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RESEARCH ARTICLE

Identification and expression of microRNA-34a-3p and its target Rapamycin-insensitive companion of mTOR (RICTOR) in polycystic ovarian syndrome in South Indian population

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Objectives: Polycystic Ovarian Syndrome (PCOS) is a complex condition affecting 4% to 26% of the world-wide population and is characterized by enlarged ovaries and cysts. These cysts are actually immature ovarian follicles that have failed to mature and release an egg, which is a process known as anovulation. This study aims to explore the potential of miRNA as therapeutic and diagnostic biomarkers for PCOS, focusing on the identification and expression analysis of novel candidates like miR-34a-3p and its target Rapamycin-insensitive companion of mTOR (RICTOR). The objective is to enhance our understanding of the molecular mechanisms associated with PCOS, particularly the roles of miRNAs in its pathogenesis. In future, we plan to test miR-34a-3p mimics/inhibitors and RICTOR downregulation to improve insulin sensitivity and ovarian function. We will also explore combined therapies and conduct trials to assess their efficacy and safety in PCOS patients, aiming to develop practical treatments for PCOS.

Methods: National Centre for Biotechnology Information (NCBI) database, TargetScan, and miRbase were explored to identify the novel miRNA candidates, resulting in the discovery of miR-34a-3p. Secondary structure was constructed using RNA Fold, and Ct and melt curve analysis assessed its statistical expression levels. Additionally, similar research was conducted to analyze the expression levels of RICTOR, a target of miR-34a-3p.

Result: The secondary structure showed miR-34a-3p has a minimum free energy of -47.20 kcal. Additionally shows dysregulation in both miR-34a-3p and RICTOR in individuals with PCOS. Furthermore, overexpression of RICTOR and decrease in miR-34a-3p levels suggest their possible role in the pathogenesis of PCOS.

Conclusion: In PCOS, miR-34a-3p is downregulated, and there's an inverse relationship between miR-34a-3p and RICTOR levels. qRT-PCR results showed high RICTOR expression in PCOS patients. RICTOR plays a crucial role in the mTOR pathway, affecting insulin signaling, metabolism, and cellular growth, which are all implicated in PCOS.

Keywords: polycystic ovary syndrome, microRNA-34a-3p, RICTOR, gene expression, diagnosis

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Introduction

PCOS is an issue that mostly impacts reproductive-age women, with hyperandrogenism, menstrual irregularities, and the presence of polycystic ovaries. The development of PCOS involves interplay among genetic, environmental, and hormonal factors. Notably, microRNAs (miRNAs) are now recognized as important controllers in diverse biological processes, including those relevant to PCOS. [1]

On the other hand, small non-coding RNAs (sncRNA) known as miRNAs control post-transcriptional gene expression [2]. MiRNAs bind to certain target mRNAs in the 3' untranslated region (UTR), which causes mRNA destruction or translational suppression. miRNAs are critical players in numerous cellular functions, like cell proliferation, differentiation, apoptosis, metabolism, and their dysregulation has been linked with the emergence and progression of diseases, including PCOS. [3] One particular miRNA of interest in PCOS is miRNA-34a-3p, which is part of the miR-34 family which additionally contains miR-34b and miR-34c. Additionally, the role of this molecule in regulating various cellular functions, such as cell cycle arrest, apoptosis, and senescence, has been well established [4]. Dysregulation of miR-34a-3p has been observed in several diseases, notably cancer, cardiovascular diseases, and metabolic disorders [5].

A study conducted by Li et al. [6] shows that when compared to healthy controls, a significant upregulation of miR-34a-3p was observed in granulosa cells of PCOS patients. In addition, this overexpression also resulted in compromised follicular development and elevated apoptosis levels, thereby suggesting miR-34a-3p's role in the development of PCOS.

A crucial element of the mammalian target of rapamycin complex 2 (mTORC2) and one of the miR-34a-3p's discovered target genes is RICTOR. RICTOR is essential in regulating cell growth, survival, and metabolism. [7] Based on studies, miR-34a-3p adversely controls RICTOR expression levels in PCOS.

miRNAs are required in controlling the expression of genes and have an impact on both the progression and remission of disease. Therefore, finding the miRNA which

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causes PCOS holds the potential to facilitate early diagnosis and treatment. This identification process was conducted using a computational approach, mainly the NCBI database and miRBase. From these sources, we retrieved both the PCOS human genome sequences and the precursor miRNAs. After a meticulous assessment of the secondary structure, we successfully pinpointed miR-34a-3p within the PCOS genome sequences.

This study aims to explore the potential of miRNA as therapeutic and diagnostic biomarkers for PCOS, focusing on the identification and expression analysis of novel candidates like miR-34a-3p and its target Rapamycin-insensitive companion of mTOR (RICTOR). The objective is to enhance our understanding of the molecular mechanisms associated with PCOS, particularly the roles of miRNAs in its pathogenesis.

Methods

Retrieval of PCOS sequences and miRNAs

Information regarding the human genome's sequence was gathered by the International Nucleotide Sequence Database Consortium from the NCBI'S web domain. With this free search engine, the query "PCOS genome sequence in Homo sapiens" was entered to acquire the PCOS genome sequence. After removing both repeated and low-quality sequences, a local nucleotide database for PCOS-specific genomic sequences was produced. miRBase was used to retrieve human pre-miRNA (38,589 as of 2022) and mature miRNA (48,885 as of 2023) data. As a reference sequence, human miRNAs were employed (http://www.mirbase.org). The miRNAs dataset was then searched for its homolog using the previously stated PCOS nucleotide database. [8]

Identification of precursor-miRNAs

Screening for homologs in the PCOS nucleotide database was done using the mature miRNAs as a reference point. This sequence was used as a query for homology searches against the recently developed regional PCOS-specific nucleotide sequence database using the Basic Local Alignment Search Tool (BLAST) 2.2.26+ and an e-value threshold of 0.01. BLAST was used with the default settings against the NCBI protein database to validate sequences for its non-protein encoding phenomena. After that, a possible pre-miRNA sequence was created from the aligned portion. [8]

Validation of candidate pre-miRNA and their target

The secondary structure of the mature miRNA sequence in the PCOS genomic sequence was derived using the RNA fold method. The following conditions must be met before confirmation: 1) RNA constructs need the proper stem-loop hairpin structure. 2) Mature miRNA must be present on one side of the hairpin structure. 3) There shouldn't be more than seven mismatches between a miRNA and its counterpart in the other arm. 4) The negative energy and A+U content of the secondary structure must be higher (40–70%). [8] TargetScan was also employed in target prediction to help identify probable targets.

Sample collection

The Institutional ethical committee granted the study their approval and samples were collected in compliance with the Helsinki declaration. The sample size for the study was calculated using Gpower and a total of 20 PCOS and normal blood samples were collected from patients after informed consent from the Department of medicine, XXXX Medical College and Hospitals. The diagnosis of PCOS was confirmed by XXXX Medical College and Hospitals. The samples collected were centrifuged and the plasma obtained was stored in -20ºC deep freezer storage for further analysis. This study was conducted between March 2022 and April 2023.

Inclusion and exclusion criteria

Participants who met the following requirements including, over 18 years of age and ability to give informed consent were recruited. Meanwhile, participants having secondary complications like hypertension, diabetes were excluded from the study.

RNA extraction and quantification

By mixing TRIzol *reagent* (Invitrogen, Carlsbad, CA, USA) to the plasma as suggested by the manufacturer, isolation of RNA was done. Purity as well as the amount of the extracted RNA was analysed using a NanoDrop 2000 Lite spectrophotometer (Thermo Fisher Scientific, Waltham, US), which was kept at -20 °C until further analysis. [9,10]

Reverse transcription

Using deoxyribonucleotide triphosphate (dNTP, 10 mM each) from New England Biolabs Inc., nuclease-free water, and oligo (dT)18 primer (Promega, 50 M) for genes and universal adapter for miRNAs, total RNA from the sample was reverse transcribed. After five minutes of incubation at 65°C for the reaction, the mixture was quickly cooled, and the total volume was 10 µl. The final volume of the template RNA primer mixture was 20 µl, and 5x prime buffer (New England Biolabs Inc.), murine RNase inhibitor (New England Biolabs Inc.), reverse transcriptase (New England Biolabs Inc.), and nuclease-free water were added. ThermoFisher's MiniAmp plus thermal cycler was utilised for PCR, and the following settings were employed to continue the incubation: 10 minutes at 30°C, 30 minutes at 42°C, and 15 minutes at 95°C. [9,10]

Expression using qRT-PCR

The expression studies for miR-34a-3p and the gene RICTOR were performed using Sybr Green (Takara, Japan). GAPDH was used as housekeeping control for RICTOR and U6 for miR-34a-3p. Primers required for the study were purchased from Origene and expression studies were

done using CFX96 Realtime System (Bio-Rad). 95°C for the initial denaturation for 30 seconds for one cycle, 95°C for 5 seconds, and annealing for 30 seconds up to 40 cycles with a melt curve are the temperatures for PCR cycling, as specified. Gene expression was calculated using the 2^-ΔΔCq approach after the experiments were performed twice. [9,10]

Statistical Analysis

In addition to the average of replicate experiments, the data also contains the standard error of the mean (SEM). Differences in the group's statistical analysis was ascertained by Student's T-test in Microsoft Excel 365 program. P values less than 0.05 were considered significant for statistics (*).

Results

Identification of pre-miRNA and its secondary structure

Using RNA fold, the mature miR-34a-3p sequence was determined, revealing a minimal free energy of -47.20 kcal. **Table I** provides comprehensive details regarding the stem loop and mature sequence of miR-34a-3p including the length of pre-miRNA, amount of free energy, mature sequence, match extent, and miR-34a-3p A+U% content, while **Figure 1** elucidates the secondary structure of this miRNA.

Identification of targets

The targets for the particular miRNA were found using a target scan. On the basis of this study, a number of other genes that miR-34a-3p targets, including RICTOR, Notch2, sirtulin1, cyclin-dependent kinase 7, and matrix metallopeptidase 17 and others were discovered. The target genes of has-miR-34a-3p are listed in **Table II** along with their biological process and molecular function.

Gene expression analysis of miR-34a-3p and RICTOR

qRT-PCR gene expression analysis revealed that there is a significant downregulation in miR-34a-3p levels and an

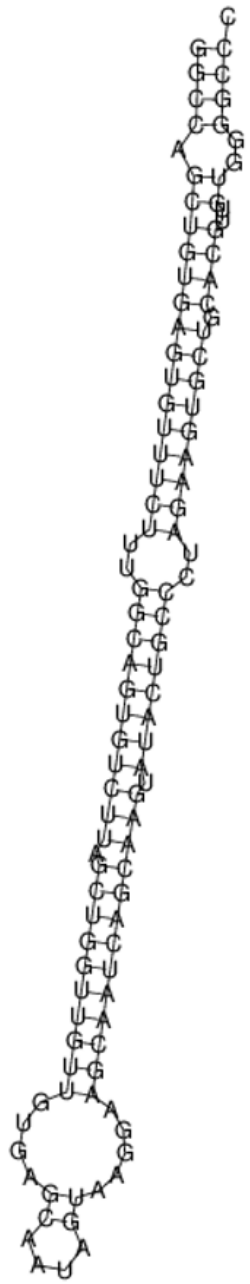

Fig. 1 represents the secondary structure for miR-34a-3p which was determined using RNA fold, revealing a minimal free energy of -47.20 kcal.

Table I. miRNA length, minimum free energy, stem loop and mature sequence, A+U percentage for miR-34a-3p

Source miRNA	Source organism	Pre-miRNA length	Minimum Free Energy	Sequence		Match Extent	Strand	A+U%
				Stem loop	Mature			
miR-34a-3p	*Homo sapiens*	110	-47.20 kcal	GGCCAGCUGUGAGUGUUUCUUUGGCAGUGUCU UAGCUGGUUGUUGUGAGCAAUAGUAAGGAAGC AAUCAGCAAGUAUACUGCCCUAGAAGUGCUGCA CGUUGUGGGGCCC	CAAUCAGCAAGUA UACUGCCCU	22/22	3p	49.09

Table II. The target genes of has-miR-34a-3p with their molecular function and biological process

S. no	Target Protein	Molecular function	Biological process
1	RICTOR	mTOR signaling	Cell growth
2	Notch2	Transmembrane signaling receptor	Organ morphogenesis
3	Sirtulin1	Histone deacetylation	Cell survival
4	Cyclin-dependent kinase 7	CK2 phosphorylation	Cell cycle control
5	Matrix metallopeptidase 17	Extracellular matrix	Tissue remodeling

elevation in RICTOR expression levels in PCOS patients when compared to normal. Hence, additional research studies could be done to confirm the function these molecules in PCOS. **Figure 2A & 2B** demonstrates the gene expression levels of miR-34a-3p and RICTOR in PCOS vs. normal blood samples.

Discussion

The findings of this study shed light on the intricate molecular mechanisms underlying polycystic ovarian syndrome (PCOS) and emphasize the crucial roles of miR-34a-3p and its target gene RICTOR. Our results indicate dysregulation of miR-34a-3p and overexpression of RICTOR in individuals with PCOS, suggesting their potential involvement in the pathogenesis of this syndrome.

Comparison with previous studies corroborates our findings and provides further insights into the significance of miR-34a-3p and RICTOR in PCOS. For instance, a study showed prominent modulation of miRNAs in ovarian follicles impacts granulosa cell apoptosis and follicular development, highlighting diverse regulatory pathways in reproductive health [11]. A separate study identified miR-34-3p as a potential serum biomarker for diagnosing endometriosis during the follicular phase, linking elevated levels to the presence of the condition [12]. Additionally, research by Luo et al. found that miR-34a-3p overexpression in PCOS was associated with insulin resistance and hyperandrogenism, further supporting our observations [13].

These studies, along with our findings, collectively highlight the dysregulation of the miR-34a-3p/RICTOR axis in PCOS and its implications for disease pathogenesis. Furthermore, the dysregulation of miR-34a-3p has been implicated in other pathologies beyond PCOS. For instance, studies have reported aberrant expression of miR-34a-3p in various cancers, cardiovascular diseases, and metabolic disorders [14]. In these contexts, dysregulated miR-34a-3p expression has been associated with tumor progression, cardiovascular dysfunction, and metabolic abnormalities, underscoring its multifaceted roles in disease pathogenesis.

Similarly, RICTOR dysregulation has been implicated in a range of diseases, including cancer, diabetes, and neu-

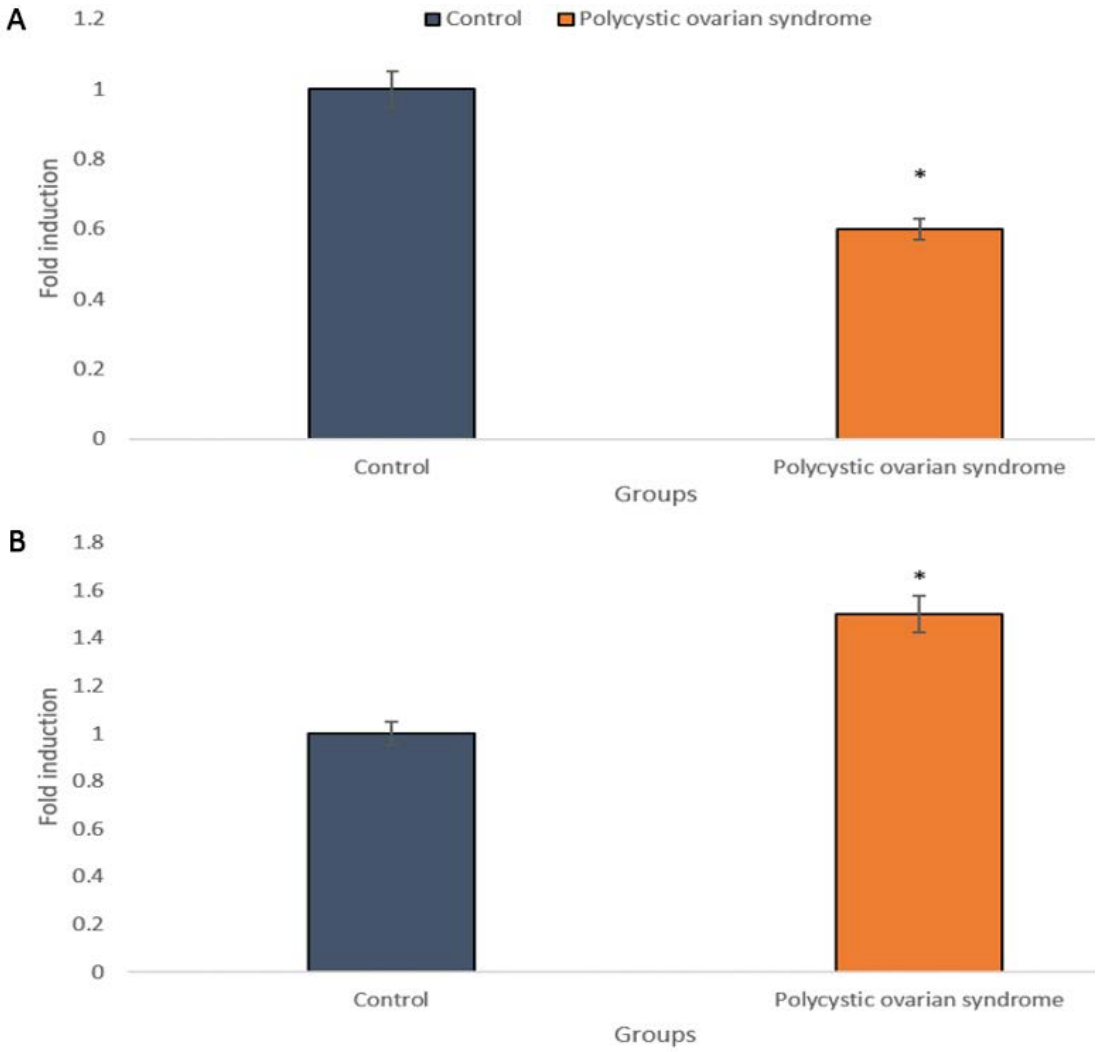

Fig. 2A represents expression levels of miR-34a-3p in polycystic ovary syndrome (PCOS) and control sample. 2B represents expression levels of the Rapamycin-insensitive companion of mTOR (RICTOR) gene in Polycystic ovary syndrome (PCOS) and control samples. P<0.05 is considered statistically significant.

rodegenerative disorders [15]. In cancer, RICTOR overexpression has been linked to increased cell proliferation, survival, and metastasis, promoting tumor progression and resistance to therapy [16]. In diabetes, dysregulated RICTOR expression contributes to insulin resistance and metabolic dysfunction, exacerbating disease severity [17]. These studies underscore the diverse functions of RICTOR in cellular processes and its involvement in disease pathogenesis across different pathological contexts.

In summary, our study provides novel insights into the dysregulation of the miR-34a-3p/RICTOR axis in PCOS and its potential implications for disease pathogenesis. By comparing our findings with previous studies and discussing the broader roles of miR-34a-3p and RICTOR in other pathologies, we highlight the significance of these molecules as potential therapeutic targets for PCOS and other related disorders. Further research is warranted to elucidate the underlying mechanisms and to explore the therapeutic potential of targeting miR-34a-3p and RICTOR in the management of PCOS and associated comorbidities.

While our findings provide valuable insights, further research is needed to confirm these associations in larger, longitudinal cohorts and to explore additional miRNA-gene regulatory networks in PCOS pathophysiology.

Moving forward, understanding the molecular mechanisms underlying PCOS may lead to the development of novel diagnostic and therapeutic strategies. Targeting miR-34a-3p or RICTOR could offer promising avenues for restoring insulin sensitivity and improving ovarian function in affected individuals. Additionally, exploring the broader miRNA landscape and conducting functional studies could unveil new therapeutic targets and provide comprehensive management strategies for PCOS. Overall, our study underscores the importance of molecular research in elucidating the complex pathophysiology of PCOS and offers potential directions for future investigations.

While our study sheds light on miR-34a-3p and RICTOR dysregulation in PCOS, it has limitations. The small sample size limits generalizability, and future research should include larger cohorts and examine other relevant tissues. Our use of qRT-PCR may miss post-transcriptional regulation, so techniques like RNA sequencing are recommended. Additionally, our observational study cannot establish causality, necessitating longitudinal studies and functional experiments for validation and deeper insights.

Conclusion

Our study contributes to the understanding of PCOS pathogenesis by elucidating the dysregulation of miR-34a-3p and its target gene RICTOR. The observed upregulation of miR-34a-3p in PCOS patients' granulosa cells suggests its involvement in disrupted follicular development and increased apoptosis, characteristic of PCOS. Furthermore, the downregulation of RICTOR, a critical component of the mTORC2 pathway, implicates dysregulated cell growth, survival, and metabolism in the syndrome.

Abbreviations

PCOS: polycystic ovarian syndrome;
NCBI: National Centre for Biotechnology Information;
sncRNA: small non-coding RNAs; **miRNA:** microRNA;
RICTOR: Rapamycin-insensitive companion of mTOR;
UTR: untranslated region;
BLAST: Basic Local Alignment Search Tool;
mTORC2: mammalian target of rapamycin complex 2;
dNTP: deoxyribonucleotide triphosphate

Authors Contribution

DS (Conceptualization; Formal analysis) MS (writing - original draft preparation) AP (writing – review and editing) AP (editing – revising). All authors read and approved the final manuscript.

Conflicts of interest

None to declare.

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