

## Original Article

# Expression of TGF- $\beta$ 1 and miR-99a in patients with first-trimester spontaneous abortion and correlation with hormone levels

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**Abstract:** Objective: The goal of this study was to investigate and analyze expression of serum transforming growth factor-beta 1 (TGF- $\beta$ 1) and microRNA-99a (miR-99a) in patients with first-trimester spontaneous abortion and correlation with hormone and cytokine levels during pregnancy. Methods: Ninety-five pregnant women with first-trimester spontaneous abortion were selected as the spontaneous abortion group (SAG), and 90 normal pregnant women with induced abortion for non-medical reasons were enrolled as the control group (CG). Enzyme-linked immunosorbent assay (ELISA) was used to detect the levels of serum hormone, TGF- $\beta$ 1, interferon  $\gamma$  (IFN- $\gamma$ ) and interleukin-4 (IL-4) in the two groups. The expression of miR-99a in the two groups was detected with quantitative real-time polymerase chain reaction (qRT-PCR), and the correlation between each index was analyzed. Results: The levels of serum  $\beta$ -human chorionic gonadotropin ( $\beta$ -HCG), progesterone, estrogen and IL-4 in the control group were significantly higher than those in the spontaneous abortion group, while the levels of serum IFN- $\gamma$  and IFN- $\gamma$ /IL-4 were significantly lower than those in the spontaneous abortion group, with statistically significant differences (all  $P < 0.05$ ). The serum TGF- $\beta$ 1 level in the control group was significantly higher than that in the spontaneous abortion group, and the difference was statistically significant ( $P < 0.05$ ). The relative expression of serum miR-99a in spontaneous abortion group was significantly higher than that in control group, and the difference was statistically significant ( $P < 0.05$ ). The serum TGF- $\beta$ 1 of pregnant women with spontaneous abortion was significantly positively correlated with  $\beta$ -HCG, progesterone, and estrogen levels ( $P < 0.05$ ), and was significantly negatively correlated with IFN- $\gamma$ /IL-4 levels ( $P < 0.05$ ). The serum miR-99a level of pregnant women with spontaneous abortion was significantly negatively correlated with the levels of  $\beta$ -HCG, progesterone, and estrogen ( $P < 0.05$ ), and was significantly positively correlated with the levels of IFN- $\gamma$ /IL-4 ( $P < 0.05$ ). The receiver operating characteristic (ROC) curve was used to analyze the predictive value of serum TGF- $\beta$ 1 and miR-99a for early spontaneous abortion. The area under the curve (AUC) of TGF- $\beta$ 1 was 0.018, while that of miR-99a was 0.992. Conclusion: The TGF- $\beta$ 1 level in pregnant women with first-trimester spontaneous abortion was significantly decreased while the miR-99a level was significantly increased. TGF- $\beta$ 1 and miR-99a levels were closely related to hormone levels and Th1/Th2 cell balance, which could be used as potential indicators for early diagnosis of pregnant women with first-trimester spontaneous abortion and are worthy of in-depth research and analysis.

**Keywords:** First-trimester spontaneous abortion, TGF- $\beta$ 1, miR-99a, hormone levels during pregnancy

## Introduction

First-trimester spontaneous abortion refers to the abortion occurring before the 12th week of gestation and is one of the common complications of pregnancy [1]. It can be induced by external physical and chemical and mechanical damages, genetic defects, genital malformation and infection, endocrine factors, etc., and the causes still remain unknown for few patients [2]. Endocrine hormone abnormalities

are mainly caused by abnormal hormone secretion level of pregnant women, which may result in spontaneous abortion. Many studies show that microRNA (miRNA) can regulate target genes and cause changes in hormone level during pregnancy and abnormal balance of Th1/Th2 cell, thus causing abortion [3, 4].

Transforming growth factor-beta 1 (TGF- $\beta$ 1), a dimer with a relative molecular mass of 25 kDa, is one of the TGF- $\beta$  family members and linked

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by two identical polypeptide chains through disulfide bonds [5]. It is a polypeptide growth factor with various biological activities, and participates in various biological processes such as proliferation and differentiation, embryonic development, angiogenesis, wound healing, extracellular matrix protein expression and immunosuppression. Research shows that TGF- $\beta$ 1 level changes during pregnancy and spontaneous abortion [6]. miRNAs have a size of 21-24 nucleotides and have maintained high conservation during evolution, and specific biological functions [7]. Embryonic development is a recurrent process of organisms from the beginning to maturity, regulated by a series of environmental factors, genetic factors and epigenetic modifications [8]. Current studies have confirmed that miRNAs are closely related to the occurrence of first-trimester spontaneous abortion. They regulate gene expression at the post-transcriptional level through complementary base binding to their target mRNAs, thus promoting mRNA degradation and inhibiting its translation. Gene chip results show that miR-99a is highly expressed in the serum of patients with endometrial cancer. Furthermore, research also suggests that miR-99a may act on some target genes and affect the secretion of estrogen and progesterone, thus causing the occurrence of diseases [9]. Therefore, it is speculated that miR-99a and TGF- $\beta$ 1 are closely related to hormonal changes during pregnancy. This study explored and analyzed expression of TGF- $\beta$ 1 and miR-99a in patients with first-trimester spontaneous abortion and their correlation with hormone levels during pregnancy, so as to provide basis for clinical diagnosis.

### Materials and methods

#### *Clinical data*

From March 2015 to March 2018, 95 women with first-trimester spontaneous abortion admitted to Fujian Provincial Maternity and Children's Hospital, Affiliated Hospital of Fujian Medical University were enrolled as the spontaneous abortion group (SAG). The diagnosis was based on the relevant diagnostic criteria of Danforth's Obstetrics and Gynecology (2004) [10]. Whereas 90 cases of normal pregnant women with induced abortion for non-medical reasons were selected as the control group (CG). Exclusion criteria: Patients with chromosome abnormality; patients with embryo dam-

age due to *Toxoplasma gondii*, *Mycoplasma* and other infections; patients with uterine anomalies. The patients in the spontaneous abortion group were 19-38 years old, with an average age of  $28.0 \pm 3.5$  years old, 5-12 weeks of pregnancy, with an average of  $6.92 \pm 1.05$  weeks, and including 41 cases with 0 times of induced abortion, 39 with 1 time, 11 with 2 times, and 4 with 3 times and above. The patients in the control group were 18-36 years old, with an average age was  $27.0 \pm 4.5$  years old, 5-12 weeks of pregnancy, with an average of  $6.89 \pm 1.21$  weeks, and including 38 cases with 0 times of induced abortion, 37 of 1 time, 9 with 2 times, 6 with 3 times and above. There was no significant difference in clinical data between the two groups ( $P > 0.05$ ). All subjects voluntarily signed informed consent forms.

#### *Specimen collection*

Five milliliter of fasting venous blood was collected in the morning for routine centrifugal separation of serum. The serum was placed in a centrifugal tube and stored in a refrigerator at  $-80^{\circ}\text{C}$  for later use.

#### *Enzyme linked immunosorbent assay*

The levels of serum TGF- $\beta$ 1,  $\beta$ -HCG, progesterone, estrogen, IFN- $\gamma$  and IL-4 in the two groups were determined by enzyme-linked immunosorbent assay (ELISA). The detection kit purchased from R&D Company in the United States was operated strictly in accordance with the instruction manual. Dilution was used to dilute the serum sample 5 times, and the sample was then added to the bottom of the ELISA Plate. After being sealed with a microplate sealer, the plate was placed in an incubator at  $37^{\circ}\text{C}$  for 30 minutes. The sealer was carefully removed, and the liquid was discarded and dried. Then washing liquid was added into the wells and discarded after 30 seconds. Each well was washed 5 times. Fifty microliter of standard reagents was added to each well (except blank well), incubated for another 30 minutes then washed. After that, color developing agent was added and mixed well, and the development was carried out at  $37^{\circ}\text{C}$  for 15 minutes. Fifty microliter of elimination solution was used to terminate the reaction, and the absorbance of each well was observed at 450 nm wavelength with a microplate reader (Beckman Kurt Inc., U.S).

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**Table 1.** Primer sequence

	Sequence
miRNA	
Forward primer	5'-ACACTCCAGCTGGCAACCCGTAGATCCG-3'
Reverse primer	5'-GGTGTCGTGCAGTCG-3'
Reverse transcription primer	5'-CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGCACAAGTT-3'
miR-99a	
Forward primer	5'-TGGCGTAGCAGCACGTAAAT-3'
Reverse primer	5'-CAGTGCAGGGTCCGAGGTAT-3'
Reverse transcription primer	5'-GTCGTATCGAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGCCAA-3'

## *Quantitative real-time polymerase chain reaction*

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect miR-99a expression in two groups. The total RNAs were extracted from the patient sera by the Trizol (Thermo Fisher Scientific Company, United States). The operation was carried out according to the instruction manual of the kit. The extracted total RNAs were placed in a refrigerator at  $-80^{\circ}\text{C}$  then reverse transcribed into complementary DNA (cDNA) and placed in a refrigerator at  $4^{\circ}\text{C}$  for later use. miR-6 was used as an internal reference, and primer sequences were designed and synthesized by Shanghai Sangon Bioengineering Co., Ltd., shown in **Table 1**. The expression of miR-99a was calculated by  $2^{-\Delta\Delta\text{CT}}$ .

## *Statistical analysis*

Statistical software SPSS 22.0 was used to process the data. The measurement data expressed by mean  $\pm$  standard deviation ( $\bar{x} \pm \text{sd}$ ) were analyzed by t test. The counting data expressed by number of cases/percentage (n/%) were analyzed by  $\chi^2$  test, Pearson correlation analysis and receiver operating curve (ROC) were used to evaluate the predictive value of TGF- $\beta$ 1 and miR-99a expression on first-trimester spontaneous abortion. The difference was statistically significant with  $P < 0.05$ .

## **Results**

### *Comparison of serum hormone levels between two groups*

$\beta$ -HCG, progesterone, estrogen, IFN- $\gamma$ , IL-4 and IFN- $\gamma$ /IL-4 in the spontaneous abortion group were ( $5,201.47 \pm 593.94$ ) mU/L, ( $10.57 \pm 2.31$ )  $\mu\text{g/L}$ , ( $469.42 \pm 85.39$ ) ng/L, ( $183.49 \pm 21.02$ ) ng/L, ( $191.47 \pm 23.60$ ) ng/L and  $0.96 \pm 0.28$ ,

respectively; whereas those in the control group were ( $13,584.29 \pm 3,695.58$ ) mU/L, ( $21.85 \pm 4.69$ )  $\mu\text{g/L}$ , ( $1,126.29 \pm 375.93$ ) ng/L, ( $150.48 \pm 24.69$ ) ng/L, ( $210.84 \pm 26.59$ ) ng/L and  $0.73 \pm 0.21$ , respectively. Therefore, the levels of  $\beta$ -HCG, progesterone, estrogen and IL-4 in the control group were significantly higher than those in the spontaneous abortion group, while the levels of serum IFN- $\gamma$  and IFN- $\gamma$ /IL-4 in the control group were significantly lower than those in the spontaneous abortion group, with statistically significant differences ( $P < 0.05$ ), as shown in **Figure 1**.

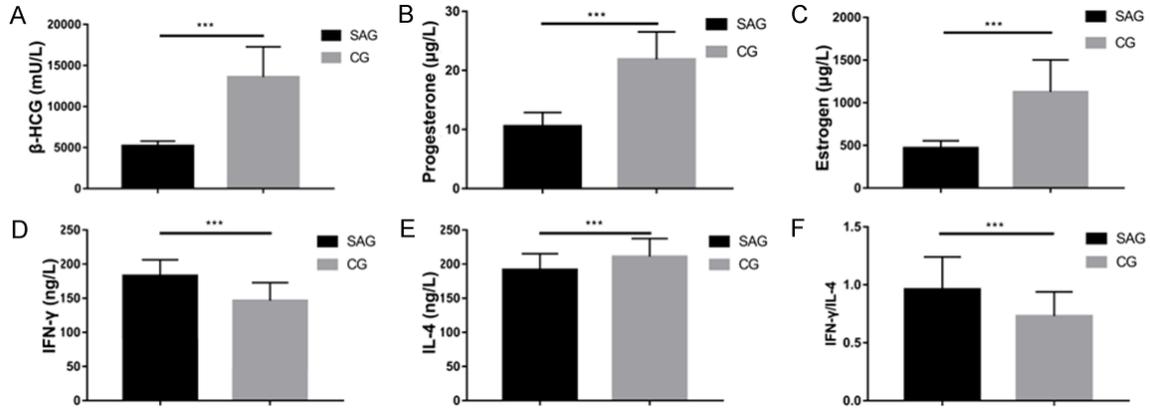
### *Comparison of serum TGF- $\beta$ 1 and miR-99a levels between two groups*

The levels of serum TGF- $\beta$ 1 and miR-99a in the spontaneous abortion group were ( $208.95 \pm 37.59$ ) pg/mL and  $1.65 \pm 0.58$  respectively, while those in the control group were ( $348.63 \pm 41.64$ ) pg/mL and  $0.82 \pm 0.24$  respectively. The serum TGF- $\beta$ 1 level in the control group was significantly higher than that in the spontaneous abortion group, and the difference was statistically significant ( $P < 0.05$ ). The relative expression of serum miR-99a in the spontaneous abortion group was significantly higher than that in the control group, and the difference was statistically significant ( $P < 0.05$ ), as shown in **Figure 2**.

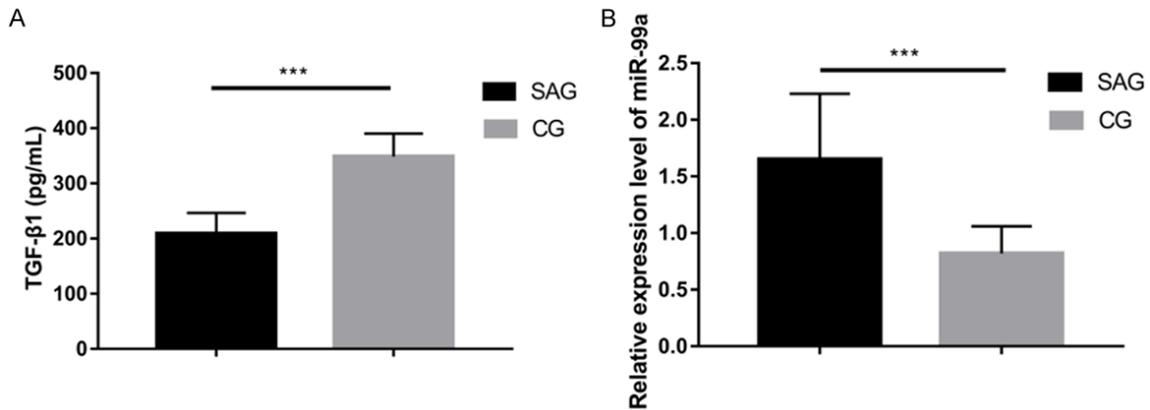
### *Correlation between serum TGF- $\beta$ 1 level and hormone level*

There was a significant positive correlation between serum TGF- $\beta$ 1 and  $\beta$ -HCG ( $r = 0.656$ ,  $P < 0.001$ ), progesterone ( $r = 0.661$ ,  $P < 0.001$ ) and estrogen ( $r = 0.615$ ,  $P < 0.001$ ) in women with first-trimester spontaneous abortion, while serum TGF- $\beta$ 1 and IFN- $\gamma$ /IL-4 were significantly negatively correlated ( $r = -0.249$ ,  $P = 0.001$ ) as shown in **Figure 3**.

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**Figure 1.** Comparison of serum hormone levels between two groups. A:  $\beta$ -HCG level; B: Progesterone level; C: Estrogen level; D: IFN- $\gamma$  level; E: IL-4 level; F: IFN- $\gamma$ /IL-4. Compared with the control group, \*\*\* $P$ <0.001. SAG, spontaneous abortion group; CG, control group;  $\beta$ -HCG,  $\beta$ -human chorionic gonadotropin; IFN- $\gamma$ , interferon  $\gamma$ ; IL-4, interleukin-4.



**Figure 2.** Comparison of serum TGF- $\beta$ 1 and miR-99a levels between two groups. A: TGF- $\beta$ 1 level; B: miR-99a level. Compared with the control group, \*\*\* $P$ <0.001. SAG, spontaneous abortion group; CG, control group; TGF- $\beta$ 1, transforming growth factor-beta 1; miR-99a, microRNA-99a.

### Correlation between serum miR-99a level and hormone level

There was a significant negative correlation between serum miR-99a level and  $\beta$ -HCG ( $r$ =-0.682,  $P$ <0.001), progesterone ( $r$ =-0.664,  $P$ <0.001) and estrogen ( $r$ =-0.628,  $P$ <0.001) in women with first-trimester spontaneous abortion, while the serum miR-99a level was significantly positively correlated with IFN- $\gamma$ /IL-4 ( $r$ =0.291,  $P$ <0.001) as shown in **Figure 4**.

### Analysis of the predictive value of serum TGF- $\beta$ 1 and miR-99a for first-trimester spontaneous abortion by ROC curve

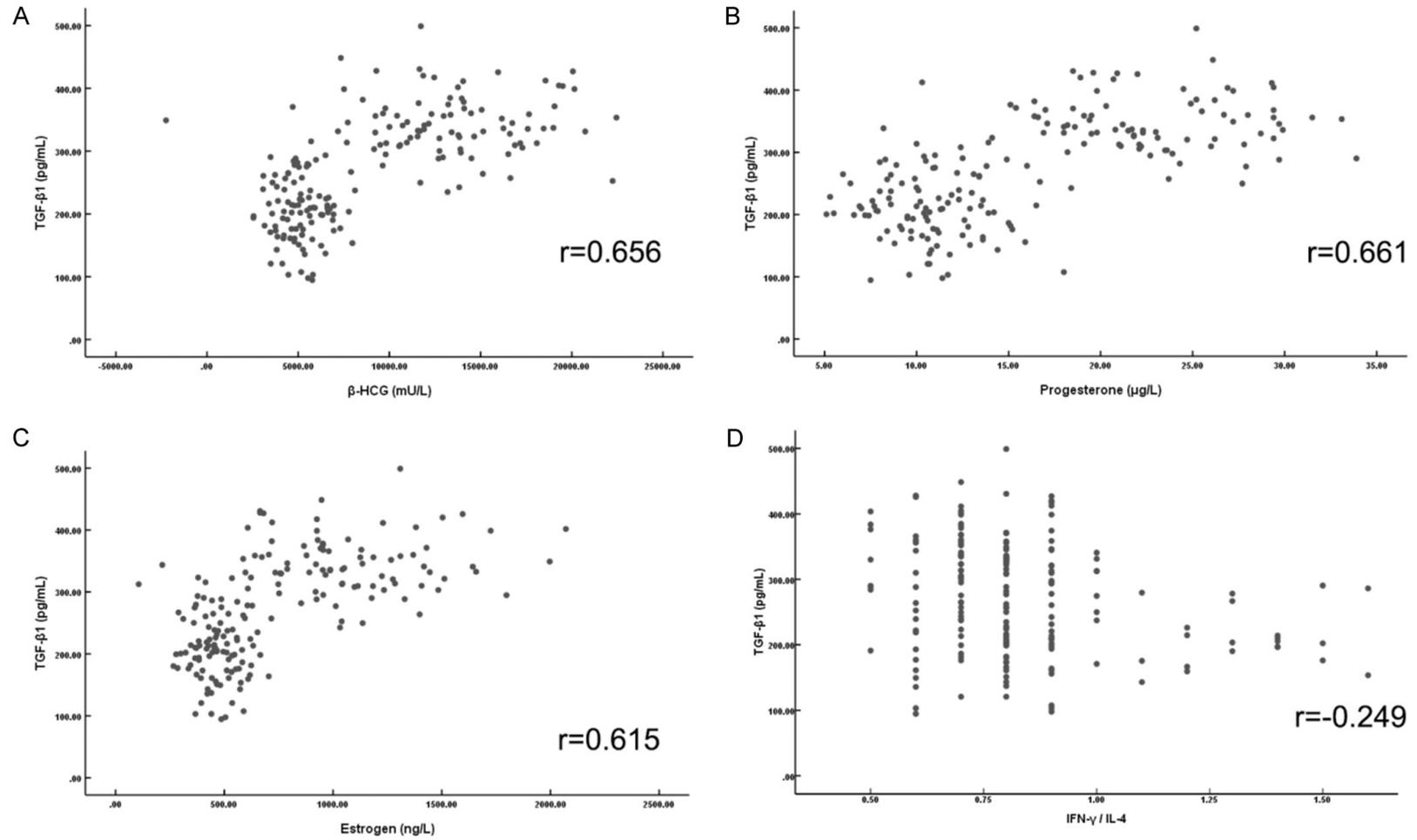
ROC curve was used to analyze the predictive value of serum TGF- $\beta$ 1 and miR-99a for first-

trimester spontaneous abortion. The results showed that the area under curve (AUC) of TGF- $\beta$ 1 was 0.018, while that of miR-99a was 0.992 as shown in **Figure 5**.

### Discussion

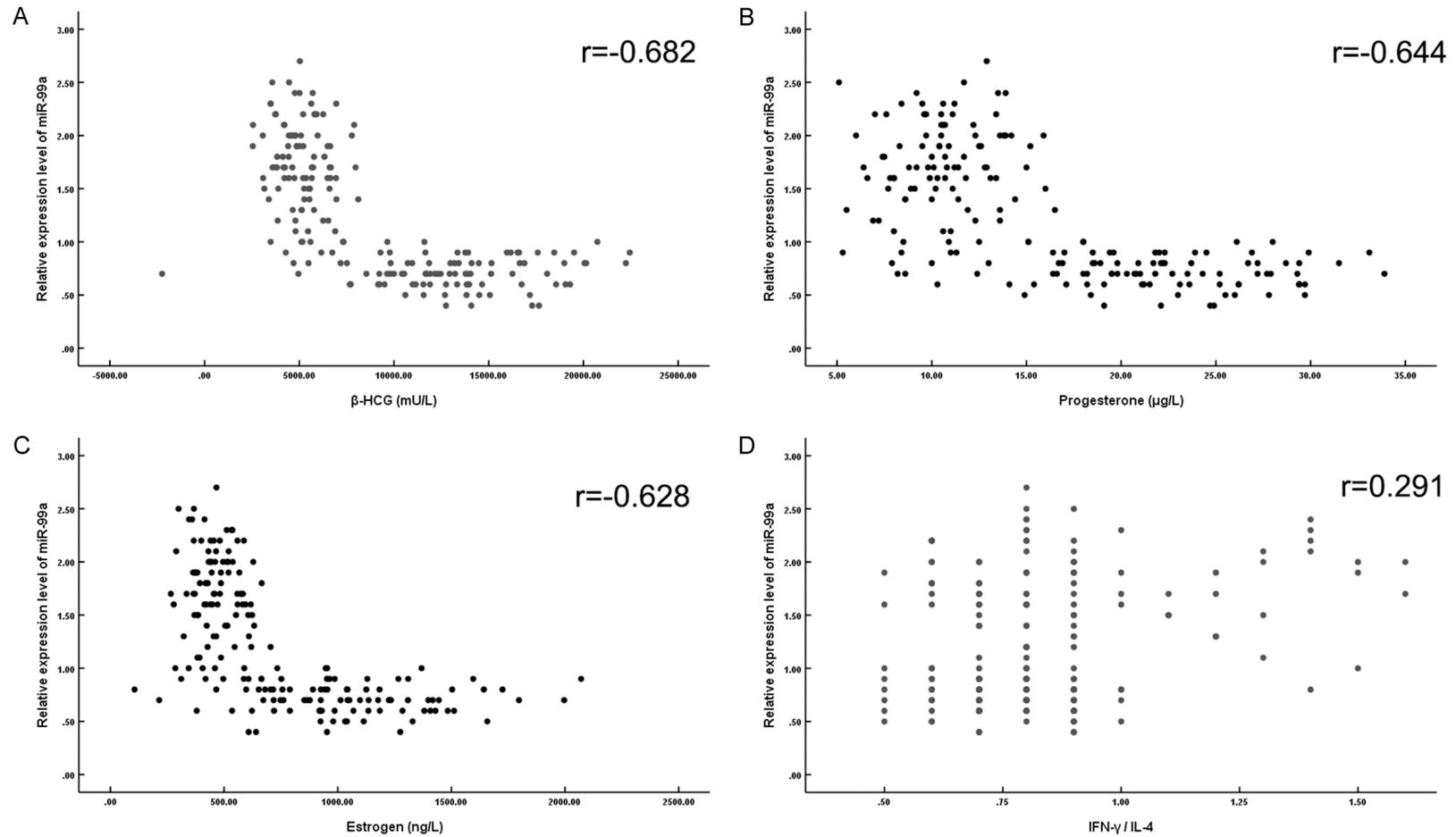
Successful implantation of mammalian embryos requires sufficient hormone secretion, syncytiotrophoblast cell formation, synchronous development and coordination of blastocyst and endometrium, so as to form the maternal-fetal interface. As a kind of glycoprotein secreted by placental trophoblast cells,  $\beta$ -HCG has an important clinical significance for the diagnosis of early pregnancy [11]. Studies have shown that  $\beta$ -HCG, progesterone and estrogen

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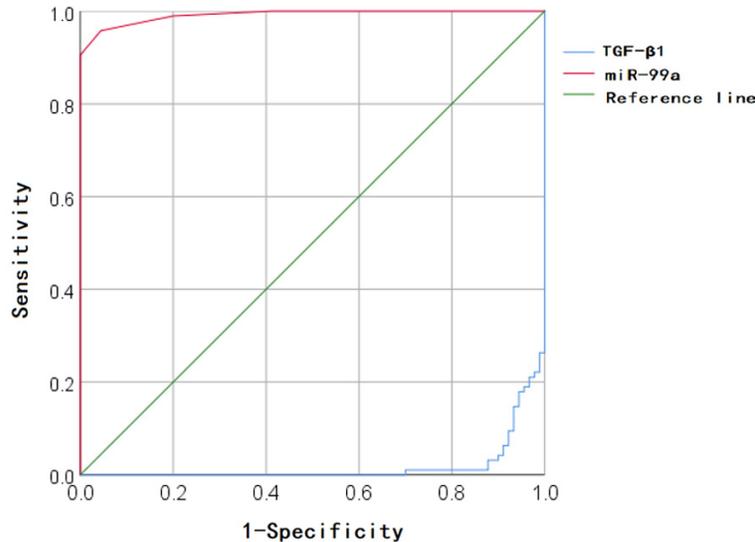
**Figure 3.** Correlation between serum TGF- $\beta$ 1 level and hormone level. A: Correlation between serum TGF- $\beta$ 1 and  $\beta$ -HCG; B: Correlation between serum TGF- $\beta$ 1 and progesterone; C: Correlation between serum TGF- $\beta$ 1 and estrogen; D: Correlation between serum TGF- $\beta$ 1 and IFN- $\gamma$ /IL-4. TGF- $\beta$ 1, transforming growth factor-beta 1; IFN- $\gamma$ , interferon  $\gamma$ ; IL-4, interleukin-4.

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**Figure 4.** Correlation between serum miR-99a level and hormone level. A: Correlation between serum miR-99a and β-HCG; B: Correlation between serum miR-99a and progesterone; C: Correlation between serum miR-99a and estrogen; D: Correlation between serum miR-99a and IFN-γ/IL-4. miR-99a, microRNA-99a; β-HCG, β-human chorionic gonadotropin; IFN-γ, interferon γ; IL-4, interleukin-4.

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**Figure 5.** Analysis of the predictive value of serum TGF- $\beta$ 1 and miR-99a for first-trimester spontaneous abortion by ROC curve. miR-99a, microRNA-99a; TGF- $\beta$ 1, transforming growth factor-beta 1.

play a vital role in the pregnancy and the occurrence of first-trimester spontaneous abortion [12, 13]. They can regulate expression of various substances in tissues and the morphology and function of tissue cells by interacting with hormone receptors, thus directly affecting the reproductive process and consequences. In addition, T lymphocytes, as a key cells of cellular immunity, can be divided into two subtypes of Th1 and Th2 according to its function. Research shows that the level of Th1/Th2 in patients with first-trimester spontaneous abortion is closely related to pregnancy outcome. IFN- $\gamma$ , a Th1 cytokine, promotes the cellular immune function and affects the growth and development of trophoblast cells and embryos. IL-4 is a Th2 cytokine, which promotes the proliferation of B cells and inhibits the secretion of Th1 cytokines, thus improving the immune tolerance and maintaining normal pregnancy [14, 15].

TGF- $\beta$ 1 is mainly secreted by CD4<sup>+</sup> T cell subsets and expresses in endometrium and gestation decidua. It can inhibit proliferation of T cells and the activity of cytotoxic T lymphocytes and natural killer cells, effectively reduce embryo toxicity. The correlation between TGF- $\beta$ 1 and pathological pregnancy has gradually attracted the attention of scholars [16, 17]. At present, many studies show that miRNAs are

related to first-trimester spontaneous abortion. It is believed that miR-99a may affect the secretion of estrogen and progesterone by acting on some target genes, thus leading to diseases [18, 19]. There are few reports on the relationship between miR-99a and spontaneous abortion, so this study speculated that miR-99a may affect hormone levels during pregnancy, thus causing spontaneous abortion.

The results of this study showed that the levels of serum  $\beta$ -HCG, progesterone and estrogen in the control group were significantly higher than those in the spontaneous abortion group, which is similar to some reports, suggesting

that the serum hormone level of first-trimester spontaneous abortion pregnant women is obviously abnormal compared with normal ones, which may be one of the important mechanisms of first-trimester spontaneous abortion [20]. In addition, the serum TGF- $\beta$ 1 level in the control group was significantly higher than that in the spontaneous abortion group, and the relative expression of serum miR-99a in the spontaneous abortion group was significantly higher than that in the control group. It has been suggested that compared with normal pregnant women, the serum TGF- $\beta$ 1 level of pregnant women with spontaneous abortion is significantly decreased and miR-99a level is significantly increased.

The serum TGF- $\beta$ 1 of pregnant women with spontaneous abortion was significantly positively correlated with the levels of  $\beta$ -HCG, progesterone and estrogen, while the serum miR-99a level was significantly negatively correlated, which suggests that TGF- $\beta$ 1 and miR-99a are closely related to the serum hormone level of pregnant women with spontaneous abortion. It has been speculated that TGF- $\beta$ 1 and miR-99a regulate the synthesis pathway of reproductive hormone or control the target gene in the signal pathway of reproductive hormone release, thus changing the hormone level *in vivo* and eventually inducing spontaneous abor-

tion [15]. In addition, TGF- $\beta$ 1 had a significant negative correlation with IFN- $\gamma$ /IL-4 levels, whereas the reverse was found in miR-99a, suggesting that TGF- $\beta$ 1 and miR-99a are closely related to the balance of Th1/Th2 cells in spontaneous abortion parturients, thus affecting the pregnancy outcome, which is similar to relevant research results [21]. Finally, an ROC curve was used to analyze the predictive value of serum TGF- $\beta$ 1 and miR-99a for first-trimester spontaneous abortion. The results show that the AUC of TGF- $\beta$ 1 was 0.018, and that of miR-99a was 0.992, indicating that serum TGF- $\beta$ 1 and miR-99a have high predictive value and can be used as predictive indicators for early spontaneous abortion.

In summary, the TGF- $\beta$ 1 level in pregnant women with first-trimester spontaneous abortion is significantly decreased while the miR-99a level is significantly increased. TGF- $\beta$ 1 and miR-99a levels are closely related to the hormone levels during pregnancy and the Th1/Th2 cell balance, which can be used as potential indicators for early diagnosis of pregnant women with first-trimester spontaneous abortion and are worthy of in-depth research and analysis.

### Disclosure of conflict of interest

None.

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