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Original Article

Unveiling Novel Biomarkers: A Clinical Study of miR-146a and miR-222 in the Diagnosis and Treatment of Polycystic Ovary Syndrome

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ABSTRACT

Introduction: Polycystic ovary syndrome (PCOS) is a prevalent endocrine disorder affecting 5%-10% of women of reproductive age. It is marked by hyperandrogenism, chronic anovulation, and polycystic ovarian morphology, with associated long-term health risks, including cardiovascular disease, type 2 diabetes, and infertility. This study investigates the potential of specific microRNAs, namely miR-146a and miR-222, as novel biomarkers for the diagnosis and treatment of PCOS.

Materials and Methods: A structured, evidence-based approach was undertaken using real-time PCR to analyze the expression levels of miR-146a and miR-222 in Wistar albino rats with DHEA-induced PCOS. Blood samples were collected for RNA extraction and subsequent miRNA expression quantification. The diagnostic potential was evaluated through receiver operating characteristic (ROC) curve analysis of the expression data.

Results: Both miR-146a and miR-222 showed upregulation in the PCOS group, compared to controls, though these differences were not statistically significant. ROC analysis indicated that miR-222 had a moderate discriminatory capability, with an area under the curve (AUC) of 0.70, supporting its potential as a biomarker for PCOS. miR-146a presented an AUC of 0.65, suggesting a less robust but relevant role in differentiating PCOS from control samples.

Conclusion: The findings propose that miR-146a and miR-222 may serve as viable biomarkers for PCOS, facilitating the advancement of non-invasive diagnostic methods and targeted therapeutic options. Nevertheless, additional studies with larger sample sizes are essential to substantiate these preliminary findings.

1. Introduction

Infertility, defined as the inability to conceive after 12 months of unprotected intercourse (or 6 months if the female partner is over 35), has posed a significant challenge for many couples and is influenced by various reproductive health condition[1]. Among these, Polycystic Ovary Syndrome (PCOS) is widely recognized as one of the most common and complex metabolic disorders impacting the reproductive and endocrine systems of women[2]. PCOS is diagnosed based on the Rotterdam criteria, requiring the presence of at least two of the following: ovarian cysts, ovulatory dysfunction, and hyperandrogenism[3]. The syndrome is characterized by chronic anovulation, hyperandrogenism, and polycystic ovarian morphology and is strongly associated with long-term health risks, including cardiovascular disease, type 2 diabetes, obesity, endometrial cancer, and menstrual irregularities[4,5].

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The condition of hyperandrogenism in PCOS has frequently been linked to insulin resistance and obesity, conditions that are sometimes compounded by androgen-secreting tumors[6]. Symptoms including acanthosis nigricans, acne, hirsutism, and seborrhea further complicate the syndrome's presentation and management[7]. Current management strategies have typically involved lifestyle modifications, hormonal contraceptives, and insulin-sensitizing agents to address the syndrome's metabolic and reproductive effects[8]. However, the prevalence of PCOS has varied significantly across populations, with studies reporting rates between 2.2% and 26%, largely due to differing diagnostic criteria and population sampling methods[9]. For instance, studies conducted in Iran have reported PCOS prevalence rates of 7.1% under the National Institute of Health (NIH) criteria and up to 14.6% when applying the Rotterdam criteria, underscoring the need for a standardized diagnostic approach to improve consistency across studies[10]. Although features, such as hyperinsulinemia, menstrual irregularities, ovarian morphology, and metabolic syndrome are commonly associated with PCOS, these alone do not serve as definitive diagnostic features[3,11]. The complex diagnostic landscape of PCOS highlights the urgent need for more precise biomarkers to facilitate early detection and management of this disorder. In recent years, microRNAs (miRNAs) have been increasingly explored as non-invasive biomarkers for various diseases, including PCOS[12]. These small RNA molecules, generally 19-25 nucleotides in length, regulate gene expression at the post-transcriptional level and can be isolated from numerous biological sources, including serum, plasma, tears, and urine[13,14]. Among the miRNAs associated with PCOS, miR-146a and miR-222 have emerged as potentially significant biomarkers due to their roles in regulating endocrine signaling pathways and their altered expression in PCOS patients[15]. miR-146a, for example, has been identified as a critical modulator of inflammation by inhibiting excessive inflammatory responses through a feedback loop in the innate immune system[16]. Both miR-146a and miR-222 have shown relevance in signaling pathways, such as MAPK, Wnt, and Jak-STAT pathways that influence cell cycle regulation, apoptosis, and other processes relevant to the pathogenesis of PCOS[17]. Notably, elevated expression levels of these miRNAs have been observed in PCOS patients, compared to healthy controls, suggesting their potential utility in diagnosing and understanding PCOS[18-20].

This study was conducted to investigate the potential of miR-146a and miR-222 as biomarkers for PCOS, thereby advancing diagnostic and therapeutic strategies. By examining the roles of these miRNAs in PCOS, we hope to contribute to a better understanding of the molecular mechanisms underlying the disorder and provide avenues for targeted interventions that may improve management and outcomes for affected individuals.

2. Materials and Methods

This study utilized an evidence-based study technique to compare the role of miR-146a and mir-222 in Wistar albino rats with polycystic ovarian syndrome.

2.1. Ethical approval

The study was carried out after approval by the ethics committee of the Islamic Azad University of Mashhad (IR.IAU.MSHD.REC.1399.209).

2.2. Animals and PCOS models

In total, 12 female Wistar albino rats were obtained prepuberty (21 days old) from the Laboratory Animal Research Center of the Ferdowsi University of Mashhad. The animals were acclimatized for one week in an animal house maintained at 23°C with 60±5% humidity, under a 12-hour light/dark cycle, and housed in separate cages under standard conditions throughout the experiment. The rats were provided with commercial feed containing specified proportions of protein, fat, fiber, ash, and nutrients, along with ad libitum access to water. All experimental protocols were conducted in accordance with the guidelines of the Animal Care Committee of the Ministry of Health. The animals were divided into two groups, each comprising 6 rats, as detailed in Table 1.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from blood samples using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) following the manufacturer's protocol. To remove any potential DNA contamination, the extracted RNA was treated with DNase enzyme (Takara, Japan) in an RNase-free environment. The first strand of complementary DNA (cDNA) was synthesized using the Hyperscript RT reagent Kit (GeneAll, South Korea) and random hexamer primers (Takara, Japan), as per the manufacturer's instructions[19].

2.4. Quantitative real-time PCR

Specific PCR primers for miR-146a and miR-222 were designed using bioinformatics tools, such as Gene Runner

Table 1. Groups participating in the study

Group Number	Group Name	Description
1	Positive control group	In the positive control group, 0.2 ml of sesame oil and 0.01 ml of 95% ethanol were injected into the back of the neck for 35 days.
2	DHEA group (PCOS group)	1mg/kg/day DHEA (using 0.2ml sesame oil and 0.01ml 95% ethanol as a tool) was injected for 35 consecutive days.

DHEA: Dehydroepiandrosterone

Table 2. The list of primers used to specifically amplify

Transcript	Primer	Sequence	PCR Product (bp)
miR-146a	F	5'- GCGGTTTTCCCAGTCACGAC -3'	20
	R	5'- GTGCAGGGTCCGAGGT -3'	16
miR-222	F	5'- AGCUACAUUCCCUACAAUGC -3'	20
	R	5'- GGGCAUUGUAGGGAAUGUAGCU -3'	22

(version 3.05), PerIPrimer (version 1.1.21), and Oligo (version 7.56). All quantitative real-time PCR reactions were performed using Real Q plus 2x master mix Green (Ampliqon, Denmark), supplemented with ROX dye, on an ABI STEP ONE real-time PCR system (Applied Biosystems, Foster City, CA). The following cycling conditions were used: initial denaturation at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 63°C for 55 seconds. The authenticity of the PCR products was confirmed through melt curve analysis and direct sequencing (Table 2) [21].

2.5. Statistics

All real-time PCR experiments were conducted in duplicate to ensure accuracy and reproducibility. PCR efficiency was calculated using LinReg PCR software (version 11), and the 2^-ΔΔCT method was applied to normalize gene expression levels in the PCOS group relative to the control group. To assess the diagnostic potential of miR-146a and miR-222, receiver operating characteristic (ROC) curve analysis was performed using GraphPad Prism (version 5.0.0.288). This analysis evaluated the discriminatory ability of the biomarkers to distinguish between PCOS and control groups and categorize PCOS severity.

3. Results

3.1. Differential Expression of miR-146a and miR-222 in PCOS

The expression levels of miR-146a and miR-222 were quantified in both the control and PCOS groups using quantitative real-time PCR (qRT-PCR). Relative expression levels, normalized to the control group, are displayed in Figures 1 and 2.

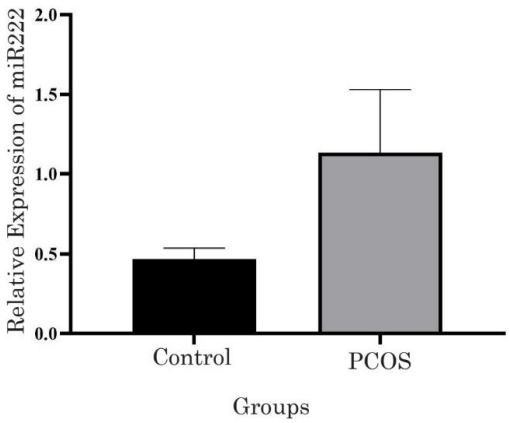

Figure 1. The chart of relative expression of miR-222 in control and PCOS groups

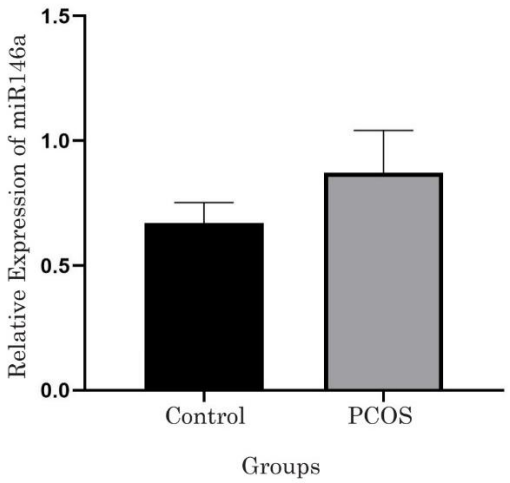

Figure 2. The chart of relative expression of miR-146a in control and PCOS groups

- **miR-222 Expression:** Figure 1 shows that the relative expression of miR-222 was higher in the PCOS group, compared to the control group.
- **miR-146a Expression:** Similarly, as illustrated in Figure 2, the expression level of miR-146a was also elevated in the PCOS group relative to the control group.

The area under the ROC curve (AUC) for miR-222 was calculated as 0.70. Sensitivity and specificity associated with this AUC were 70% and 65%, respectively, within a 95% confidence interval (Figure 3).

The area under the ROC curve (AUC) for miR-146a was calculated to be 0.65. Sensitivity and specificity associated with this AUC were 65% and 60%, respectively, within a 95% confidence interval (Figure 4).

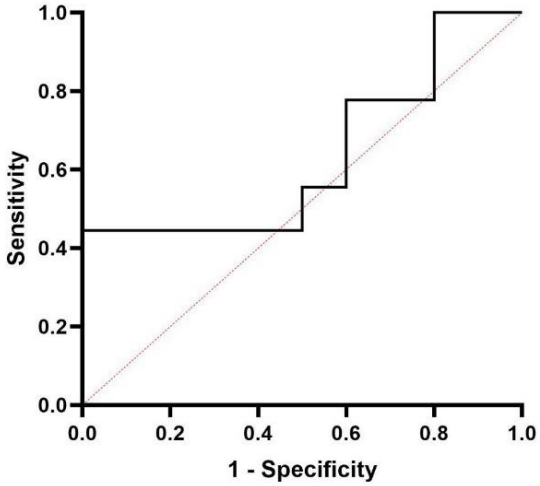

Figure 3. The Receiver Operating Characteristic (ROC) curve of miR-222

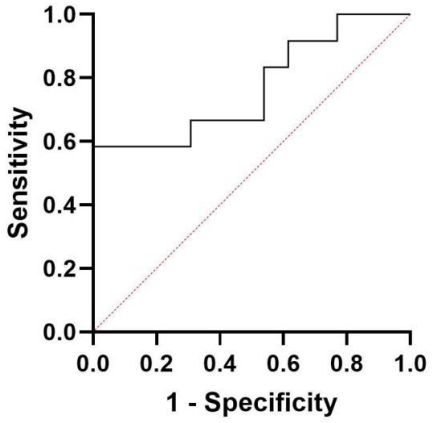

Figure 4. The Receiver Operating Characteristic (ROC) curve of miR-146a

4. Discussion

This study investigated the expression levels of miR-146a and miR-222 as potential biomarkers for PCOS, a condition known for its complex etiology and impact on reproductive health. Our findings revealed an upregulation of both miR-146a and miR-222 in the PCOS group, compared to the control group, although these differences were not statistically significant. This lack of statistical significance may stem from the study's limited sample size, which could have reduced the power to detect more subtle differences between groups. However, the observed trends align with previous research, suggesting that miR-146a and miR-222 may still hold relevance in PCOS pathophysiology[18,22]. The upregulation of miR-146a in the PCOS group aligns with previous studies indicating that miR-146a plays a critical role in regulating inflammatory responses. miR-146a has been shown to modulate inflammation by targeting key molecules in the Toll-like receptor (TLR) signaling pathway, such as IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6), both of which are involved in inflammatory signaling[23,24]. This regulatory function is particularly relevant to PCOS, which is frequently associated with low-grade chronic inflammation[25]. Although our results did not reach statistical significance, the observed trend toward increased miR-146a expression in PCOS supports its proposed role in modulating inflammation in this disorder.

Similarly, the elevated levels of miR-222 in the PCOS group observed in our study align with findings from other studies that have reported a positive association between miR-222 expression and insulin resistance, a common feature in PCOS patients[26,27]. miR-222 is known to influence insulin signaling pathways, which are critical in the context of PCOS pathophysiology, where insulin resistance often exacerbates reproductive and metabolic symptoms[28]. Our findings, though not statistically significant, are consistent with those of these studies, suggesting that miR-222 may contribute to the insulin resistance observed in PCOS. In this study, the discriminatory potential of miR-146a and miR-222 as biomarkers was further evaluated through ROC curve analysis. The AUC for miR-222 was calculated at 0.70, with a P-value of 0.04, indicating moderate discriminatory ability and statistical significance. This result suggests that miR-222 may serve as a potential biomarker for distinguishing PCOS from control samples, although its discriminatory power remains moderate. In contrast, the AUC for miR-146a was 0.65, with a P-value of 0.08, suggesting weaker discriminatory ability that did not reach statistical significance. These findings are in line with those of other research indicating that while miRNAs can offer insight into PCOS pathophysiology, their diagnostic power may improve when combined with other clinical markers or additional miRNAs[29]. Despite the promising potential of miR-146a and miR-222 as PCOS biomarkers, the current study's limitations, such as the small sample size, suggest that further research with larger cohorts is essential to confirm these findings. Future studies should consider expanding the sample size to increase statistical power and exploring these miRNAs in combination with other established clinical markers. Such approaches may enhance the accuracy and reliability of miRNAs as diagnostic tools, potentially leading to the development of non-invasive, miRNA-based diagnostic methods for PCOS.

In summary, our study adds to the growing body of evidence supporting the role of miR-146a and miR-222 in PCOS. Although statistical significance was limited by sample size, the trends observed are consistent with other studies highlighting the involvement of these miRNAs in inflammation and insulin signaling pathways. With additional research, miR-146a and miR-222 could contribute to innovative diagnostic and therapeutic strategies for managing PCOS, ultimately improving patient outcomes.

5. Conclusion

This study highlights the potential roles of miR-146a and miR-222 as biomarkers for PCOS, reflecting their involvement in inflammatory and insulin-related pathways relevant to PCOS pathophysiology. Although statistical significance was limited, the observed trends align with previous findings, suggesting that miR-146a and miR-222 may contribute to non-invasive diagnostic methods for PCOS. To confirm these findings, further research with larger cohorts is essential to enhance statistical power and validate the diagnostic potential of miR-146a and miR-222. Future studies should also examine the combination of these miRNAs with other clinical markers to improve diagnostic accuracy. Clinically, integrating miRNA-based diagnostics could aid in early detection and personalized treatment strategies for PCOS, potentially improving patient outcomes.

Declarations

Competing interests

The authors of this study declined any competing interests.

Authors' contributions

Rezazadeh Khabaz MJ, Fattahpour A and Faravani F collected samples, processed the data, analyzed and interpreted the data generated. Heidari B and Aghajani A designed and supervised the research and finalized the manuscript. The authors revised and approved the final manuscript.

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Availability of data and materials

Data from the current study will be available upon reasonable request.

Ethical considerations

The authors have made necessary ethical considerations (e.g., plagiarism, consent to publish, misconduct, datafabrication, and/or falsification, double publication and/or submission, and redundancy).

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