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The correlation between the expression of the growth differentiation factor 9B (*GDF9B*) gene and *miR-378-5p* in infertile women

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Abstract

The normal expression of follicle-producing genes, as well as the hormone balance, and reproductive physiology all have a significant impact on female fertility. Infertile Iraqi women were compared to controls who appeared to be in good health to assess the gene expression of the growth differentiation factor B9 (*GDF9B)* gene. The second goal was to quantify the gene expression of a particular miRNA (*miRNA 378-5p*), that specifically targets the *GDF9B* gene. The study included 50 samples from infertile women diagnosed with polycystic ovarian syndrome and hyperprolactinemia, along with an additional 50 samples from healthy fertile women The ELISA technique was used to measure the levels of the hormones FSH, LH, AMH, and prolactin. Also, real-time qPCR was used to measure the gene expression of the *GDF9B* gene and of its target's *miRNA 378-5p* gene. The results showed an increase in the levels of hormones in the patients compared with the healthy females, with almost three-fold of change. There was also a decrease in the expression of the *GDF9B* gene and an increase in the expression of *miRNA 378-5p* gene by 0.2- and 0.3-fold change, respectively, in the patients compared with the healthy subjects. The relevance of the *GDF9B* gene and its function in ovarian development were confirmed by the lower expression of this gene in infertile women. Moreover, the high level of *miRNA 378-5p* suggests that the expression of genes is controlled post-transcriptionally and that their functions are restricted. Hence, excessive hormone levels may be linked to ovarian abnormalities that interfere with reproduction.

Keywords: Follicle-producing genes, Hormone balance, Post-transcriptional control, Female infertility, Prolactin, Ovarian development.

العلا قة بين التعبير الجيني لعامل تمايز النمو ب 9 (*GDF9B*) و*miRNA 378-5p* في النساء العقيمات

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الخلاصه

إن التعبير الطبيعي عن الجينات المنتجة للجريب ، وتوازن الهرمونات ، وعلم وظائف الأعضاء التناسلية جميعها لها تأثير كبير على خصوبة المرأة. تمت مقارنة النساء العراقيات المصابات بالعقم بالضوابط اللاتي بدون بصحة جيدة لتقييم التعبير الجيني لجين عامل تمايز النموب9 (GDF9B),هذا كاول هدف من الدراسة اما تحديد التعبير الجيني لميرنا 378- 5 (miRNA 378-5p) الذي يستهدف جين عامل تمايز النموب9 (GDF9B) فكان الهدف الثاني.شملت الدراسة 50 عينة من نساء يعانين من العقم وتم تشخيص إصابتهن بمتلازمة المبيض المتعدد الكيسات وفرط بروﻻكتين الدم بالإضافة إلى 50 عينة من نساء يتمتعن بصحة جيدة وخصيبات. تم استخدام تقنية ELISA لقياس مستويات الهرمونات FSH و LH و AMH والبروﻻكتين. كما استخدم qPCR في الوقت الفعلي لقياس التعبير الجيني لجين GDF9B و-378 miRNA 5p. أظهرت النتائج زيادة في مستوى الهرمونات لدى المرضى في كلا المجموعتين مقارنة بالإناث الأصحاء ، مع ما يقرب من ثلاثة أضعاف التغيير ، بينما كان هناك انخفاض في مستوى التعبير الجيني لجين GDF9B في المرضى مقارنة مع الأصحاء ، بالإضافة إلى زيادة التعبير الجيني لـ 378-5p miRNA في المرضى مقارنة بالأشخاص الأصحاء. إن الاعتبارات القائلة بأن خصوبة الإناث تتأثر بشدة بعلم وظائف الأعضاء التناسلي ، والتوازن الهرموني ، والتعبير الجيني الفعال لتكوين الجريبات ، قادتنا إلى استنتاج مفاده أن أهمية جين GDF9B ووظيفته في تطورالمبيض قد تم تأكيدهما من خلال التعبير الجيني المنخض في النساء العقيمات. علاوة على ذلك ، يشير المستوى العالي من 378-5p miRNA إلى أنه يتم التحكم في التعبير عن الجينات بعد النسخ وأن وظيفة الجين مقيدة. ومن ثم ، قد ترتبط المستويات المفرطة للهرمونات لدى المريضة بخلل في المبايض ، مما يتداخل مع التكاثر.

1. Introduction

One of the disorders that affect women of reproductive age and cause infertility and sub-infertility is polycystic ovarian syndrome (PCOS). Also known as a hyperandrogenic disorder. POCS appears to have a complex etiology, indicating that a variety of factors, such as genetic, environmental, and lifestyle factors, can all have an effect [1]. Chronic oligo-anovulation and polycystic ovarian morphology are two features of PCOS. which, along with hypothyroidism and hyperprolactinemia, can have major consequences, including the risk of infertility [2]**.** PCOS is recognized as a complex genetic disorder, caused by a number of predisposing genes, rather than one, combined with environmental circumstances [3].

A physiological, pathological, or idiopathic origin may be responsible for the prevalent endocrinological condition, Known as hyperprolactinemia. Hyperprolactinemia's primary physiological impact is the suppression of pulsatile gonadotropin-releasing hormone (GnRH). Depending on the patient's age and gender, the clinical manifestations of the disease vary substantially. In women, it frequently results in gonadal dysfunction, including ovulatory difficulties, menstrual irregularity, galactorrhoea, and infertility [4]. A polypeptide hormone called prolactin is pulsating secreted from the anterior pituitary gland. Along with lactation, it is crucial for a variety of reproductive processes. Physiological factors influence and modulate human prolactin secretion in response to diverse stimuli. A challenge in diagnosing and managing hyperprolactinemia is the presence of the macro-prolactin molecule, which is physiologically inactive but recognized as such in the majority of immunological assays [5].

The growth differentiation factor B9 (*GDF9B*) gene, also known as the bone morphogenetic protein 15 (*BMP15*) gene, has a unique correlation to female reproductive development. As a multifunctional latent factor specific to oocytes, this gene has been reported to regulate follicle

growth and ovulation frequency, and so it governs female fertility and follicular development. [6]. *GDF9B* is required for folliculogenesis and female fertility, as well as inhibiting LH-stimulated androstenedione synthesis in theca cells [7].

BMP15 belongs to the transforming growth factor beta superfamily, with a location on the chromosome Xp11.2. It encodes 392 amino acid proteins [8, 9]. Nearly 1,361 distinctive gene transcripts are expressed in human oocytes. The genetic alteration of these genes, and their regulatory molecules, such as miRNAs, all work together to control oogenesis [10]. The cause of infertility is unknown (i.e., idiopathic) in more than half of infertile women; however, a few factors are involved, including genetic, immunological, and environmental factors, as well as the hormonal issue; however, the pathway of interaction has not been precisely defined and is under consideration [11, 12]. The *MicroRNA 378a* gene is a non-coding RNA gene and is affiliated with the miRNA class; Diseases associated with miRNA include the myelodysplastic syndrome and arterial standstill 1[13]. miRNA expression in the ovary varies according to cell type, function, and estrous cycle stage. Primordial follicle development, follicular recruitment and selection, follicular atresia, oocyte-cumulus cell interaction, granulosa cell activity, and luteinization are all influenced by miRNAs [14]. They were recognized as significant posttranscriptional regulators of gene expression more than 20 years ago. Recently, studies are commonly focusing on the regulatory function of these small RNA molecules in the human reproductive system. The control of reproductive processes, including oocyte maturation, folliculogenesis, corpus luteum function, implantation, and early embryonic development, has been demonstrated to be significantly influenced by mRNAs [15]. The current study aimed to evaluate the gene expression of the GDF9B gene and its associated miRNA 378-5p in Iraqi women with PCOS, in addition to measuring levels of some hormones, including FSH, LH, AHM, and prolactin.

2. Materials and Methods

2.1 Subjects, sample size, and study design

A total of one hundred blood samples were collected from Iraqi females and classified into two groups: 50 samples of infertile women (divided into 25 with polycystic ovarian syndrome (PCOS) and 25 with hyperprolactinemia (HPL)), and 50 samples of healthy fertile women. Patients were between 24 and 45 years old who attended Al-Elwiya Educational and Kadhimiya Teaching Hospitals in Baghdad, Iraq, from April to August 2022. Seven to ten millilitres of venous blood. were collected and each sample was promptly divided into two tubes: one in an EDTA tube for gene expression and the other in a gel tube for hormone determination using the enzyme-linked immunosorbent assay (ELISA).

2.2 Enzyme-linked immunosorbent assay (ELISA)

The quantitative sandwich ELISA kits were used for in vitro quantitative determination of the human follicle-stimulating hormone (FSH) (Cat. No. EL10013; ANOGEN Co., Canada) concentration in serum. Additionally, measurements of the levels of human luteinizing hormone (LH) (Cat. No. EL10011; ANOGEN Co., Canada), human prolactin (Cat. No. EL10014; ANOGEN Co., Canada), and human anti-Mullerian hormone (AMH) (Cat. No. MBS262919; MyBioSource Co., USA) were conducted.

2.3 RNA Analysis

RNA Extraction: Total RNA was directly extracted from the whole blood sample using the Easy Pure® Blood RNA Kit (Cat. No. ER401) (TransGen Biotech Company, China) by following the manufacturer's instructions. The Nanodrop spectrophotometer 2000c (Thermo Fisher Scientific, USA) was used to measure the concentration and purity of the extracted RNA and thereby assess the quality of samples for later RT-qPCR analysis. The samples' RNA

concentrations ranged from 50 to 100 ng/µl. The sample's absorbance was measured at two distinct wavelengths to determine RNA purity (260 and 280nm). The presence of an A260/A280 ratio between 2.0 and 2.1 indicates optimal purity of the nucleic acid sample, with minimal contamination by proteins or other impurities.

Reverse transcription: Total RNA was reverse-transcribed to complementary DNA (cDNA). The EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (Cat. No. AE311-02) (TransGen Biotech Company, China) was used according to the manufacturer's instructions. The process was performed in a reaction volume of 20 µl. (4 µl) with a concentration of 80 µg/µl of total RNA, which had to be reversely transcribed. After being subjected to thermal conditions of 25 °C for 10 minutes and 42 °C for 15 minutes in a thermal cycler, the enzyme was inactivated at 85 °C for 5 seconds.

RT-qPCR primer design: The primers of *GDF9B* gene were designed and the specific sequences were sent to Alpha DNA Company in Canada. The received primers in their lyophilized form were dissolved with nuclease-free water until they reached a concentration of 10 pmol/µl. The primers of the other genes used in the study were organized according to their reference sequence, as shown in Table 1.

Table 1: qPCR primer pairs and sequences.

Primer	Sequence (5′→3′ direction)	Tm (°C)	Product size (bp)
Growth differentiation factor 9B (*GDF9B*) gene			120
Forward	CTAGAAGAATCCCCTGGCGA	62	
Reverse	ATGGTGCGGTTCTCTCTAGG	62	
Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)* gene			150
Forward	GAATCCATCACCATCTTCCAGG	66	
Reverse	GAGCCCCAGCCTTCTCCATG	66	
miRNA 378- 5p			70
Forward	TGG CTCCTGACTCCAGGTC	62	
Reverse	CGAGGAAGAAGACGGAAGAAT	62	
miRNA U6			73
miRU6 F.P.	AGAGAAGATTAGCATGGCCCCT	66	
miRNA-universe R.P.	GCGAGCACAGAATTAATACGAC	64	

qPCR genes expression**:** The reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to estimate gene expression levels using the TransStart® Top Green qPCR Super Mix kit (TransGen, biotech. AQ131-01). According to the manufacturer's instructions, the reaction was carried out with a final volume of 20 µl. The amount of the Master mix containing SYBR Green dye needed for preparing the required number of the reactions was calculated to be 10 µl. To complete the final volume of the reaction, 3 µl of cDNA as a template, 1 µl each of the forward and reverse primers, and 5 µl of nuclease-free water were added. The cycling routine was created using the thermal profile presented in Table 2.

Table 2: RT-qPCR thermal conditions.

Stage		Temperature °C	Time /Sec.	Cycle
Stage 1	Denaturation	94	60	1
Stage 2	Denaturation	94	5	35
	Annealing	58	15	
	Extension*	72	20	
Stage 3	Dissociation	65-95	1	1

2.4 Statistical analysis

The means of both genes and miRNA in the current study were measured using Excel 2010. Fold expression was detected depending on the $\mathbf{2^{-\Delta\Delta Ct}}$ method. The correlation between the *GDF9B* gene and miRNA378-5P was determined using SPSS version 26.

3. Results

3.1 Quantitative determination of hormone levels

The present study employed the quantitative ELISA technique to measure hormone concentrations in the serum of PCOS and hyperprolactinemia patients, along with a control group. The mean values of FSH, LH, AMH, and prolactin were found to be significantly different among the groups with a p-value of 0.0001. The results showed higher hormone concentrations (mIU/mL) in 25 samples from infertile women diagnosed with PCOS and another 25 samples from those with HPL compared to the control group, as presented in Table 3. Specifically, the average values of FSH, LH, AMH, and prolactin in PCOS patients were (19.33±0.77, 19.62±2.05, 10.35±0.80, and 32.51±2.21, respectively). Similarly, the average values of hormone concentrations in serum in hyperprolactinemia patients were (27.06±1.97, 21.89±1.83, 4.91±0.48, and 43.45±3.87, respectively). In contrast, the control group (healthy females) exhibited lower mean values of FSH, LH, AMH, and prolactin (7.44±1.26, 4.29±0.55, 2.33±0.17, and 17.22±1.77, respectively).

Table 3: Mean ±SE values of serum hormone concentration in study groups. PCOS; polycystic ovarian syndrome, HPL; hyperprolactinemia, FSH; human follicle-stimulating hormone, LH; luteinizing hormone, AMH; anti-müllerian hormone, LSD; Least Significant Difference

Study Groups	Hormone concentration (mIU/mL) Mean ± SE			
	FSH	LH	AMH	Prolactin
PCOS	19.33±0.77	19.62±2.05	10.35±0.80	32.51 ±2.21
HPL	27.06±1.97	21.89±1.83	4.91±0.48	43.45 ± 3.87
Control	7.44±1.26	4.29±0.55	2.33±0.17	17.22± 1.77
LSD	4.00	4.64	1.55	7.91
P-Value	0.0001	0.0001	0.0001	0.0001

3.2 GDF9B Gene and miRNA378-5P Expression Patterns

The levels of fold expression for the *GDF9B* gene and *miRNA378-5P* in female patients diagnosed with PCOS and hyperprolactinemia compared to healthy control subjects are displayed in Table (4). The findings revealed that both patient groups exhibited a notable down-regulation of the *GDF9B* gene expression, as evidenced by fold expressions of (0.353) and (0.204) for PCOS and hyperprolactinemia, respectively. Moreover, the study measured the expression of the *miRNA378-5P* gene, which is known to regulate the *GDF9B* gene, and the results indicated a significant up-regulation in its expression in both patient groups, with fold expressions of (3.409) and (2.088) for PCOS and hyperprolactinemia, respectively, compared to healthy controls. These findings shed light on the molecular mechanisms underlying the pathogenesis of hormonal disorders and may contribute to the development of novel diagnostic and therapeutic interventions

Table 4: Comparison and Expression Analysis of *GDF9B* and *miRNA378-5p* Genes in the Study Groups

Study Groups	Mean of target gene Ct Values	Mean of reference gene Ct Values	Δct	ΔΔct	$2^{-\Delta\Delta Ct}$ Fold of gene expression
Growth differentiation factor 9B (*GDF9B*) gene					
PCOS	24.78	18.03	6.75	1.50	0.35
HPL	25.07	17.53	7.54	2.29	0.20
Control	23.72	18.47	5.25	0.00	1.00
miRNA 378- 5p					
PCOS	26.03	17.69	8.34	-1.77	3.41
HPL	26.30	17.25	9.05	-1.06	2.08
Control	27.37	17.26	10.11	0.00	1.00

The correlation between the gene expression of *GDF9B* and *miRNA 378-5p* was assessed through a Pearson correlation coefficient test. The results presented in Table 5 revealed a negative correlation between the two genes in all study groups, indicating that higher expression of *miRNA 378-5p* is associated with lower expression of the *GDF9B* gene, which confirmed the findings of the fold expression analysis. The Pearson correlation coefficient values were -0.025, -0.194, and -0.145 for the PCOS, hyperprolactinemia, and control groups, respectively, with corresponding p-values of 0.9, 0.6, and 0.8.

Table 5: Pearson correlation coefficients between *GDF9B* gene expression and *miRNA378-5p* in study groups

Correlation between *GDF9B* gene & *mi-RNA378-5p*			
Study Group	Pearson Correlation Coefficient factor	Results	*P*-Value
PCOS	-0.025	Negatively associated	0.9
HPL	-0.194	Negatively associated	0.6
Control	-0.145	Negatively associated	0.8

4. Discussion

Female infertility is a worldwide complex disorder that has an impact on several facets of family life and society. Early identification and control of the various genetic (chromosomal abnormalities, mutations in genes involved in reproductive function), epigenetic, hormonal, and environmental factors such as exposure to toxins or radiation that can contribute to female infertility are essential in improving the chances of successful pregnancy and reducing the impact of infertility on individuals and society [16].

This report showed an increase in the levels of hormones in the sera of both PCOS and hyperprolactinemia patients, compared with healthy females, with almost 3-fold change, which supports the relationship between this increase and the important roles of these hormones in the formation of the ovaries and reproduction. These findings are consistent with a previous study conducted by Falah (2022), which reported that women with PCOS may have higher serum prolactin levels and may experience ovulatory disruption, which can negatively affect their chances of pregnancy. Besides, women with PCOS were more likely to experience hyperprolactinemia than normal fertile women [17]. The current study identified that LH serum levels were significantly different in infertile females compared to healthy ones, which is

consistent with the results of a previous study published by Hashemi and colleagues [18]. Other studies also reported that hormonal imbalances, including elevated levels of FSH, LH, and prolactin, can contribute to female infertility [19]. BMP15 was reported to inhibit FSH expression in human granulocytes through Smad and non-Smad mechanisms, which could be used to regulate follicular expansion. Incomplete puberty and impaired ovarian function can result from an increase in the level of follicle-stimulating hormone, causing the ovarian follicles to develop abnormally and fail to produce an ovum, leading to infertility [20]. A previous study reported by Saadia found that PCOS, a common endocrinological problem among women of reproductive age, is characterized by chronic ovulatory dysfunction, hyperandrogenism, and an elevated LH:FSH ratio, which is consistent with the current study's findings [21]. Overall, these findings have significant clinical implications for the diagnosis and treatment of female infertility, particularly in PCOS and hyperprolactinemia.

Anti-Müllerian hormone (AMH) is a reliable predictor of ovarian reserve and can significantly enhance the efficiency of in vitro fertilization (IVF), as it correlates with the number of follicles that can potentially generate oocytes for ovulation. AMH has the potential to become a standard diagnostic marker for ovarian diseases, particularly for detecting ovarian malignancies. In addition, it may serve as a therapeutic tool for specific tumors and could help prevent oocyte loss due to chemotherapy or radiation treatment [22]. Overall, AMH is a valuable versatile biomarker and a strong predictive tool for personalized medicine that shows promising prospects for improving fertility treatments and managing ovarian diseases.

The present study identified that the expression of the *GDF9B* gene was notably down-regulated in both hyperprolactinemia and PCOS patients compared to healthy females, with fold expression values of 0.2 and 0.3, respectively. These results are consistent with previous studies, which reported down-regulation of the *GDF9B* gene in primary and secondary infertile women compared to fertile women. Hormonal influences were also found to have a significant impact on the expression of the gene [23, 24]. Another study published by Riepsamen and coworkers reported lower serum levels of the *GDF9B* gene in females with infertility factors, while levels were higher in fertile control females [25]. These findings suggest that *GDF9B* may be involved in the pathogenesis of both PCOS and hyperprolactinemia conditions, as it plays a critical role in oocyte maturation and follicular development. Hence, a defect in this gene may lead to abnormalities in the ovaries, resulting in infertility. These findings could have important clinical implications for the diagnosis and treatment of female infertility.

According to recent studies, miRNAs are crucial in the onset and progression of problems connected to female infertility. The key to improving the prognosis of these illnesses and lowering the risk of infertility and other adverse effects is early identification and control [26]. Based on our results, the expression of the *miRNA378-5p* gene was notably up-regulated in infertile females compared to healthy controls, with fold expression increases of two and three times in hyperprolactinemia and PCOS patients, respectively. This finding is consistent with a previous report that linked the down-regulation of miR-378a-3p to increased apoptosis via increased caspase-3 expression in the decidua of women with early pregnancy loss, suggesting that it could be a potential underlying mechanism of EPL. Progesterone has been demonstrated to increase the expression of miR-378a-3p, which could be a potential mechanism for the treatment of EPL [27]. Another study showed that miR-378a-5p can reduce cell proliferation in colorectal cancer (CRC) cells by decreasing the expression of cell cycle-dependent protein kinase 1 (CDK1), suggesting that this miRNA could be a potential therapeutic target for CRC [28]. These findings further demonstrate the potential clinical significance of miR-378a-5p and its potential application in the treatment of different diseases. Collectively, our data suggest that the up-regulation of *miRNA 378-5p* in infertile females may have an implication in

understanding the underlying mechanisms of female's infertility, or EPL, and potentially improving its treatment. The findings also suggest that miR-378a-5p could be a therapeutic target during the treatment of this disorder. However, further research is necessary to fully understand the role of miRNA 378-5p in these conditions.

5. Conclusions

The present study suggest that women diagnosed with PCOS and hyperprolactinemia exhibit elevated levels of FSH, LH, AMH, and prolactin, which can negatively impact oocyte development and follicle formation, potentially leading to infertility. Furthermore, it provides valuable insights into the molecular mechanisms underlying the pathogenesis of PCOS and hyperprolactinemia-related infertility. The upregulation of miRNA 378-5p, which inhibits the expression of the GDF9B gene, suggests a potential role for miRNAs in modulating ovarian function and reproductive health. These findings have important clinical implications, as identifying reliable biomarkers for female infertility could help improve the diagnosis, treatment, and management of this condition. Moreover, the study highlights the potential for targeted therapies aimed at modulating miRNA378-5p expression levels to help restore ovarian function and fertility in women diagnosed with PCOS and hyperprolactinemia. This study opens a way to further identify the exact mechanism of the complex interplay between miRNAs, gene expression, and ovarian function, ultimately leading to new treatment strategies and improved outcomes for patients with female infertility.

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Conflict of interest

There are no conflicts of interest to be declared.

Author contributions

Conceptualization, study design, sample collection, qRT-PCR: Al-Kafagi and Abdul Hussein. Data analyses, manuscript drafting, and manuscript finalization: Mohammed, Hussein and Al-Tameemi.

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