REVIEW ARTICLE

# miRNA-99a and TGFβ1 (C/G +915) Gene SNP in Females with Secondary Unexplained Infertility

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## Abstract

Background: Women infertility is a big health problem worldwide and genetic disorders are considered underlying causes of unexplained female infertility. Objective: The current study aims to find out the role of miR-99a and TGFβ1 SNP (G/C +915) in the development of secondary unexplained infertility in women and the prognostic of miR-99a. To achieve this goal, the fold change of miR-99a had been measured by using Real-time PCR. In addition to the identification of TGFβ1 (G/C +915) SNP by ARMS- PCR. Material and methods: The study is a case-control study which had been conducted by collecting blood samples from a population including 50 female patients with secondary unexplained infertility along with apparently 50 healthy controls. The samples were collected during the period from November 2021 to July 2022. The genomic RNA and DNA were extracted from serum and blood respectively for molecular assay. Results: The mean of miR-99a fold change in the patients group was 3.70 ±1.18 versus 0.84 ±0.34 in the control group and $p < 0.001$. TGFβ1 SNP +915 (GC) genotype CC was more frequent significantly in the patients group than in the control group $p < 0.001$ and the OR was 19.06. While genotype GG was significantly less frequent in the patients group than in the control group $p = 0.046$ and the OR was 0.22. Conclusion: The present study demonstrated a diagnostic and prognostic value for miR-99a, since it showed high sensitivity 100%, specificity 98%, and accuracy 100% in secondary unexplained infertility, TGFβ CC genotype as a predisposing risk factor for developing secondary unexplained infertility.

**Keywords:** miRNA-99a, TGFβ1 SNP, mTOR, and women with secondary unexplained infertility.

## Introduction:

Infertility is a disease of the female or male reproductive system, defined by the failure of couples to achieve a clinical pregnancy after 12 months or more of regular timed unprotected sexual intercourse, which can cause psychological, physical, mental, spiritual, and medical detriments to the patient (1). Infertility of unknown origin comprises both idiopathic and unexplained infertility (2). Genetic disorders considered the underlying causes of unexplained female infertility so if some critical genes are not properly expressed during window of implantation of the regular menstrual cycle such as adhesion molecules, growth factors, cytokines, chemokines, and lipids, embryo implantation will not take place and this accounts, in a major part, for the low pregnancy rates found in patients with repeated implantation failures (3).

The miRNAs are potential regulators of endometrial receptivity and embryo implantation which control gene expression that involves cell-cycle progression, proliferation and differentiation, and expression of numerous molecules that are known to occur during the cyclic changes in the endometrium (4). The aberrant miRNA expression such as miR-99a has been associated with down-regulating the expression of some genes which play an important role in the window of implantation and cause implantation failure (5). TGFβ1 is a pleiotropic cytokine and a potent immune regulator and is engaged in the embryo's implantation process and the binding of trophoblast cells to the extracellular matrix (6). This cytokine is involved in immune deviation from Th1 to Th2 and plays an essential role in maintaining pregnancy (7), single nucleotide polymorphism (SNP) in the exon 2 of the human TGFβ1 gene has been reported (8). This SNP was indicated to be related to the basal levels of this cytokine (8).

## The Material and Methods

A total of 50 women with secondary unexplained infertility were recruited in this case/control study during the period from November 2021 to July 2022 who were previously diagnosed with secondary unexplained infertility and confirmed by a gynecologist and internal medicine specialist. The patients and control subjects were included in this study with age ranges between 18 and 35 years. The inclusion criteria for secondary unexplained infertile females included normal results of ovaries reserve, ovulation, luteal phase, normal results of fallopian tube, uterus, normal results of sex hormone analysis, normal results in semen analysis of their partners, normal anatomical and physiological, did not have infectious, autoimmune disorders, or obesity as well as with no a history of using a particular drug. All the patients had at least one previous successful pregnancy and the infertile women of known cause were excluded. The control group had a history of normal third pregnancy or more, without a previous abortion and they were in the first trimester when blood was collected.

Each blood sample of the two groups was divided into two parts two milliliters of blood collected directly in a sterile tube containing EDTA for DNA extraction, then use (ARMS-PCR) technique application to detect TGFβ1 SNP (G/C +915). Three milliliters were collected in a gel tube and the serum was separated then 250 µl of serum had been added to an Eppendorf tube containing 750 µl of triazole was used for the quantified expression of miR-99a.

The Genomic DNA Extraction from Fresh Blood

The procedure was achieved according to the method recommended by the manufacturing company (ReliaPrep Blood gDNA Miniprep System) promega-USA and the DNA was quantified by NanoDrop spectrophotometer and checked for purity and concentration.

### The *TGFβ1* Genotyping

The genotyping of TGFβ1 polymorphism was done by ARMS-PCR. The primer sequences with respective annealing temperature and amplicon size are shown in Table (1). In each 25 µl PCR reaction tube: Go taq Green Master Mix 12.5µl (Promega), upstream primer (generic) 1.5 µl, downstream primer 1.5 µl, DNA template 3.0 µl and Nuclease-free water 6.5 µl. The PCR cycling condition for SNP was the initial denaturation at 95 C̊ for 5 min. followed by 30 cycles for 30 sec. at 95 C̊ and annealing temperature 60 C̊ for 30 sec. and extension at 72 C̊ for 30 sec. with final extension at 72 C̊ for 5 min.

The PCR products were separated into 1% agarose gel containing 0.5 µg/ml ethidium bromide and observed under UV light. Figure (1) shows TGFβ1 genotypes.

Total RNA Extraction and Quantification of miR-99a Expression

Total RNA was extracted from serum samples by using the TRIzol® reagent kit (Bioneer. Korea) and checked by using a Nanodrop spectrophotometer that checks RNA concentration and estimation of RNA purity.

The Primers for miR-99a were designed by using (The Sanger Center miRNA database Registry) to select the miRNA sequence and using the miRNA Primer Design Tool, while the primer of U6 which was used as an internal control in the present study according to (9) Table (2). Reverse transcription was performed using a one-step Prime Script miRNA cDNA synthesis kit (Thermo Fisher Scientific). The stem-loop RT-qPCR was used in the quantification of miR-99a expression analysis that normalized by small nuclear RNA U6 gene in the serum of patients and control samples by using the qReal-Time PCR technique.

The data results of qRT-PCR for miR-99a and U6 gene were analyzed by the relative quantification gene expression levels (fold change) (The ΔCT Method Using a reference gene) that was described by (10).

The Statistical Analysis

Data were collected, summarized, analyzed, and presented using Statistical Package for Social Sciences (SPSS) version 23 and Microsoft Office Excel 2010. Qualitative (categorical) variables were expressed as numbers and percentages, whereas, quantitative (numeric) variables were first evaluated for normality distribution using the Kolmogorov-Smirnov test, and then accordingly normally distributed numeric variables were expressed as mean (an index of central tendency) and standard deviation (an index of dispersion).

## The Results

The Demographic Characteristics of the Patients and the Control Groups

The mean age of the patients and the control groups was 29.24 ±4.82 years versus 28.90 ±4.13 years respectively and there was no significant difference in the mean age between the two groups (P= 0.311). This result is mandatory for such a case-control study to exclude the impact of age factor on the bias of the studied parameters.

### The *TGFβ1* Genotypes

This study revealed a significant association for alleles and genotypes of TGFβ1 (+915 G/C) SNP between women with secondary unexplained infertility and healthy pregnant women as control individuals.

Table (3). shows a comparison of genotypes and alleles frequency distribution between the patients and control groups. Genotype CC was more frequent significantly in the patients group than in the control group, 28 % versus 2 % ($p < 0.001$) and the OR was 19.06 (so CC genotype is a risk factor). There was no significant difference in the frequency rate of genotype CT between study groups ($p = 0.106$). In addition, genotype GG was less frequent significantly in the patients group than in the control group, 4 % versus 16 % ($p = 0.046$), and the OR was 0.22, thus the GG genotype was a protective factor.

Allele C was more frequent and allele G was less frequent in the patients group 62 % and 43 % respectively, while allele C was less frequent and allele G more frequent in the control group 43% and 57 % respectively and the difference was significant ($p = 0.007$). The OR for allele C was 2.16, thus it was a risk factor and the OR for allele G was 0.46, thus it was a protective factor. The results appeared to support the association of the +915 CC genotype with the risk of developing secondary unexplained infertility.

The Expression of miR-99a in the Patients and the Control Groups and ROC Analysis

A comparison of the mean fold change of miR-99a between the patients and control groups is shown in Table (4) and Figure (2). The mean serum miR-99a of the patients group was significantly higher than that of the controls group, 3.70 ±1.18 versus, 0.84 ±0.34 respectively ($p < 0.001$).

(ROC) analysis was done and the results are shown in Table (5) and Figure (3). The cutoff value of miR-99a >1.99 sensitivity was 100 %, the specificity 98%, and the accuracy was 100%.

## The Discussion

This study revealed a significant association for alleles and genotypes of TGFβ1 +915 G/C SNP between women with secondary unexplained infertility and healthy pregnant women as control individuals. These results were consistent with (11) that found C allele and CC genotype were associated with an increased risk of unexplained infertility in females in the Iranian population. A previous study by (12) showed C allele in heterozygote and homozygote of TGFβ1 +915 G/C polymorphism increases the risk of unknown etiology recurrent miscarriage in the first trimester in Tunisian women. While (13) failed to identify the association of TGFβ1 polymorphism +915 G/C with miscarriage in women of the Brazilian population. These contradictory results were due to genetic heterogeneity and population stratification observed within each ethnic studied group in these studies. The present study indicated that the defect in the production and function of TGFβ1 in women with (+915) CC genotype contributed to the probability of increased incidence of secondary unexplained infertility.

The +915 G/C SNP, located in the signal peptide sequence at amino acid 25 corresponds to a change from a large polar amino acid (arginine encodes by CGT codon ) to a small apolar one (proline encoded by CCT codon), and is located close to the cleaved region of proTGF-β1 which gives rise to latency associated protein (LAP) and mature TGF-β1(11). The evaluation of protein hydrophobicity revealed that the presence of proline at a locus (+915) increases protein and hydrophobicity shortens the structure of the helix α and can cause a disruption of protein function. It also has a role in unstable protein secondary structure and leads to destructive change in the secondary structure of mRNA. One of the mRNA arms removed the structure of the other arm severely disrupted and two new loops were added to the molecule (11). This can illustrate why the CC genotype and allele C are more frequent in infertile women, and the GG genotype and G alleles are more frequent in healthy fertile control. Moreover, this study showed that the mean serum miR-99a of the patients group was significantly higher than that of the control group this result corresponded with (5) that found up-regulation of miR-99a in women with repeat implantation failure. The predictive target for miR-99a is the mammalian Target Of Rapamycin (mTOR) (14). In female reproduction, the mTOR pathway regulates events that involve follicle quiescence, proper oocyte maturation, ovulation, and embryo and placenta development ( 15). It also regulates the translation of proteins required for trophoblast differentiation and endometrial receptivity (16,17). So, disruption of mTOR signaling could cause unexplained infertility (18,19).

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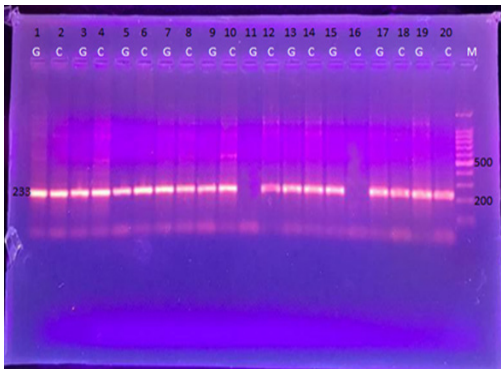


Figure 1: Agarose gel electrophoresis (1%, 1X TBE) show three genotypes by ARMS (~233bp) of TGFβ1 genotypes for 10 patients each patient loaded two PCR products for G and C alleles. lanes of (1,2) to (9,10), (13,14), (17,18) and (19,20) exhibit GC, lanes (11,12) exhibit CC, and (15,16) exhibit GG.

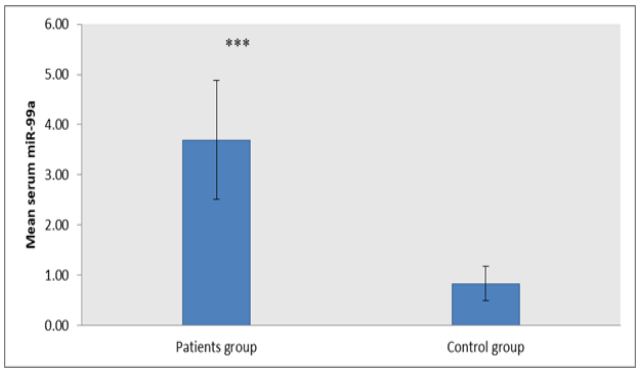


Figure 2. Bar chart showing a comparison of mean serum miR-99a between patients and control group

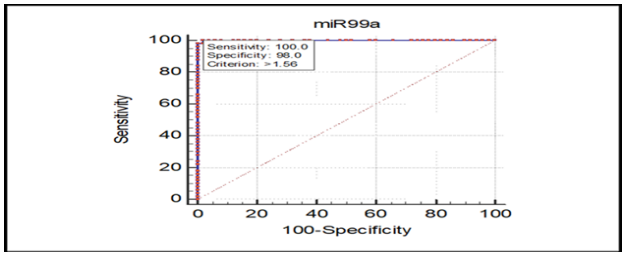


Figure 3. ROC curve analysis to find the cutoff value of miR-99a that can predict a positive diagnosis

Table 1: primers sequences used for detection of TGF-β1gene

| Polymorphism, location of SNP | Primers sequences | Product of allele | | Method | Annealing temperature |
|---|---|---|---|---|---|
| TGF-β1 exon1 Locus +915 | 5′-GTGCTGACGCCTGGCCC-3′ | G | 233 | ARMS-PCR | 60 C̊ |
| | 5′-GTGCTGACGCCTGGCCG-3′ | C | 233 | | |
| | 5′-GGCTCCGGTTCTGCACTC-3′ | Generic | | | |

Table 2. shows primers of 99a and U6 which were used in this study

| Primer | Sequence | |
|---|---|---|
| hsa-miR-99a RTprimer | | 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCACAAG -3′ |
| hsa-miR-99a | F | 5′- AACAAGATCACATTGCCAGGG -3′ |
| | R | 5′- GTCGTATCCAGTGCAGGGT -3′ |
| U6 (snRNA) | F | 5′-CTCGCTTCGGCAGCACATAT-3′ |
| | R | 5′-TTGCGTGTCATCCTTGCG-3′ |

Table 3: Comparison of TGFβ1 SNP 915 (GC) genotypes and alleles frequency distribution between patients and control group

| **TGFβ1 SNP 915 (CG)** | **Patients group** *n* = 50 | **Control group** *n* = 50 | ***p*** | **OR** | **95% CI** |
|---|---|---|---|---|---|
| **Genotypes** | | | | | |
| CC, *n* (%) | 14 (28 %) | 1 (2 %) | <0.001 *** | 19.06 | 2.40 -151.60 |
| CG, *n* (%) | 34 (68 %) | 41 (82 %) | 0.106 C NS | 0.47 | 0.18 -1.19 |
| GG, *n* (%) | 2 (4 %) | 8 (16 %) | 0.046 C * | 0.22 | 0.04 -1.09 |
| **Alleles** | **Patients group** *n* = 100 | **Control group** *n* = 100 | ***p*** | **OR** | **95% CI** |
| C, *n* (%) | 62 (62 %) | 43 (43 %) | 0.007 C *** | 2.16 | 1.23 -3.81 |
| G, *n* (%) | 38 (38 %) | 57 (57 %) | | 0.46 | 0.26 -0.81 |

C: chi-square test; NS: not significant; ***: significant at p ≤ 0.001; OR: odds ratio; CI: confidence interval

Table 4: Comparison of mean fold change of miR-23b between patients and control groups.

| **Characteristic** | **Patients group** *n* = 50 | **Control group** *n* = 50 | ***p*** |
|---|---|---|---|
| **miR-99a** | | | |
| Mean ±SD | 3.70 ±1.18 | 0.84 ±0.34 | < 0.001 *** |
| Range | 1.59 -6.39 | 0.2 -1.69 | |

n: number of cases; SD: standard deviation; t-test; ***: significant at p ≤ 0.001

Table 5: The statistical indexes of ROC curves

| **Characteristic** | **miR-23b** |
|---|---|
| Cutoff value | >1.99 |
| AUC | 1.000 |
| 95% CI | 0.964 to 1.000 |
| *p* | <0.001 *** |
| Sensitivity % | 100.00 |
| Specificity % | 98.00 |
| Accuracy % | 100.00 |