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Complete List of Authors:	Butler, Alexandra E.; Qatar Biomedical Research Institute, Diabetes Research Hayat, Shahina; Weill Cornell Medical College in Qatar, Medicine Dargham, Soha; Weill Cornell Medical College in Qatar, Medicine Malek, Joel; Weill Cornell Medical College in Qatar, Medicine Abdullah, Silvana; Weill Cornell Medical College in Qatar, Medicine Mahmoud, Yasmin; Weill Cornell Medical College in Qatar, Medicine Suhre, Karsten; Weill Cornell Medical College in Qatar, Medicine Sathyapalan, Thozhukat; Hull York Medical School, Academic Endocrinology, Diabetes and Metabolism Atkin, Stephen L; Weill Cornell Medical College in Qatar, Research
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Alterations in long non-coding RNAs in women with and without Polycystic Ovarian Syndrome

Alexandra E. Butler[1], Shahina Hayat[2], Soha R Dargam[2], Joel A Malek[2], Silvana A Abdullah[2], Yasmin A Mahmoud[2], Karsten Suhre[2], Thozhukat Sathyapalan[3], Stephen L. Atkin[2,4]

[1]Diabetes Research Center, Qatar Biomedical Research Institute, Hamad Bin Khalifa University, Qatar Foundation, PO Box 34110, Doha, Qatar

[2]Weill Cornell Medicine-Qatar, Education City, PO Box 24144, Doha, Qatar

[3]Academic Diabetes and Endocrinology, Hull York Medical School, Hull, UK

[4]Royal College of Surgeons, Bahrain

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Corresponding author: Stephen Atkin, Weill Cornell Medicine Qatar, PO Box 24144, Doha, Qatar. Email: sla2002@qatar-med.cornell.edu. Phone: +97455639807. Fax: +4497444928422

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Abstract. Long non-coding RNAs (lncRNAs) are RNA transcripts over 200 nucleotides long that are not translated into protein; however, there is increasing evidence of their regulatory functions. To date, there are few studies measuring lncRNA in control women or women with polycystic ovary syndrome (PCOS).

Objective. To determine lncRNA differences between PCOS and control women.

Methods LncRNA were measured in 24 anovulatory women with all three diagnostic features of PCOS compared to 24 control women in the follicular phase of their menstrual cycle from a PCOS biobank.

Results. Women with PCOS were age and weight matched compared to the control women but were significantly insulin resistant and hyperandrogenemic ($p<0.01$). Eight lncRNA ($p<0.05$) were detected that differed between PCOS and control women, but only MIRLET7BHG correlated with body mass index ($r=0.66$, $p<0.05$). No lncRNA correlated with anti-mullerian hormone (AMH) levels, insulin resistance (HOMA-IR) or the free androgen index (FAI). Ingenuity pathway assessment (IPA) did not identify any functional pathways for the lncRNAs.

Conclusion. LncRNAs differ between anovulatory PCOS and control women in the follicular phase of the menstrual cycle. It is unclear if this is due to inherent differences between PCOS and control women or due to changes in lncRNA that are menstrual cycle dependent. However, their IPA did not identify linked pathways, likely because few functions are as yet assigned to these lncRNAs.

Introduction

Polycystic ovarian syndrome (PCOS) affects 9-21% of the female population, making it one of the most common endocrine disorders amongst women of reproductive age, and it is the major cause of anovulatory infertility (1). The clinical and biochemical hyperandrogenism characteristic of PCOS are associated with insulin resistance (IR), obesity, type 2 diabetes, and hypercholesterolemia (2). Though the etiology of PCOS is poorly understood, genetic abnormalities, such as mutations, epigenetic alterations and changes in noncoding RNAs, are considered to be major underlying etiological factors (3-5).

The ENCODE project established that, in both humans and rodents, only approximately 2% of the genome is transcribed into coding sequences, the vast majority being transcribed into noncoding sequences. These noncoding sequences include microRNAs, small interfering RNAs and long noncoding RNAs (lncRNAs) (6, 7). Long noncoding RNAs are transcripts greater than 200 nucleotides in length that lack protein-coding capacity. An understanding of their mechanistic role in diverse biological processes is accumulating (8). Evidence indicates that they have regulatory role in development, differentiation, proliferation and apoptosis and their involvement in diseases such as diabetes (9), cardiovascular disease (10), neurological disorders (11), and cancer (8). More recently, differential expression of 862 lncRNA transcripts (692 upregulated, 170 downregulated) was reported in granulosa cells from Chinese women with PCOS undergoing *in vitro* fertilization (IVF) therapy (12); however, the number of women in the study (7 PCOS, 7 controls) was limited.

Given the limited data on lncRNA in PCOS, our study was designed to use serum samples from the PCOS Biobank collected within the follicular phase in normal women to compare circulating levels of lncRNAs in Caucasian women with PCOS versus controls. The lncRNA were not preselected but rather those that were detected from each of the RNA libraries constructed.

Materials and methods

Study design: This was a cross sectional study undertaken in 24 medication naïve women with PCOS and biochemical hyperandrogenaemia (age 18-45 years) who presented sequentially to the department of endocrinology and those who fulfilled the criteria of the study were recruited into the local PCOS biobank at United Kingdom (ISRCTN70196169). Twenty-four control women (age 20-44 years) who were age and body mass index (BMI) matched to the PCOS women were included into this study. Subject demographics are shown in Table 1. The diagnosis of PCOS was based on fulfilling all three diagnostic criteria of the Rotterdam consensus, namely clinical and biochemical evidence of hyperandrogenemia (Ferriman-Gallwey score >8; free androgen index >4 respectively), oligomenorrhea or amenorrhea and polycystic ovaries on transvaginal ultrasound (classical phenotype) (13). Liver ultrasound was performed at the same time to exclude non-alcoholic fatty liver disease. Study participants had no concurrent illness, and were not on any medication for the preceding nine months. None of the women had successful pregnancy or miscarriage at least five year prior to study entry. Diabetes was excluded by a 75g oral glucose tolerance test. Non-classical 21-hydroxylase deficiency, hyperprolactinaemia, Cushing's disease and androgen-secreting tumours were excluded by appropriate tests. All women gave written informed consent. This study was approved by the Newcastle & North Tyneside Ethics committee, UK.

Sample collection: Blood samples were taken after an overnight fast during the follicular phase of the menstrual cycle in control women and serum was stored frozen at -80^{O}C pending analysis. All PCOS women were anovulatory (3 months amenorrhea; random progesterone less than 5nmol/l). Serum testosterone and androstenedione were measured by isotope dilution liquid chromatography-tandem mass spectrometry (Waters Corporation, Manchester, UK). Sex hormone binding globulin (SHBG) was determined by an immunometric assay with fluorescence detection on the DPC Immulite 2000 analyzer using the manufacturer's

recommended protocol. The free androgen index was obtained as the total testosterone x100/SHBG. Serum insulin was assayed using a competitive chemiluminescent immunoassay performed on the manufacturer's DPC Immulite 2000 analyzer (Euro/DPC, Llanberis, UK). The analytical sensitivity of the insulin assay was 2 μU/ml, the coefficient of variation was 6%, and there was no stated cross-reactivity with proinsulin. Plasma glucose was measured using a Synchron LX20 analyzer (Beckman-Coulter), using the manufacturer's recommended protocol. The coefficient of variation for the assay was 1.2% at a mean glucose value of 5.3 mmol/liter during the study period. The insulin resistance was calculated using the HOMA method [HOMA-IR=(insulin x glucose)/22.5], and pancreatic beta cell sensitivity measured by HOMA—β [HOMA-β=(20 x insulin)/glucose -3.5].

RNA preparation and analysis Following RNA extraction: Approximately 20ng of total RNA was used to generate strand-specific paired end 75bp Illumina libraries with NEXTFLEX Rapid Directional RNA-Seq Library Prep Kit (Bio-Scientific, Austin, TX) according to the manufacturer's protocol. Briefly, the protocol included the following steps: first strand cDNA synthesis, second strand synthesis, A- tailing, adaptor ligation and purification, followed by UDG treatment, library amplification and purification. Library quality and quantity were analyzed with the Bioanalyzer 2100 (Agilent, Santa Clara, CA) on a High Sensitivity DNA chip. Six libraries were then pooled in equimolar ratios and sequenced on one lane of an Illumina HiSeq 4000 run (Illumina, San Diego, CA). Average sequencing depth for the libraries is 16 million reads.

RNA-seq data analysis: We performed quality check for the read with FastQC (14). Reads with adaptors and rRNA contamination were removed using BBMap (15). After filtering, the reads were mapped to human reference genome from Ensembl GRCh38 release 93 (16) with STAR (17) using Ensembl 93 gene annotation(16). For mapped read quantification, we used featureCounts function from Rsubread package (18) in R (19).

Following quantification, lncRNA were identified and the lncRNAs were then quantified using Wald test from DESeq2 (20). P value <0.05 was taken as the cutoff for significance.

Statistical analysis: There was no information for lncRNA on which to base a sample size calculation. For such pilot studies, Birkett and Day (14) suggest a minimum of 20 degrees of freedom to estimate variance from which a larger trial could be powered. We recruited 24 for each group to allow for drop-outs and covariate adjustment. Statistical analysis was performed using SPSS (v22, Chicago, Illinios). Descriptive data is presented as mean ± SD for continuous data and n (%) for categorical data. T-tests or Mann Whitney tests were used to compare means/medians where appropriate. Linear associations were assessed using the Pearson's correlation test.

Results:

Baseline characteristics of the 24 PCOS and 24 control women are shown in **Table 1.** The PCOS and control women did not differ for age or BMI; however, the PCOS women showed greater insulin resistance and hyperandrogenemia ($p<0.01$), androstenedione did not differ. There were 8 significant lncRNAs between the PCOS and control women (Table 2). Of those, MIRLET7BHG significantly correlated with BMI (r 0.66, $p<0.05$). No lncRNA correlated with age, AMH, HOMA-IR, testosterone, androstenedione or the FAI. All of the lncRNA detected are shown in Supplementary Table 1.

When Ingenuity pathway analysis was undertaken to look at the functional relationships, no pathway could be constructed when taking the top 10, top 25, top 50 or top 70 lncRNAs into account.

Discussion

Next generation sequencing (NGS) has provided a wealth of novel information about genomic organization and regulation of gene expression, with non-coding (non-translated) RNAs being identified as important regulators of gene expression (15). Whilst thousands of

lncRNAs have now been identified, functional characterization has only been determined in a small subset but has indicated that they are involved in numerous physiological processes, working at the epigenetic, transcriptional and post-transcriptional level (16). Information to date has shown that their dysregulation is associated with a wide variety of diseases including cancer (8), neurological disorders(11), cardiovascular disease(10) and diabetes (9). At least 19 lncRNAs are associated with diabetes in humans (17), some directly affecting pancreatic beta-cells, with others acting as a link between insulin signaling and insulin resistance (18). Insulin resistance is an integral component of PCOS, and the prevalence of T2DM is increased in women with PCOS (2), therefore aberrant expression of lncRNAs in serum of women with PCOS might be anticipated, and indeed the findings are not unexpected.

This study suggests that there are significant alterations of lncRNAs in subjects with PCOS compared to age and BMI matched control subjects in the normal women in the follicular phase of the menstrual cycle compared to the anovulatory PCOS.

In women with PCOS compared to controls, there were 8 lncRNAs that differed significantly. Using www.genecard.org, MALAT1 (metastasis associated lung adenocarcinoma transcript 1) is thought to provide molecular scaffolds for ribonuclear proteins and is associated with cancer progression. MIR181A1HG is associated with acute myeloid leukaemia. The functions of the other lncRNAs (AC005332.6, AC009404.1, PSMG3-AS1, MIRLET7BHG, AC016831.6 and AC012313.1) are unknown.

Reported phenotypes associated with the lncRNA include BMI (AP001999.1 and IQCH-AS1), systolic blood pressure (AC093459.1 and AC016831.6), type 2 diabetes (AC016831.6) dyslipidemia (AC016831.6) and PCOS (LINC01828) (www.genecard.org). Given that little function has to date been ascribed to lncRNAs, it may therefore be understood why no functional pathways could be constructed with the Ingenuity Pathway assessment tool.

Correlation was undertaken for the significant lncRNAs for the follicular phase with age, BMI, AMH, HOMA-IR and FAI, but only MIRLET7BHG correlated with BMI in the follicular phase. However, the sample size was too small to exclude a type 2 statistical error. This pilot study was limited by the small number of subjects but the strength of the study was that all of the PCOS women fulfilled all 3 of the diagnostic Rotterdam criteria providing homogeneity. A comparison with PCOS subjects without the metabolic phenotype would have been of value. The study clearly showed that lncRNAs may be found in PCOS and that they differ from control women. However, given that the lncRNA did not correlate to either insulin resistance or androgen levels then it may suggest that the differences could be more related to the menstrual cycle, but there is no data on lncRNA throughout the menstrual cycle to answer this.

In conclusion, lncRNA were found to differ between PCOS and control women in the follicular phase of the menstrual cycle, though functional pathway analysis was limited by the unknown functions of many of the lncRNAs. The phase of the menstrual cycle may be important for lncRNA expression and that needs to be taken into account in future studies.

Author contributions

A.E. Butler contributed to data analysis and wrote the manuscript. Shahina Hayat and Soha R Dargham analyzed the data. Joel Malek, Silvana Abdullah and Yasmin A. Mahmoud performed the LNC RNA measurements. Karsten Suhre contributed to data analysis. Thozhukat Sathyapalan supervised sample collection. Stephen L. Atkin designed the studies, supervised the work, contributed to data analysis and was involved in preparation of the manuscript.

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work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Table 1. Demographics, biochemical and clinical markers (mean±SD) for the PCOS and control group 1 from biobank women.

	Control women (n=24)		PCOS women (n=24)		P-value
	Mean	SD	Mean	S D	
Age (years)	32.4	7.8	31.6	8.8	0.735
BMI	27.2	6.0	28.8	5.6	0.324
Glucose (mmo/l)	4.6	0.5	4.8	0.6	0.202
Insulin mIU/ml)	7.3	4.9	12.0	6.1	0.012
HOMA IR	1.5	0.9	2.6	1.7	0.006
Androstenedione (nmol/l)	8.3	4.8	10.6	6.6	0.21
Testosterone (nmol/l)	1.0	0.4	2.5	1.6	0.007
SHBG (mmol/l)	76.4	76.1	46.6	51.0	0.167
FAI	2.0	1.0	12.4	18.0	0.013

(BMI, body mass index; FAI, free androgen index; SHBG, sex hormone binding globulin; HOMA-IR, homeostatic model assessment-insulin resistance)

Table 2. Significant long non coding RNA (n=8) in patients unselected for date of menstrual cycle for patients with PCOS and control women.

LNC RNA	Fold Change	p value
AC005332.6	0.444	0.008
MALAT1	-0.095	0.016
AC009404.1	0.561	0.026
MIR181A1HG	0.617	0.034
PSMG3-AS1	0.547	0.038
MIRLET7BHG	0.334	0.040
AC016831.6	-0.669	0.045
AC012313.1	0.427	0.048

Supplementary Table 1. All long non coding RNA (significant n=8) in patients unselected for date of menstrual cycle for patients with PCOS and control women.

LNC RNA	Fold Change	p value
AC005332.6	0.444	0.008
MALAT1	-0.095	0.016
AC009404.1	0.561	0.026
MIR181A1HG	0.617	0.034
PSMG3-AS1	0.547	0.038
MIRLET7BHG	0.334	0.040
AC016831.6	-0.669	0.045
AC012313.1	0.427	0.048
AC103718.1	1.046	0.057
AC015813.1	0.220	0.085
LINC01588	-0.420	0.106
TP53TG1	-0.401	0.157
XIST	0.723	0.159
LINC00667	-0.269	0.159
MIR22HG	-0.290	0.195
CYTOR	-0.317	0.196
SMIM25	-0.620	0.196
LINC00513	-0.414	0.226
MIAT	-0.609	0.228
HCG11	0.334	0.251
APTR	0.315	0.257
AC245041.2	0.393	0.266
AC245041.1	0.541	0.277
AC060780.1	0.243	0.278
MIR222HG	0.340	0.278
SNHG15	-0.243	0.280
LINC00665	0.371	0.298
OLMALINC	0.434	0.313
BAIAP2-DT	-0.223	0.366
AL160272.1	0.265	0.378
EPB41L4A-AS1	0.263	0.390
AC006504.5	0.196	0.393
SERPINB9P1	0.286	0.397
LINC01089	-0.282	0.402
NEAT1	-0.066	0.410
LINC01934	-0.268	0.414
AC092171.2	0.143	0.462
AC090114.2	0.164	0.465

RAB30-AS1	-0.211	0.468
MIR34AHG	-0.251	0.477
CASC15	0.289	0.501
LINC-PINT	-0.190	0.503
AC092490.1	-0.540	0.514
FP671120.4	-0.330	0.530
AC078846.1	-0.198	0.542
AL161431.1	0.401	0.545
PVT1	0.127	0.562
AP003486.1	0.127	0.568
LINC00294	0.157	0.573
MIR29B2CHG	-0.143	0.592
NUP50-DT	-0.156	0.597
AC232271.1	-0.154	0.605
AC006058.1	-0.198	0.611
LINC01503	0.131	0.617
AC083843.2	-0.136	0.630
LINC00472	-0.234	0.653
LINC00342	-0.090	0.655
LINC02015	0.164	0.655
LINC01963	-0.129	0.665
LINC00958	0.197	0.668
AL512274.1	0.177	0.671
LINC00630	0.118	0.694
LINC00861	-0.249	0.704
MIATNB	-0.161	0.717
AC007878.1	-0.098	0.721
AC005261.1	0.047	0.723
GMDS-DT	-0.063	0.763
LINC02035	-0.049	0.764
LINC00662	-0.104	0.765
AC058791.1	0.101	0.792
ILF3-DT	0.064	0.808
MIR4435-2HG	-0.047	0.829
EIF3J-DT	-0.052	0.844
AF117829.1	0.034	0.877
SNHG8	-0.042	0.886
MUC20-OT1	0.017	0.887
LINC01184	0.025	0.889
AC008124.1	-0.045	0.892
EBLN3P	-0.014	0.899
AC016831.4	-0.031	0.902

AC048341.1	0.025	0.919
LINC00265	0.017	0.936
NORAD	0.004	0.970
FTX	-0.006	0.978
LNC RNA	Fold change	p value
NEAT1	0.972784308	0.001318918
MALAT1	-0.095207667	0.012134396
AC103718.1	1.077682776	0.0369768
MIRLET7BHG	0.317374986	0.043351359
TP53TG1	-0.619755201	0.071335597
PSMG3-AS1	0.453541106	0.081348578
HCG11	0.603643213	0.081929868
LINC00667	-0.341448173	0.087503347
AC015813.1	0.227320011	0.092930753
SNHG15	-0.683187137	0.103497955
SERPINB9P1	0.731681883	0.111523219
AL160272.1	0.583857724	0.116441763
AC245041.2	0.523006704	0.13472168
AC012313.1	0.328641312	0.143946995
MIR181A1HG	0.435739735	0.15073483
AC005332.6	0.279377116	0.164287339
MIAT	-0.646798865	0.174631999
MIR222HG	0.41222115	0.18000658
LINC01503	0.427762913	0.200340291
AC009404.1	0.361729735	0.208276474
AC245041.1	0.578277167	0.216315965
AC078846.1	-0.460605791	0.218564928
AC058791.1	0.574462618	0.245814576
SNHG8	-0.560413955	0.246369192
MUC20-OT1	0.187348995	0.260903116
NORAD	-0.140427453	0.263529997
LINC02015	0.438662764	0.265375365
EBLN3P	-0.170719196	0.268378424
AC016831.4	0.461698707	0.276379729
LINC01963	-0.363631346	0.282413238
MIR29B2CHG	0.473979602	0.307546848

AL161431.1	0.646113968	0.309886358