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Role of circulation miRNA in patients suffering with polycystic ovary syndrome (PCOS)

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Abstract
Introduction: Polycystic ovary syndrome (PCOS) is a hormonal disorder common among women of reproductive age. Women with PCOS may have infrequent or prolonged menstrual periods or excess male hormone (androgen) levels.
Material & Methods: A total of 110 patients with PCOS and 125 healthy controls were included in the present study. Total miRNA were isolated for 4mL of blood samples followed by cDNA construction. Furthermore, selected miRNAs (miR-21, miR-146a, miR-200a, miR-92a & miR-92b) were validated by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).
Results: In the present study, we demonstrated that women with PCOS had significantly increased expression level of miRNA 21 and miRNA146 compared to healthy controls. Our study revealed that the expression level of miR‐92a, miR‐19b and miR‐200a were significantly down regulated in patients with PCOS compared with controls.
Conclusions: miRNAs are differentially expressed between PCOS patients and controls. We identified and validated five different miRNAs. They are significantly up and down regulated and may be involved in the pathogenesis of PCOS.

Keywords: Circulating miRNA, PCOS, QRT-PCR, markers

Introduction
Polycystic ovary syndrome (PCOS) is one of the common metabolic and endocrine disorders characterized by hyperandrogenemia and ovulation failure (Glintborg & Andersen, 2010). Approximately 5-10% of women with reproductive age affected with PCOS worldwide. Several investigations have suggested that PCOS is multifactorial disease but the exact mechanisms for the development of PCOS are still not clear. Several studied suggested that various genetics factor, impaired insulin resistant and dysregulation of hypothalamic pituitary-adrenal (HPA) axis responsible for the development of PCOS (Pasquali *et al*. 2011). The clinical manifestations of PCOS characterized by different investigators which is vary between them due to set of communications between environmental and genetic factors (Bazarganipour *et al*. 2013).
MicroRNAs (miRNAs) are a novel class of 22 nucleotide long, non-coding, single-stranded RNA molecules highly conserved which negatively regulate gene expression. miRNAs bind to the 3′ un-translated regions of messenger RNAs (mRNAs), and induce inhibition or degradation of protein translation. The expression of various targeted genes involves in the pathological and physiologic conditions are significantly influenced by miRNAs. miRNAs are also known to contribute to Female reproductive function in also influenced by various miRNAs (Carletti *et al*. 2009) [3]. Therefore, miRNAs signify the potential regulators of metabolic processes and also play an important role to targets and modulating the complex pathways involved in PCOS and associated disorders (Flynt *et al*. 2008) [9].
Various studies mainly concentrated on the relationship of miRNAs expression and the reproductive disorders of PCOS patients (Sang *et al*. 2013; Roth *et al*. 2014) [26, 25]. miRNAs serve as significant intracellular mediators of normal follicular growth and function and target various intra-ovarian receptors and regulators (Lim *et al*. 2005; Hossain *et al*. 2013) [16]. miRNA-103, miRNA-27b, miRNA-155 and miRNA-21 have been associated with various metabolic disorders such as obesity and diabetes, which are also associated with PCOS (Murri *et al*. 2013) [19].
Herein, we hypothesized that the circulating miRNA profile in PCOS patients were different from healthy individuals. We screened various miRNAs expression level in serum of PCOS and controls by quantitative reverse transcriptase polymerase chain reaction (qRT‐PCR) to validate

them. The precise targets of those differentially expressed miRNAs were identified. Results of the present study demonstrated that PCOS is associated with differential expression of regulatory noncoding miRNAs.

Materials & Methods

Selection of Subjects

The study protocol was approved by the Institutional Ethics Committee. Written informed consent was obtained from all participants who were enrolled in the study. Total of 110 patients who were suffering with PCOS were enrolled in the present study. A total of 125 healthy controls were included in the present study referred for routine general medical check-up and from voluntary blood donors from Hyderabad who willingly participated in the study.

Blood Sample Collection

4mL peripheral blood samples were collected from the patients and healthy controls. The basic characteristics and demographic features of the enrolled participants were collected. All samples were processed on the same day and within few hours after collection. Serum was separated from blood samples of each subject by centrifugation at 1,500 x g for 10 min and used for miRNA isolation.

Extraction of miRNA

miRNA from each subject was extracted immediately after serum separation by using miRNeasy Serum/Plasma Kit (Qiagen), cDNA was prepared using reverse transcriptase enzyme (MMuLVRT), Universal Stem Loop Primer (USLP) and U6 RT primer (Yang *et al*. 2014) [30]. Constructed cDNAs were stored at -80 °C for further experimental purposes.

miRNA expression analysis

Five selected miRNAs (miR-21, miR-146a, miR-200a, miR-92a & miR-92b) (Table 1) expression levels were quantified by using real time-quantitative polymerase chain reaction (CFX96, Bio Rad) based on SYBR-Green microRNA relative quantification assay. The amounts of all miRNAs were calculated relative to the amount of U6SnRNA (used as internal control) in the same sample.

Statistical Analysis

Sample size was determined by open Ep1 strategies 95% confidence level with 90% of sample power. Gene expression was expressed as relative quantification ($RQ = 2^{-\Delta\Delta CT}$) as per Livak method (Livak *et al*. 2001) [18] and further validated by Pfaffl method (Pfaffl *et al*. 2001) [23]. Results were expressed as mean ± standard error of the mean (SEM). Data was analyzed by using Graph Pad Prism software (version V; San Diego, CA). Mann-Whitney U test was used to draw comparisons between groups. Spearman test was used for correlation studies. P values ≤0.05 was considered statistically significant for all the variables.

Results and Discussion

Quantification of selected miRNA's revealed differential expression in PCOS patients as compared to healthy controls. All 5 allied miRNAs displayed significant difference in fold change among healthy controls and PCOS patients.

Table 1: List of primers used for the study

S. NO	Primer Name	Sequence
1	Universal stem-loop primer	5'-GAAAGAAGGCGAGGAGCAGATCGAGGAAGAAGACGGAAGAATGTGCGTCTCGCCTTCTTTCNNNNNNNN- 3'.
2	U6RT primer	5'-CGCTTCACGAATTTGCGTGTCAB- 3'
3	miR-21	FP: 5'-CGGGATCCTGGGGTTCGATCTTAACAGGC- 3' RP: 5'-CGGAATTCCCACAATGCAGCTTAGTTTTCC- 3'
4	miR-146a	FP: 5'-CTAGCCTGCAGGCTGCCCTTGGACCAGCAGTC- 3' RP: 5'-ATCCGGCCGGCCGCTCTCTTTTTCTTCTTGAC- 3'
5	miR-200a	FP: 5'-TGCGTGTCGTGGAGTC- 3' RP: 5'-GGGGTAACACTGTCTGGTAG- 3'
6	miR-92a	FP: 5'-TGCGTGTCGTGGAGTC- 3' RP: 5'-CACCTATATTGCACTTGTCC- 3'
7	miR-92b	FP: 5'-TGCGTGTCGTGGAGTC- 3' RP: 5'-CGGTATTGCACTCGTCC- 3'

Micro RNAs expression analysis

Analysis of relative fold change in miR-21 and miR-146a expression revealed significant up regulation in PCOS patients compared to healthy controls. The expression levels of miR-21 between healthy controls and PCOS patients displayed an increasing tendency towards statistical significant fold reduction with *p* value <0.0001. MiR-146a expression also revealed significant up regulation during the PCOS displayed statistical significant fold change with *p* value <0.0001 (Figure 1). In addition, qRT‐PCR validation confirmed down regulation of miR‐92a, miR‐19b and miR‐200a in PCOS patients compared to healthy controls (Figure 2).

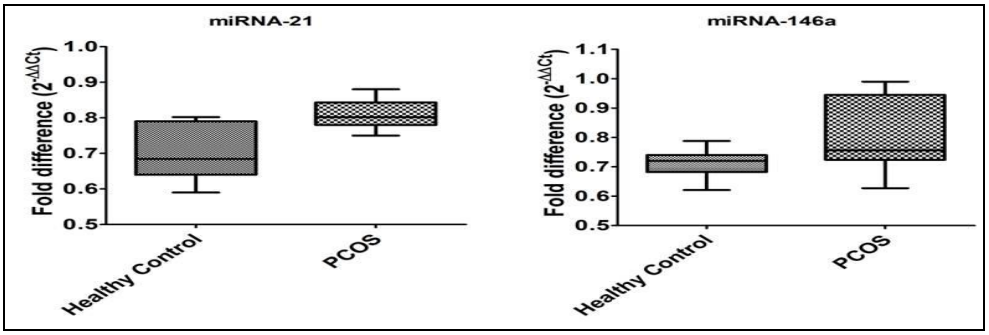

Fig 1: Relative expression levels of miRNA-21 and miRNA-146a in PCOS

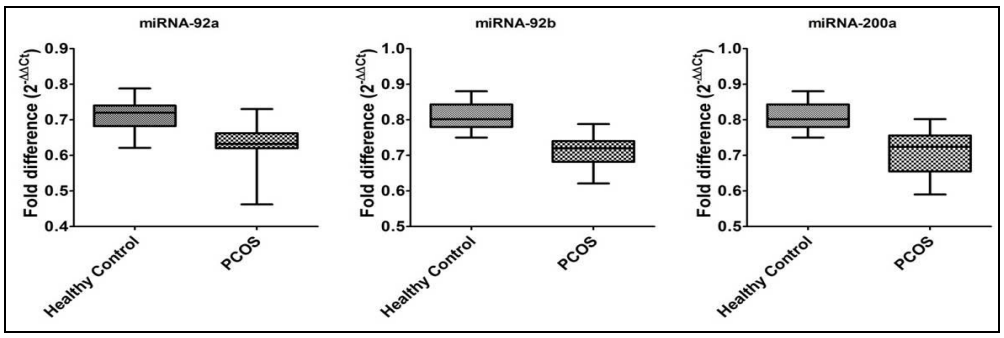

Fig 2: Relative expression levels of miRNA-92a, miRNA-92b and miRNA-200a in PCOS

PCOS is a multifactorial endocrine disorder and 75% of anovulatory infertility is because of PCOS (Dunaif 1997; Norman *et al*. 2007; Azziz *et al*. 2009) [7, 21, 2]. PCOS is a assorted condition with multifaceted pathologies with minimal granulosa cell proliferation, follicle growth arrest at the small antral stage, chronic anovulation hyperthecosis and hyperandrogenemia (Dunaif 1997) [7]. Patients with PCOS also have chance to develop metabolic diseases such as diabetes, cardiovascular disease, obesity and insulin resistant along with infertility (Revised 2003 consensus 2004) [24]. Several investigators suggested that altered gene expression involve in metabolism, cell division, apoptosis is one of the major causes of PCOS (Franks *et al*. 2006) [10] but actual etiology of PCOS is still not clear.

Recently, various investigators reported that human plasma/serum could serve as a class of novel noninvasive biomarkers for diseases (Chen *et al*. 2008; Hu *et al*. 2010; Ng *et al*. 2009; Fichtlscherer *et al*. 2010) [4, 3, 20, 8]. Previous report suggested that the expression levels of circulating miR-92 were significantly high in patients with colorectal cancer and well illustrious from gastric cancer, inflammatory bowel disease and healthy subjects (Ng *et al*. 2009) [20]. Ai *et al*. demonstrated that circulating miR-1 expression level was found to be significantly elevated in acute myocardial infarction (AMI) patients compared with non-AMI subjects (Ai *et al*. 2010) [1].

miRNA-21, Let-7 family, miRNA-125b, miRNA-143, miRNA-126, miRNA-99a, miRNA-145, and miRNA-199b were found to be most predominant in the ovary, regardless of the species in mammals (Hossain *et al*. 2012) [12]. Additionally, elevated expression of miRNA has been demonstrated in various ovarian-derived disorders, such as PCOS, premature ovarian failure and ovarian cancer (Li *et al*. 2015) [15].

miR-146a, miR-22, miR-132, miR-200c, miR-141, and miR-21 were differentially expressed in ovary tissues of patients with PCOS (Teague *et al*. 2010) [28]. There are only few studies available which demonstrated that serum/plasma miRNA level in patient with PCOS. Wei *et al*. 2010 demonstrated the various expression of nine different miRNA in patients with PCOS. They also demonstrated that the expression levels of miRNA in ovary tissue were not modulated in serum of patients with PCOS. The exact machinery how miRNAs go into the serum and whether they are biologically functional or simply biomarkers for unidentified etiological factors is still a big fascination.

Kyeong *et al*. 2010 demonstrated that the expression of serum miRNA-4522, miRNA-324-3p, and miRNA-6767-5p was down-regulated in the women with PCOS compared with the control subjects. In the present study, we demonstrated that women with PCOS had significantly increased expression level of miRNA 21 and miRNA146 compared to healthy controls. Our study revealed that the expression level of miR‐92a, miR‐19b and miR‐200a were significantly down regulated in patients with PCOS compared with controls.

Both miR‐92a and miR‐92b belong to the miR‐17‐92 miRNA cluster located at 13q31.3. MiR‐92a has been associated with cancer pathogenesis and has been reported being significantly downregulated in patients with acute myeloid leukemia (AML) and acute lymphocytic leukemia (ALL), hepatocellular carcinoma (HCC), (Shigoka *et al*. 2010) [27] ovarian cancer, (Ohyagi *et al*. 2013) [22] chronic lymphatic leukemia, (Fulci *et al*. 2007) [11] and during myeloid differentiation. (Chen *et al*. 2008) [4] Recently, miR‐92a was reported to play a role in non‐tumor diseases, such as ischemia (Daniel *et al*. 2014) [6]. MiR‐200a expression results in decreased expression of dual‐specificity phosphatase‐2 that subsequently results in prolonged extracellular‐signal‐regulated kinases activation through hypoxia‐inducible factor, contributing to inflammation in the pathogenesis of endometriosis (Lin *et al*. 2012) [17].

Conclusion

This finding suggests that circulating miRs profiles may indicate a potential role of miRs as non-invasive biomarkers, and also demonstrates that the PCOS has an impact. It has been

demonstrated that miR-146a, miR‐92a, miR‐19b and miR‐200a & miR-21 is associated with PCOS and it may use as good biomarkers for the diagnosis and prognosis of PCOS.

Acknowledgement
None

Conflict Of Interest
Authors are not having any conflict of interest

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