Androgen receptor signalling transactivator IncRNAs PRNCR1 and PCGEM contribute to PCOS pathogenesis

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Article

Keywords: LncRNA, PRNCR1, PCGEM1, Hyperandrogenism and polycystic ovary syndrome

Posted Date: March 2nd, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2598360/v1

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Abstract

Polycystic ovary syndrome is the most common endocrine, reproductive disorder of women in their reproductive years. It is commonly considered as an androgenic disorder. Apart from high circulating testosterone levels, hyperandrogenism can also be attributed to the hyperactive androgen receptor (AR). Androgen receptor mediated signalling involves the association of multiple coactivators and corepressors and any aberrant activity of these factors may disrupt the normal transcriptional activity of AR and hence may act as contributing factors in the pathophysiology of various diseases. LncRNAs prostate cancer associated non-coding RNA 1 (PRNCR1) and prostate cancer gene expression marker 1 (PCGEM1) act as androgen receptor coactivator IncRNAs. The study was aimed at determining the association of IncRNAs PRNCR1 and PCGEM1 with PCOS. In this case-control study, a total of 178 participants were recruited these included 105 PCOS cases and 73 age-matched healthy controls. The Anthropometric, Metabolic and hormonal characteristics of the participants were determined. Total RNA was isolated from peripheral mononuclear blood cells and reverse transcribed into cDNA. Quantitative real-time PCR (qPCR) was done to study the expression pattern on IncRNAs. The association between different hormonal and metabolic parameters with the expression of PRNCR1 and PCGEM1 was determined by correlation analysis. Our study for the first time demonstrated that IncRNA PRNCR1 and PCGEM1 are overexpressed in Kashmiri women with PCOS. The expression levels of PRNCR1 (PRNCR1, 5.26 ± 1.77 versus 1.22 ± 0.73; P < 0.001) and PCGEM1 (PCGEM1, 3.39 ± 1.88 versus 1.23 ± 0.74; P < 0.001) were significantly higher in PCOS women compared to controls. Furthermore, IncRNA PRNCR1 showed a positive correlation with testosterone (P < 0.001).

Introduction

Polycystic ovary syndrome is the most common endocrine, reproductive disorder affecting 5–20% of women in their reproductive years. PCOS is a complex multifactorial and multidimensional disorder characterized by the combination of androgen excess, chronic anovulation and polycystic ovarian morphology. Different criteria were proposed to diagnose PCOS like National Institutes of Health 1990 criteria, Rotterdam 2003 criteria and Androgen Excess and PCOS Society 2006 criteria. However, the Rotterdam criteria is most widely used among different diagnostic criteria to diagnose this complex syndrome. The clinical symptoms of PCOS are complex and show enormous diversity. Classical symptoms of PCOS include the simultaneous presence of hyperandrogenism/hirsutism, infertility, obesity and polycystic ovarian morphology.

Owing to the complex nature and the variability in clinical characteristics of PCOS, the etiology of the syndrome remains highly obscure. Although the etiology of PCOS is multifactorial, it is commonly considered as androgenic disorder. Apart from high circulating testosterone levels, hyperandrogenism can also be attributed to the hyperactive androgen receptor (AR). Androgen receptor mediated signalling involves the association of multiple coactivators and corepressors and any aberrant activity of these
factors may disrupt the normal transcriptional activity of AR. Thus, the aberrant expression of AR coactivators may contribute to the development of androgenic disorders like PCOS.

Long Non Coding RNAs (lncRNAs) constitute a significant proportion of noncoding RNAs. The LncRNAs have a tremendous role in normal cellular physiology predominantly in regulating transcription, and post-transcriptional events besides being involved in signal transduction and cellular organisation. Thus, the dysregulation of these LncRNAs would result in aberrant cellular physiologies and consequently their involvement in the pathophysiology and progression of various diseases.

LncRNAs prostate cancer associated non-coding RNA 1 (PRNCR1) and prostate cancer gene expression marker 1 (PCGEM1) act as androgen receptor coactivator lncRNAs. These lncRNAs successively bind to AR and cause transactivation of AR-mediated genes both in presence of ligand and ligand independently. Thus, these lncRNAs may play an important role in AR related disease progression and accordingly, a hypothesis was generated that lncRNAs PRNCR1 and PCGEM1 might play an important role in the pathophysiology of PCOS.

The aim of this study was to evaluate the association of androgen receptor coactivator lncRNAs PRNCR1 and PCGEM1 with the risk of PCOS in ethnic Kashmiri women. We also attempted to find the correlation of lncRNA PRNCR1 and PCGEM1 with various clinical and biochemical parameters of PCOS.

Materials And Methods

Recruitment of Subjects

A total of 178 participants were recruited for this case-control study. Among these 178 participants, 73 were healthy controls and 105 were drug naïve PCOS women. PCOS cases were recruited from the patients attending the outpatient clinic of the Department of Obstetrics and Gynaecology, Govt. Medical College, Srinagar, Jammu and Kashmir, and Department of Endocrinology, Sher-e-Kashmir Institute of Medical Sciences, Srinagar, Jammu and Kashmir, India for PCOS related complications from October 2017 to March 2020. PCOS patients were recruited based on the Rotterdam criteria. For exclusion criteria, participants who presented with PCOS mimicking disorders like hyperprolactinemia, Cushing’s syndrome thyroid dysfunction, androgen secreting tumours and congenital adrenal hyperplasia were excluded from the study. Controls recruited for the study were age matched healthy women without any biochemical or clinical signs of hyperandrogenism, had regular cycles, with no history of autoimmune or endocrine disorders. All of the participants were ethnic Kashmiris living in Kashmir province and did not receive any hormonal therapy for at least past six months.

Ethics statement

The study was ethically approved by the ethical committee Government Medical College Srinagar under ethical approval no. 94/ETH/GMC/ICMR. The participants were recruited only after written informed
consent was obtained from them. All methods and protocols were performed in accordance with the relevant guidelines and regulations.

**Anthropometric and clinical evaluation**

The study participants recruited underwent general anthropometric measurements that include height, weight, waist, hip, waist-hip ratio (WHR), body mass index (BMI) and extent of hirsutism (FG score). A detailed history of clinical symptoms like menstrual cycles, hirsutism, acne, alopecia, and acanthosis nigricans was also taken from all participants. Weight was determined using a weighing balance while wearing light clothing and height was determined in a standing position without shoes. In order to measure waist circumference, the minimum measurement between the iliac crest and lateral costal border was established while the measurement for hip circumference was the maximum measurement over the buttocks. The waist-hip ratio was determined by dividing the waist circumference by the hip circumference. BMI was calculated as weight(kg)/Height(m²). The Ferriman-Gallwey scoring system was applied to measure the hirsutism score. Transabdominal ultrasonography was performed on all PCOS patients to determine the ovarian volume and/or number of peripheral ovarian cysts.

**Hormonal and Biochemical assessment**

The peripheral venous blood samples were collected from the participants on the second or third day of their menstrual cycle after an overnight fast in clot activator vials for hormonal and biochemical assessment. The hormones include testosterone, luteinizing hormone, follicle stimulating hormone, thyroid stimulating hormone and Prolactin were estimated on Beckman coulter UniCelDxI 800 (Access Immunoassay system) by Radioimmunoassay (RIA) using RIA kits (Immunotech S.R.O, Prague, Czech Republic). Fasting insulin, androstenedione, dehydroepiandrosterone sulphate, and Sex Hormone-Binding Globulin were measured by enzyme-linked immunosorbent assays using Calbiotech, CA, USA, and DGR Instruments GmbH Marburg ELISA kits using Skanlt RE 4.0 software on Thermo Scientific Multiskan FC ELISA reader.

The biochemical parameters done included oral glucose tolerance test, Lipid profile, Liver function and kidney function tests. All the biochemical parameters were determined by using ERBA bioassay diagnostic kits ERBA Chemtouch 7, Semi-Automatic Biochemistry Analyzer, Wiesbaden, Germany.

Insulin resistance was determined by calculating HOMA IR

\[
\text{“HOMA IR = fasting glucose (mg/dL) x Fasting Insulin (IU/L)/405”}. \]

**Isolation Peripheral blood mononuclear cell**

Total RNA was isolated from peripheral blood mononuclear cells (PBMCs). For PBMCs separation 2ml of whole blood sample in Na₂-EDTA vials was obtained from all the subjects. The whole blood sample was
diluted by the addition of an equal volume of phosphate buffer saline (PBS). The density gradient centrifugation was applied for the isolation of PBMCs. Ficoll-Paque plus (GE Healthcare Bio-Sciences Sweden) having a density of 1.077 g/ml was used as a density gradient medium. PBMCs have density slightly lesser than Ficoll-Paque plus (<1.077 g/ml) thus allowing PBMCs to form a buffy coat at the interface of plasma and Ficoll. After density gradient centrifugation PBMCs were isolated and washed twice with PBS to remove any contamination of Ficoll-Paque and plasma. The PBMCs thus obtained were stored at -80°C for further processing.

**RNA isolation and cDNA Synthesis**

Total RNA was isolated from PBMCs using TRIzole reagent (Life Technologies, Carlsbad California). Traces of genomic DNA in extracted RNA samples could potentially interfere with expression analysis studies. So, all of the RNA samples were subjected to DNase treatment before cDNA synthesis. Sigma Aldrich DNase treatment kit (DNase I) was used as per the manufacturer’s protocol in order to eliminate any traces of genomic DNA. All of the RNA samples were analyzed qualitatively and quantitatively using NanoDrop (Thermo Scientific). RNA samples having absorbance $A_{260/280}$ ratio between 1.9–2.0 were subsequently used for cDNA synthesis. Thermo Scientific RevertAid First Strand cDNA Synthesis Kit was used to reverse transcribe 1.5 g of total RNA into cDNA according to the manufacturer’s protocol in an applied biosystems thermal cycler.

**Quantitative real-time polymerase chain reaction**

The relative expression levels of IncRNAs PRNCR1 and PCGEM1 were measured by real-time quantitative polymerase chain reaction (qPCR). SYBR Green I assay was employed for qPCR quantification of IncRNAs. 10ml pre-formulated real-time master mix containing buffer, dNTPs, DNA polymerase and SYBR Green I dye was used (KAPA SYBR® FAST), 0.3ml of forward and 0.3ml of reverse primers and cDNA less than 100ng (1ml) in a 20ml reaction following manufacturer’s protocol. The relative quantification was performed in Roche LightCycler® 480 Instrument II on 96 well plate having the following reaction protocol pre-incubation at 95°C for 5mins, a 40 cycle amplification at 95°C for 20 sec, 58°C/56°C for 15 sec (for PRNCR1 and PCGEM1 respectively) and 72°C for 15 sec. Amplification was followed by melting curve analysis with the following conditions 95°C for 5sec, 60°C for 60sec and 95°C continuous. The product size was confirmed by running the amplified products on 2% agarose gel. The relative expression of PRNCR1 and PCGEM1 was estimated by the Livak method and Beta-Actin was used as a reference gene. Each reaction of qPCR was performed in triplicates.

The following primers were used to quantify the IncRNA PRNCR1 levels: 5’-CTCTGTGGAAGCATTGTGGAG-3’ (forward) and 5’-TATCAGCCCTTGGAATCTGG -3’ (reverse), For IncRNA PCGEM1 following primers were used 5’-GGTGCCCTTGCAATGTTAT-3’ (forward) and 5’-AGCATGCTCTCTGCAAAGGT-3’ (reverse) and β-Actin RNA was quantified as a control to normalize differences in total RNA levels using the following primers 5’-ATCGGAACGGTGAAGGTGACA-3’ (forward) 5’-TGGCAAGGGACTTCTGTAAACG-3’ (reverse)
Statistical Analysis

The quantitative baseline variables were expressed as number percentage and mean ± SD. All parametric variables including hormonal, anthropometric and biochemical were compared by unpaired students t-test between PCOS and controls. The anthropometric, hormonal and biochemical parametric variables between PCOS and controls. The association of expression of IncRNAs PRNCR1 and PCGEM1 with PCOS was evaluated by using the Chi-square test. The relation between various metabolic and hormonal parameters with the expression of IncRNA PRNCR1 and PCGEM1 was determined by Spearman or Pearson rank correlation coefficient. The distribution of expression of IncRNA PRNCR1 and PCGEM1 were divided into binary groups among both PCOS and healthy controls. The limits for binary groups were derived from the control group. For PRNCR1 limits derived for binary group cut offs were <1.46 for the low expression, >1.46 for the high expression and for PCGEM1 binary groups cut offs were <1.54 for the low expression, >1.54 for the high expression. Sigma Plot 10 was used to determine the Receiver operator characteristic (ROC) and area under curve (AUC). The statistical computing tool vassarstats (http://vassarstats.net/) was used for statistical analysis. Data at a P-value of <0.05 was considered statistically significant.

Results

Clinical characteristics of study participants

Most of the baseline characteristics were significantly deranged in PCOS women except for age, menarche, and height which were comparable between controls and PCOS women. PCOS women had significantly deranged anthropometric, hormonal and biochemical parameters compared to controls. The results of general Biochemical and hormonal characteristics have been reported in our previous publication19. 88.57%, 87.61% and 81.90% of PCOS women had hirsutism, menstrual dysfunction and polycystic ovarian morphology, respectively. Acne and alopecia were present in 61.95% and 49.50% of cases. The clinical marker of insulin resistance acanthosis nigricans was present in 28.57% of PCOS women. The results are summarized in Table 1.

Table 1: Clinical Characteristics of Controls and Patients With PCOS.
<table>
<thead>
<tr>
<th>Variables</th>
<th>PCOS cases (n=105)</th>
<th>Controls (n=73)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>23.23 ± 3.88</td>
<td>24.07 ± 2.21</td>
<td>0.15</td>
</tr>
<tr>
<td>Menstrual dysfunction</td>
<td>(92) 87.61%</td>
<td>(0) 0%</td>
<td></td>
</tr>
<tr>
<td>Acne</td>
<td>(65) 61.90%</td>
<td>(7) 9.58%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Alopecia</td>
<td>(52) 49.50%</td>
<td>(3) 4.10%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Acanthosis</td>
<td>(30) 28.57%</td>
<td>(2) 2.73%</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Hirsutism</td>
<td>(93) 88.57%</td>
<td>(0) 0%</td>
<td></td>
</tr>
<tr>
<td>PCOM</td>
<td>(86) 81.90%</td>
<td>(0) 0%</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>25.62 ± 3.39</td>
<td>22.44 ± 1.83</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>TT (ng/dL)</td>
<td>59.26 ± 21.41</td>
<td>26.00 ± 8.79</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>3.80 ± 1.26</td>
<td>1.31 ± 0.78</td>
<td>&lt; 0.001*</td>
</tr>
</tbody>
</table>

Data is presented as (number) percentage and Mean ± SD. *P-value <0.05 significant. P value calculated by chi-square test and by Independent Student’s t-test.

PCOS; polycystic ovary syndrome, BMI; body mass index, TT; Total Testosterone, PCOM; Polycystic ovarian morphology, HOMA IR; homeostasis model assessment-estimated insulin resistance.

**Relative expression of lncRNA PRNCR1 and PCGEM1 in study subjects**

Figure 1A shows the fold change in the expression of lncRNA PRNCR1 in cases and controls. The mean fold change in the expression level of PRNCR1 in the PCOS women was significantly higher (5.26 fold) than that in the healthy women (PRNCR1, 5.26 ± 1.77 versus 1.22 ± 0.73; P < 0.001). Likewise, the mean fold change in the expression of lncRNA PCGEM1 in PCOS women was also significantly higher compared to healthy controls (PCGEM1, 3.39 ± 1.88 versus 1.23 ± 0.74; P < 0.001) (Figure 1B).

**Association between expression of lncRNA PRNCR1 and PCGEM1 presence of PCOS**

To evaluate the relationship between different expression levels of PRNCR1 and the risk of PCOS, the subjects were grouped as high expression and low expression based on restrictions set by the control group. Tables 2A and 2B show subjects with a higher expression level of PRNCR1 and PCGEM1 had a significantly higher risk of PCOS (P < 0.001).

Table 2A: Odds ratio of PCOS events by expression of PRNCR1
Table 2B: Odds ratio of PCOS events by expression of lncRNA PCGEM1

<table>
<thead>
<tr>
<th>PCGEM1 Expression</th>
<th>Cases</th>
<th>Controls</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low &lt;1.46</td>
<td>7</td>
<td>56</td>
<td>46.11</td>
<td>18.02-117.99</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>High &gt;1.46</td>
<td>98</td>
<td>17</td>
<td>11.45</td>
<td>1.89-44.04</td>
<td>&lt; 0.001*</td>
</tr>
</tbody>
</table>

Table 2B: Odds ratio of PCOS events by expression of lncRNA PCGEM1

<table>
<thead>
<tr>
<th>PCGEM1 Expression</th>
<th>Cases</th>
<th>Controls</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low &lt;1.54</td>
<td>18</td>
<td>54</td>
<td>13.73</td>
<td>6.629-28.466</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>High &gt;1.54</td>
<td>87</td>
<td>19</td>
<td>11.45</td>
<td>1.89-44.04</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

PCGEM1; Prostate cancer gene expression marker 1, PCOS; polycystic ovary syndrome, OR; odds ratio, 95% CI; confidence intervals. PCGEM1 expression for binary groups cut offs were <1.54 for the low expression, >1.54 for the high expression. The OR, 95% CI, and P value were estimated for PCOS events in the high expression group compared to the low expression using χ² Test; *P-value < 0.05 is significant.

Receiver operator characteristic of lncRNA PRNCR1 expression between PCOS cases and controls

The area under curve (AUC) was constructed by performing Receiver operator characteristic curve analysis to assess the diagnostic capability of lncRNA PRNCR1 and PCGEM1 (Figures 2A and 2B). The healthy controls were used as reference. The AUC was found to be higher for PRNCR1 = 0.988 (SE = 0.006 and 95% CI = 0.976-0.99) compared to PCGEM1 = 0.86 (95% CI = 0.81-0.92, SE = 0.02).

Correlation of lncRNA PRNCR1 and PCGEM1 levels with clinical, biochemical and hormonal parameters in study subjects

Spearman rank or Pearson correlation coefficient was calculated in order to determine the correlation between the expression of IncRNAs PRNCR1, PCGEM1 and different clinical, and biochemical characteristics in PCOS. Clinical parameters were used as independent variables both in cases and in controls while PRNCR1 expression as a dependent variable. Results are shown in Table 3. Our data showed a positive association of PRNCR1 only with testosterone (r = 0.20, P = 0.03). However, no
significant association was found between the expression of lncRNA PRNCR1 and PCGEM1 with any other biochemical characteristics both in PCOS women and healthy controls.

Table 3: Partial Pearson or Spearman rank correlation coefficients of the expression of IncRNAs PRNCR1 and PCGEM1 with Participant characteristics
<table>
<thead>
<tr>
<th>Parameter</th>
<th>LncRNA PRNCR1</th>
<th></th>
<th>LncRNA PCGEM1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCOS cases (n=105)</td>
<td>Controls (n=73)</td>
<td>PCOS cases (n=105)</td>
<td>Controls (n=73)</td>
</tr>
<tr>
<td></td>
<td>r</td>
<td>P-value</td>
<td>r</td>
<td>P-value</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.03</td>
<td>0.68</td>
<td>-0.14</td>
<td>0.22</td>
</tr>
<tr>
<td>WHR</td>
<td>-0.01</td>
<td>0.86</td>
<td>-0.05</td>
<td>0.66</td>
</tr>
<tr>
<td>FG score</td>
<td>-0.05</td>
<td>0.59</td>
<td>-0.09</td>
<td>0.41</td>
</tr>
<tr>
<td>DHEAS</td>
<td>0.06</td>
<td>0.53</td>
<td>-0.17</td>
<td>0.13</td>
</tr>
<tr>
<td>Andro</td>
<td>0.12</td>
<td>0.19</td>
<td>0.05</td>
<td>0.64</td>
</tr>
<tr>
<td>SHBG</td>
<td>0.12</td>
<td>0.19</td>
<td>0.08</td>
<td>0.98</td>
</tr>
<tr>
<td>Testo</td>
<td>0.20</td>
<td>0.03*</td>
<td>0.05</td>
<td>0.66</td>
</tr>
<tr>
<td>FAI</td>
<td>0.08</td>
<td>0.37</td>
<td>-0.08</td>
<td>0.49</td>
</tr>
<tr>
<td>LH</td>
<td>0.04</td>
<td>0.65</td>
<td>0.13</td>
<td>0.23</td>
</tr>
<tr>
<td>FSH</td>
<td>0.04</td>
<td>0.67</td>
<td>-0.10</td>
<td>0.39</td>
</tr>
<tr>
<td>INSULIN</td>
<td>0.05</td>
<td>0.59</td>
<td>0.03</td>
<td>0.80</td>
</tr>
<tr>
<td>GLU F</td>
<td>-0.07</td>
<td>0.41</td>
<td>-0.002</td>
<td>0.98</td>
</tr>
<tr>
<td>GLU 2HR</td>
<td>0.08</td>
<td>0.40</td>
<td>0.05</td>
<td>0.65</td>
</tr>
<tr>
<td>CHOL</td>
<td>0.16</td>
<td>0.08</td>
<td>0.10</td>
<td>0.38</td>
</tr>
<tr>
<td>TG</td>
<td>-0.01</td>
<td>0.87</td>
<td>0.15</td>
<td>0.19</td>
</tr>
<tr>
<td>LDL</td>
<td>-0.05</td>
<td>0.56</td>
<td>0.06</td>
<td>0.59</td>
</tr>
<tr>
<td>HDL</td>
<td>-0.02</td>
<td>0.77</td>
<td>-0.04</td>
<td>0.70</td>
</tr>
<tr>
<td>HOMA</td>
<td>0.02</td>
<td>0.78</td>
<td>0.01</td>
<td>0.88</td>
</tr>
<tr>
<td>HIRSUTISM</td>
<td>0.02</td>
<td>0.77</td>
<td>0.01</td>
<td>0.92</td>
</tr>
<tr>
<td>CYCLES</td>
<td>-0.06</td>
<td>0.53</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ALOPECIA</td>
<td>0.05</td>
<td>0.59</td>
<td>0.13</td>
<td>0.25</td>
</tr>
<tr>
<td>ACNE</td>
<td>-0.04</td>
<td>0.66</td>
<td>0.009</td>
<td>0.93</td>
</tr>
<tr>
<td>ACANTHOSIS</td>
<td>0.10</td>
<td>0.26</td>
<td>0.063</td>
<td>0.59</td>
</tr>
</tbody>
</table>

r and P-values were calculated by Pearson and Spearman rank correlation coefficient in parametric and ordinal data respectively. *P-value <0.05 significant.
Association of IncRNAs PRNCR1 and PCGEM1 expression with testosterone

To validate our previous results where we found a positive correlation of IncRNA PRNCR1 with testosterone, we stratified our data to further explore the association of IncRNA PRNCR1 expression with testosterone. The PCOS subjects were grouped into high testosterone (>50ng/dl) and low testosterone (<50ng/dl) groups and T-test was used to compare the expression levels of IncRNA PRNCR1 between the two groups. As shown in Table 4 PCOS women with high testosterone (>50ng/dl) had significantly high PRNCR1 expression levels compared to PCOS women having low testosterone concentration (P = 0.0093). While no such association was found in case of IncRNA PCGEM1 (P = 0.161).

Table 4: Association between expression of IncRNAs PRNCR1 and PCGEM with testosterone

<table>
<thead>
<tr>
<th>PCOS</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Testosterone</strong></td>
<td></td>
</tr>
<tr>
<td>&lt;50ng/dl (n=36)</td>
<td>&gt;50ng/dl (n=69)</td>
</tr>
<tr>
<td>PRNCR1 Expression (Fold change)</td>
<td>4.65 ± 1.58</td>
</tr>
<tr>
<td>PCGEM1 Expression (Fold change)</td>
<td>3.68 ± 1.61</td>
</tr>
</tbody>
</table>

P-value was calculated by student’s T-test. *P-value <0.05 significant.

Abbreviations: PRNCR1; prostate cancer associated non-coding RNA 1, PCGEM1; Prostate cancer gene expression marker 1, PCOS; polycystic ovary syndrome

Association between IncRNAs PRNCR1 and PCGEM1 expression and hirsutism

To assess any possible association of expression levels of PRNCR1 IncRNA with the clinical hyperandrogenic trait, hirsutism in PCOS women, the cases were divided into binary groups based on the presence and absence of hirsutism and expression levels of IncRNA PRNCR1 and PCGEM1 were compared by T-test between these two groups. Results shown in Table 5, revealed that both IncRNAs PRNCR1 (P = 0.79) and PCGEM (P = 0.512) had not any significant association with hirsutism.
Table 5: Association between expression of IncRNAs PRNCR1 and PCGEM1 with hirsutism

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PCOS</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hirsutism</td>
<td>Present (n=93) Absent (n=12)</td>
<td></td>
</tr>
<tr>
<td>PRNCR1 Expression (Fold change)</td>
<td>5.259 ± 1.779</td>
<td>0.79</td>
</tr>
<tr>
<td>PCGEM1 Expression (Fold change)</td>
<td>3.43 ± 1.66</td>
<td>0.512</td>
</tr>
</tbody>
</table>

P-value was calculated by student's T-test

Abbreviations: PRNCR1; prostate cancer associated non-coding RNA 1, PCGEM1; prostate cancer gene expression marker 1, PCOS; polycystic ovary syndrome

Association between IncRNAs PRNCR1 and PCGEM1 expression and obesity

To evaluate the association of IncRNAs PRNCR1 and PCGEM1 expression with obesity, the subjects were categorized into two groups those having BMI <25 kg/m² and those having BMI >25 kg/m². As shown in Table 6, no significant difference in the expression of IncRNAs PRNCR1 (P = 0.897) and PCGEM1 was found between the two groups (P = 0.286).

Table 6: Association between expression of IncRNAs PRNCR1 and PCGEM1 with obesity

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PCOS</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>&lt;25 kg/m² (n=52) &gt;25 kg/m² (n=53)</td>
<td></td>
</tr>
<tr>
<td>PRNCR1 Expression (Fold change)</td>
<td>5.35 ± 1.66</td>
<td>0.64</td>
</tr>
<tr>
<td>PCGEM1 Expression (Fold change)</td>
<td>3.53 ± 1.80</td>
<td>0.286</td>
</tr>
</tbody>
</table>

P-value was calculated by student’s T-test

Abbreviations: PRNCR1; Prostate cancer associated non-coding RNA 1, PCGEM1; Prostate cancer gene expression marker 1, PCOS; polycystic ovary syndrome

Discussion

To the best of our knowledge, this study is the first of its kind that investigated the association of androgen receptor co-activator IncRNAs PRNCR1 and PCGEM1 with the risk of PCOS and its clinical manifestation in North Indian PCOS women. The IncRNAs PRNCR1 and PCGEM1 were significantly
overexpressed in PCOS women than in healthy controls. We also found that high expression lncRNAs PRNCR1 and PCGEM1 are risk factors for PCOS but do not have a significant role in driving various clinical manifestations associated with PCOS.

PCOS is a multisystemic disorder that begins in the pubertal stage and presents as hormonal and reproductive imbalance with menstrual dysfunction and cutaneous findings as the main concerns. Further, it evolves to develop metabolic and psychological complications in affected women as age advances. The complexity of the syndrome affects the understanding of the onset and the mechanism of development of this disorder. However, Hyperandrogenism, insulin resistance, genetic, epigenetic and environmental factors play a significant role in the pathogenesis of PCOS. The primary signs of clinical hyperandrogenism that affect PCOS women include acne, hirsutism and alopecia. In PCOS women these hyperandrogenic cutaneous manifestations remain a major concern. In our study, we reported 88.57%, of PCOS women had hirsutism, 61.95% had acne and 49.50% had alopecia. These clinical signs indicate androgen excess in these women. All major androgens were significantly higher in PCOS women than in controls. Besides the levels of SHBG were significantly lower in PCOS women compared to healthy controls thus further increasing the bioavailable androgens as SHBG binds and limits the availability of free androgen. The increased androgens and decreased SHBG levels explain hyperandrogenism and its cutaneous manifestations in women with PCOS. The characteristic feature of PCOS is chronic oligo-anovulation, which presents as menstrual dysfunction in affected women. Studies have summarized that 75%-85% of PCOS women have clinically evident menstrual abnormalities (oligo/anovulation) across different ethnicities. Our results show 87.61% of women with PCOS had delayed menstrual cycles. Similar to our findings, Chang and colleagues reported 83.90% of PCOS women had menstrual dysfunction and 85.90% were reported by Daimanti-Kandkaris and Danidis. Chronic oligo-anovulation is associated with polycystic ovarian morphology. Our study showed 81.9% of PCOS women had PCOM. Studies carried out on PCOS women of different ethnicities consistently showed similar results and no significant variations were reported in PCOM across different ethnicities.

Hyperandrogenism is one of the characteristic traits of PCOS. It is associated with most of the reproductive, metabolic and endocrine complications of PCOS besides being the primary factor involved in its pathophysiology. Hyperandrogenism results in the hyperactive AR and overexpression of AR regulated genes. Studies have established that hyperactive AR in the ovaries or at extra-ovarian regions, especially at neuroendocrine sites plays a critical role in PCOS pathophysiology. The hyperactive AR in the neuroendocrine site leads to abnormal GnRH pulsation, ovulatory dysfunction and classic polycystic ovaries. In the present study, we investigated lncRNA PRNCR1 and PCGEM1 expression in study participants. According to our data, lncRNAs PRNCR1 and PCGEM1 are associated with PCOS in Kashmiri women. Both these IncRNAs were significantly overexpressed in PCOS women compared to healthy controls ($P<0.001$). The IncRNAs PRNCR1 and PCGEM1 are the best studied androgen receptor co-activator IncRNAs. They have been shown to cause transactivation of androgen receptor both in a ligand dependent and ligand independent fashion. Both IncRNAs PRNCR1 and PCGEM1 bind to the androgen receptor and then subsequently they bind to the promoter region of androgen receptor regulated
genes and thereby turn on their expression. Besides IncRNA PCGEM1 independently forms a complex with AR and reprograms the transcriptional network of AR regulated genes and some important metabolic genes thus playing an important role in AR related disease progression. The overexpression of these IncRNAs has been reported to have a positive association with diseases linked to AR like prostate cancer. Studies have also demonstrated that the knockdown of IncRNAs PRNCR1 and PCGEM1 genes attenuated the transactivation of AR and significantly compromised AR induced gene expression. Together these studies suggest IncRNAs PCGEM1 and PRNCR1 play an essential role in the expression of AR regulated genes and hence may be involved in the progression of related diseases like PCOS. Moreover, we found higher expression of both IncRNAs PRNCR1 and PCGEM1 was seen more frequently in PCOS women possibly increasing androgenic effects in these women, while lower expression was more frequent in controls. This indicates that women with higher expression of these IncRNAs have a higher risk of PCOS which is consistent with the role of hyperandrogenism in PCOS development.

The correlation between various biochemical and hormonal parameters and expression of both IncRNAs PRNCR1 and PCGEM1 was determined. Although, these IncRNAs were associated with PCOS. They had no significant correlation with different clinical manifestations of PCOS. The expression of PRNCR1 and PCGEM1 was found to be independent of obesity and no significant difference in their expression was observed between normal and obese PCOS women. Likewise, other clinical features Hirsutism (P > 0.05) Acne (P > 0.05) Alopecia (P > 0.05) did not show any significant correlation with the expression of IncRNAs PRNCR1 and PCGEM1. Similar results were found in different studies carried out on different IncRNAs. The various clinical manifestations or phenotypic conditions that arise in PCOS women are the outcomes of multiple factors acting together like genetic and epigenetic as well as environmental factors. In the present study since we did not find any significant correlation between IncRNAs PRNCR1 and PCGEM1 with different clinical characteristics of PCOS. This further suggests that these manifestations of PCOS are the result of the concurrent presence of multiple factors. Given the role of IncRNAs PRNCR1 and PCGEM1 in the hyperactivity of AR and overexpression of AR regulated genes, we presume that these androgen receptor coactivator IncRNAs may be acting synergistically with other factors in contributing to the development of different clinical manifestations associated with PCOS.

Among different circulating androgens, testosterone is the primary circulatory androgen in females. Testosterone is mainly responsible for different hyperandrogenic complications in PCOS women. Studies have reported positive feedback of increased androgen activity on ovarian androgen synthesis. Thus, increased androgen activity via hyperactive androgen receptor stimulates ovarian androgen synthesis. Similar results were observed in the present study, PRNCR1 showed a significant positive correlation with total testosterone (P < 0.001) in PCOS women while no such association was observed in healthy controls. Further, upon stratification of data, we found PCOS patients with high total testosterone had significantly higher PRNCR1 expression levels compared to PCOS patients with lower levels of total testosterone. Our study is consistent with different earlier studies showing a positive association of
serum total testosterone with androgen responsive lncRNA CTBP1-AS and different genetic studies on CAG repeat polymorphisms\textsuperscript{35,44–46}.

In conclusion, our study demonstrated that AR coactivator lncRNAs PRNCR1 and PCGEM1 are associated with PCOS women and their overexpression is a possible risk factor for PCOS in Kashmiri women. LncRNAs PRNCR1 and PCGEM1 may act as important factors for the hyperactivation of AR and subsequent overexpression of AR regulated genes and thus may contribute towards the pathophysiology of PCOS. In the present study we for the first-time observed AR coactivator lncRNAs overexpression in PCOS women. However, further studies clarifying the mechanistic action and signalling pathways of these lncRNAs may further help in better understanding of PCOS pathophysiology, its clinical manifestations and in identifying novel diagnostic therapeutic targets.

**Abbreviations**

**AR:** Androgen Receptor  
**AUC:** Area under curve  
**BMI:** Body mass index  
**CHOL:** Cholesterol  
**DHEAS:** Dihydroepian-androstenedionesulphate  
**FAI:** Free androgen index  
**FG Score:** Ferriman Gallwey Score  
**Glu F:** Glucose fasting  
**FSH:** Follicle stimulating hormone  
**HOMA IR:** Homeostatic model assessment of insulin resistant  
**LH:** luteinizing hormone  
**LncRNAs:** Long non coding ribonucleic acid  
**PBMCs:** Peripheral blood mononuclear cells  
**PCOM:** Polycystic ovary morphology  
**PCGEM1:** Prostate cancer gene expression marker 1  
**PRNCR1:** Prostate cancer-associated non-coding RNA 1
PCOS: Polycystic ovary syndrome

ROC: Receiver operator characteristic

SHBG: sex hormone binding globin

TG: Triglycerides

TT: Total testosterone

qPCR: Real-time quantitative polymerase chain reaction

WHR: Waist-hip ratio

Declarations

Data availability: The data and materials analysed will be provided by the corresponding author upon reasonable request.

Consent for publication: All authors have approved the manuscript for submission.

Acknowledgements

We thank all the study subjects. We are also thankful to Mr. Suhail Murtaza technical assistant, who assisted in the sampling of PCOS cases and control women. We are thankful to our Co-scholars for their help during the course of experimental studies.

References


**Figures**
Figure 1

A: The expression profile of lncRNA PRNCR1 in peripheral blood leukocytes in PCOS cases and healthy controls measured by quantitative real-time PCR. Data is expressed as mean ± SD

B: The expression profile of lncRNA PCGEM1 in peripheral blood leukocytes in PCOS cases and healthy controls measured by quantitative real-time PCR. Data is expressed as mean ± SD

Figure 2

A: ROC curve analysis of lncRNA PRNCR1 in PCOS cases and healthy controls
B: ROC curve analysis of IncRNA PCGEM1 in PCOS cases and healthy controls