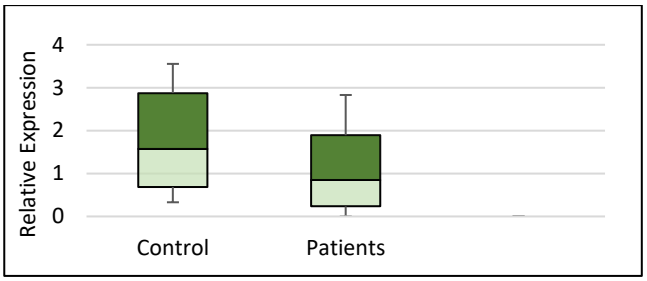

Fig. (3): miR- 101-3p expression in different studied groups.

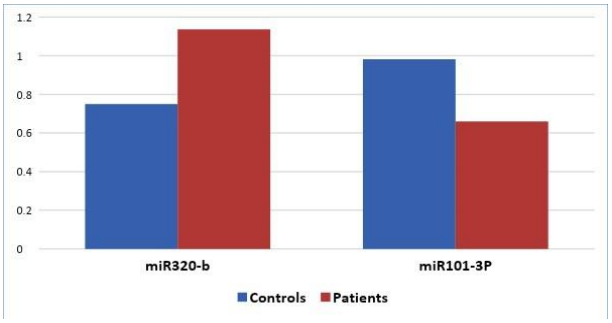

Fig. (4): Mean of Expression levels of miRNAs in different studied groups

3.3. Sensitivity, specificity, and ROC curve for miR 320-b and miR 101-3p

The diagnostic value of circulating miR 320-b and miR101-3p, as a potential non-invasive biomarker for diagnosis of RPL, was assessed by receiver operating characteristic curve (ROC) curve analysis (Fig 5). For miR 320-b, the area under the curve (AUC) is 0.806 (95% CI: 0.694-0.91, P=0.000) having a sensitivity of 88.5% and specificity of 65.5% at a cut-off of 0.573. miR101-3p had (AUC) of 0.702 (95% CI: 0.226–0.840, p=0.010) having a sensitivity of 90 % and specificity of 37.5% at a cut-off of 0.325 as listed in Table 3.

Table (3): Diagnostic performance of miRNA-101=3p and miRNA 320-b between studied groups.

Test Variable(s)	AUC	P value Sig.	Asymptotic 95% CI		Cut off	Sensitivity	Specificity %
			Lower Bound	Upper Bound			
miR-320.b	0.806	**0.000***	0.694	.919	0.5731	88.5%	65.5%
miR 101-3p	0.702	**0.010***	0.226	0.840	0.3205	90 %	37.9%

Receiver Operator Characteristic (ROC) analysis

AUC: Area under curve, CI: Confidence Interval, miR-3206: MicroRNA320b, miR101-3p: MicroRNA 101-.3p

* p< 0.05: significant.

3.4. Detection of Parvovirus B19 antibodies

35/40 (87.5%) tested negative for IgG and 5/40 (12.5%) tested positive for IgG among the **control group**. In the **case group,** 22/40 (55%) tested negative for IgG and 18/40 (45%) tested positive for IgG. IgG results were highly significant in the case group compared to the control group p<0.05. On the other hand, Anti-B19 IgM antibodies (recent infection) tested negative in all subjects as shown in Table 4.

Table (4): Correlation between human parvovirus B19 IgG and IgM.

Parvovirus B19	Control	Case	P VALUE
IgG Negative N (%)	35 (87.5%)	22 (55.0%)	0.001 *
IgG Positive N (%)	5 (12.5%)	18 (45.0%)	
IgM Negative N (%)	40 (100%)	40 (100%)	–
IgM Positive N (%)	Nil	Nil	

Ig**:** immunoglobulin

The data is presented in numbers (N) and percentages.

* p< 0.05: significant

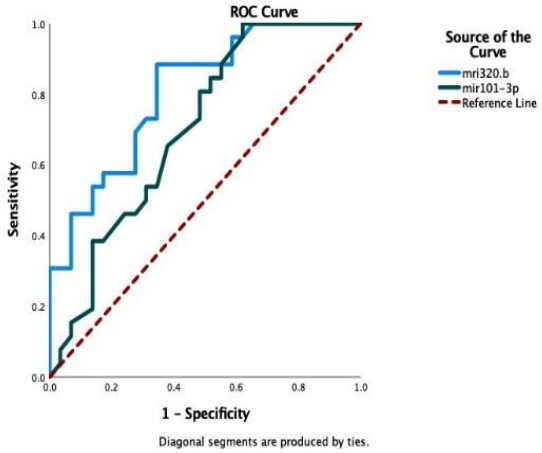

Fig. (5): ROC curve of miR-320.b and miR101-3p between different studied groups.

4. Discussion

Miscarriage has been identified as a public health issue and is considered one of the common pregnancy complications as around 30% of all pregnancies end with miscarriage (17-18). Some studies showed that miscarriage and stillbirth occur due to various factors including genetic factors, thrombophilia, immune factors, endocrine factors, lifestyle factors, and infections (19-20). prognosis of recurrent miscarriage is specifically difficult due to no functional diagnostic biomarker available. However, the finding of miRNAs in maternal circulation has not only simplified the understanding of their role during pregnancy but also opened a new era of other biomarkers for early detection of pregnancy complications (9). miRNAs are stable in circulating body fluids ie: plasma, serum, and saliva. Many miRNAs are released from the placenta and are responsible for trophoblast differentiation, propagation, apoptosis, migration as well as angiogenesis, indicating that miRNAs have a vital role in the growth of the placenta (21). Circulating miRNAs can be assessed through non-invasive techniques when compared with current standard invasive tests (22). miR-320b, an important member of the miR-320 family and involved in several biological processes. The role of miR-320 in disease progress and its molecular pathways are well established. Studies focused on the impact of miRNA-320b as an important molecular modulator in recurrent miscarriage (23).

Our study showed that miR-320b was significantly upregulated and the expression level in the case group was higher than that in the control group. Our results agreed with a case-control study in Iraq which documented that miR-320b was upregulated with a highly significant difference in miR 320-b between the RPL group compared to the control group (23). Our results also agreed with a study done by Qin and his colleagues. The study observed the expression level of some circulating miRNAs in 27 RPL patients and 28 women with healthy outcomes the results of their study showed that miR-320b was upregulated with a high significant difference among women having RPL compared with the control group (9). Another study involving 200 Kurdish females studied the relationship of miR - 320b with in vitro fertilization miscarriage rate, they concluded that miR-320b was more abounded among IVF miscarried females compared to fertile control and mir-320b might influence implantation of embryo throughout IVF cycles (24). Research by Machtinger and colleagues found that among 207 examined microRNAs, miR-320b proved to be the most predominant and greatly expressed in follicular fluid highlighting the possible role of this miRNA in oocyte quality (25).

miR 101-3p control the expression of steroid hormone synthesis-associated genes such as steroidogenic acute regulatory protein, cytochrome P450 family 19 subfamily A member 1 and 3 β-hydroxysteroid-dehydrogenase through stanniocalcin-1 depletion, which increase the excretion progesterone and estrogen (10). In our study, miR 101–3p was downregulated and the expression level in the case group was lower than that in the control group. Our results agreed with another study which found that the level of miRNA-101-3p in the plasma of RPL women was significantly lower than those in controls (26). Also, Qin and colleagues studied the expression of miRNAs in the serum of 27 women having RPL and observed that the serum level of miRNA-101-3p was significantly down-regulated among RPL women (9). Another study identified that miRNAs in uterine tissue examined from women with recurrent implantation failure demonstrated that miR 101-3p was down-regulated when compared to healthy fertile women (27). In disagreement with our results, Bahia and colleagues reported that among 72 differentially expressed miRNAs in RPL cases miR-320b was expressively upregulated in comparison to healthy controls (28). In our study, the ROC curve analysis showed that miR320-b could be a dependable diagnostic factor as it had a specificity of 65.5% and a sensitivity of 88.5%.

Parvovirus B19 (B19V) is a widespread infection that may affect 1-5% of pregnancies (29). fetuses B19 V infected fetuses mostly have healed spontaneously without negative effects but in 5 to 10% of cases, fetal death may happen (30). Enders and colleagues in their study reported the incidence of miscarriage associated with maternal infection at 8, 12, 16, and 17 weeks of gestation was 17.2%, 9.9%, 12.7%, and 5.7%, respectively (31).

In our study, the frequency of B19V IgG detected was 28.7 %. Our results were in alignment with a study done in Iraq by Buraa and colleagues investigating the seroprevalence of B19V among women with miscarriage about 23 % tested positive for B19V IgG (32). Recently in Egypt, a study done by Abd El-Salam and colleagues showed that among their studied cases (N=60), there were 26 cases of abortion eight cases (30.7%) were positive for B19V IgG (33). Also, De Paschale and colleagues reported that 69.5 % of RPL women tested positive for B19V IgG (34). Other researchers gave the same results (35-13). On the other hand, in our study, all pregnant women tested negative against B19V IgM. Other studies also showed a very low incidence rate of B19V IgM. For example, Karami and colleagues in their study on 110 pregnant women, although 2 cases were positive against B19V IgM, further investigations using PCR failed to verify infection by B19V, these data showed that the incidence of infection with Parvovirus B19 was 0 %, and 2% by using PCR and IgM, respectively (13). In contrast to our results, a study done in Iraq observed that among 90 pregnant women who had previously experienced abortion. The frequency rate for parvovirus B19V IgM was 11.11% among women who experienced recurrent abortions (14), other studies showed similar incidences of B19V IgM among women suffering from recurrent miscarriage (36-37-38).

5. Conclusion and recommendations

miR320-b and miR101-3p could be used as non-invasive indicators of adverse outcomes of pregnancies including RPL. On the other hand, no direct correlation was detected between B19V and RPL incidences as anti-B19V IgG among pregnant women was about 28.7% and anti-B19V IgM was not detected among all subjects. This data indicated pregnant women are more immune to B19 due to previous infections and no recent infections were found.

To validate circulating miR 320b and miR 101-3p as potential biomarkers for RPL, we recommend that screening a large number of pregnant women and comprehending the etiological role of B19 prevalence during pregnancy, a larger randomized study is required.

6. List of abbreviations

AUC: Area under curve.

B19V: Parvovirus B19.

C.I: Confidence Interval.

CT: the threshold cycle.

EDTA: ethylenediaminetetraacetic acid.

ELISA: Enzyme-linked Immunosorbent Assay.

Ig: Immunoglobulin.

IVF: In Vitro Fertilization.

miRNA: micro-RNA.

qPCR: quantitative real-time polymerase chain reaction.

RPL: Recurrent Pregnancy Loss.

ROC: Receiver Operator Characteristic

SD: standard deviation.

7. Conflict of interest

No conflict of interest was reported by the authors

8. Funding

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9. Data availability

Not applicable.

10. References

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