Long Non-Coding RNA Expression in Nonobese Polycystic Ovary Syndrome and Weight Matched Controls

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Abstract.

Research Question. Long non-coding RNAs (lncRNAs) do not show protein translation but do have gene regulatory functions in several disease states. Studies have shown that lncRNA differ in overweight PCOS women with increased insulin resistance and hyperandrogenemia.

The objective of this study was to determine lncRNA in serum in age and weight matched non-obese women with and without PCOS.

Methods In this prospective pilot cohort study, lncRNA was measured in serum in 13 non-obese women with PCOS and 10 control women undergoing IVF.

Results. There was no difference between groups for age, BMI or insulin resistance; PCOS women showed a higher free androgen index ($p=0.03$) and AMH ($p=0.001$). A total of 29 lncRNA ($p<0.05$) differed between PCOS groups. LncRNA AC095350.1 correlated with age ($r=0.79$, $p<0.05$), but there was no correlation seen between the significantly different lncRNA and free androgen index (FAI) or anti-mullerian hormone (AMH). Functional pathway assessment through the Ingenuity Pathway Assessment (IPA) tool showed no relationships for the lncRNA.

Conclusion. LncRNA in serum differed between non-obese PCOS and control women and the pattern of expression differed to that reported in obese PCOS from the same ethnic population but did not correlate with androgen or insulin resistance.

Introduction

Polycystic ovary syndrome (PCOS) affects 9-21% of premenopausal women and is a leading cause of anovulatory infertility (Azziz et al. 2004). Metabolic dysregulation is a major feature of PCOS, with the characteristic clinical and biochemical hyperandrogenism being associated with metabolic syndrome and type 2 diabetes (Franks 1995). Noncoding RNA changes, epigenetic changes and mutations are genetic changes that may contribute to the pathogenesis of PCOS (Shi et al. 2012, Sorensen et al. 2014, Wang et al. 2014).

Only 2% of the human genome is transcribed into coding sequences, with the remainder being noncoding sequences including long noncoding RNA (lncRNA) (Marchese et al. 2017). Most of them are transcribed by RNA polymerase II like messenger RNAs; however, they lack coding capacity (Marchese et al. 2017, Yao, et al. 2019). These noncoding sequences, defined as transcripts greater than 200 nucleotides that are not translated into protein, are responsible for the formation of differing noncoding RNAs such as lncRNA (Djebali et al. 2012, Hangauer et al. 2013). There is increasing evidence of lncRNA working in a coordinated regulatory fashion in biological networks with other noncoding RNAs, such as microRNA and circularRNA that may then effect multiple downstream biological processes (Li and Chen 2013, Kleaveland et al. 2018). Diverse multiple effects may then involve regulation of development, differentiation, proliferation and apoptosis, with resulting effects in differing cardiometabolic diseases (Broadbent et al. 2008, Gopalakrishnan, et al. 2015). We have recently reported the expression of lncRNA in PCOS women and showed the differential expression of lncRNA in obese weight matched PCOS and control women (Butler et al. 2019), and others have reported differential lncRNA expression in PCOS granulosa cells (Liu et al. 2017), with the suggestion that

01572.28 can inhibit granulosa cell growth through decreased p27 degradation (Zhao, et al. 2018). It is also clear that there is differential expression of lncRNA in follicular fluid derived from mature and immature follicles (Jiao et al. 2018), and that these lncRNA derived from granulosa cells may relate to androgen status (Jin et al. 2018). Others have shown that lncRNA associated with insulin resistance are altered in PCOS with downregulation of lncRNA Growth-arrest specific transcript 5 (Lin et al. 2018), and it has been shown that lncRNA RP11-151A6.4 was associated with PCOS women with increased BMI and insulin resistance (Zhao et al. 2019). However, it is unclear if the lncRNA findings reflect underlying changes in BMI through obesity or are a direct association with PCOS. This study was designed to determine if lncRNA expression differed between control and PCOS women that were weight matched and nonobese in order to remove this major confounding factor. We used serum samples collected within the follicular phase from PCOS and normal women to compare lncRNA circulating levels. 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 prospective cohort study and was performed from January 2015 to January 2016 within the Hull IVF Unit, UK following approval by the Yorkshire and The Humber NRES ethical committee, UK (reference number 10/H0906/17; approval date May 6, 2010). All participants gave their written informed consent. The 13 PCOS subjects and 10 normal controls were all undertaking assisted reproduction. Subjects presenting sequentially were age and weight matched. All women were on folic acid 400mcg daily but not any other medication. Exclusion criteria were patients with diabetes, renal or liver insufficiency, acute or chronic infections, systemic inflammatory diseases, age <20 or >45 years, or with known immunological disease.

The diagnosis of PCOS was based on fulfilling two of the three diagnostic criteria of the Rotterdam consensus (classical phenotype) namely oligomenorrhea or amenorrhea, clinical and biochemical evidence of hyperandrogenemia (Ferriman-Gallwey score >8; free androgen index >4 respectively), and polycystic ovaries on transvaginal ultrasound (Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group. 2004). For the PCOS subjects, diabetes, non-classical 21-hydroxylase deficiency, hyperprolactinaemia, Cushing's disease and androgen-secreting tumours were excluded by appropriate testing.

Sample collection. A fasting blood sample was taken before commencing IVF treatment, centrifuged 3000rpm for 10 minutes and the serum was stored frozen at -80^{O}C pending analysis. Analysis was undertaken according to that reported previously (Butler et al. 2019). Serum testosterone was 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. The free androgen index was obtained as the total testosterone x100/SHBG. Serum insulin was assayed using a competitive chemiluminescent immunoassay performed on a 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). 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]. AMH was measured using a Beckman Coulter Access automated immunoassay; between run precision was <3% across the range measured.

RNA preparation and analysis following RNA extraction. This has been described before (Butler et al. 2019). Briefly, ~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). 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. This was performed as previously reported (Butler, Hayat et al. 2019). Briefly, the read was quality checked with FastQC (Andrews 2010) and rRNA contamination removed with BBMap (Bushnell 2015); subsequently, the reads

were mapped to the Ensembl GRCh38 release 93 human reference genome (Speir et al. 2016) with STAR (Dobin et al. 2013). FeatureCounts function from Rsubread package (Liao, Smyth et al. 2019) in R (Team 2013) were used for mapped read quantification. Following quantification, lncRNA features were selected and differential gene expression analysis was performed on these features using Wald test from DESeq2 (Love et al. 2014). P value <0.05 was taken as the cut off for significance.

Statistical analysis: This was a pilot study as there was no information regarding lncRNA changes in nonobese Caucasian PCOS patients to undertake a formal power calculation; therefore, Birkett and Day (Birkett and Day 1994) suggest a minimum of 20 degrees of freedom to estimate variance from which a larger study could be powered from pilot data: a minimum of 10 for each group to allow for drop-outs and covariate adjustment. Statistical analysis was performed using SPSS (v23, Chicago, Illinois). Descriptive data are presented as mean ± SD for continuous 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

The two groups were well matched for age and weight (Table 1) and there was no difference in insulin or insulin resistance between the 2 groups. Androgen levels did differ between the two groups with both total testosterone and FAI being elevated in PCOS women, and AMH was significantly elevated as expected (Table 1).

There were 29 lncRNA that passed FDR and that differed between the anovulatory PCOS subjects and controls, as shown in Table 2. All of the lncRNA are shown in

Supplementary Table 1. LncRNA AC095350.1 significantly correlated with age (r 0.79, $p<0.05$). None of the lncRNA significantly correlated with BMI, AMH, HOMA-IR or the FAI (data not shown).

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 lncRNA into account (based on p values).

Discussion

In this study, 29 lncRNA passed the false discovery rate indicating differences between the normal weight PCOS and control subjects; however, only AC095350.1 correlated with age. For those 10 lncRNA that were shown to most significantly different between the PCOS and weight and age matched control women, no specific function was found to be assigned to AC095350.1, LINC01539, LINC00616, AL591368.1, AC139749.1, AL160272.1, AC093459.1, C1orf220 and AL450332.1. In comparison with our previous study in the same population and ethnicity, in age and weight matched obese subjects (Butler et al. 2019), only AC016831.6 was common to both studies in showing a significant difference between groups, whilst AC005332.6, MALAT1, PSMG3-AS1, MIRLET7BHG and AC012313.1 were detected but did not differ between groups, and both AC009404.1 and MIR181A1HG found in the previous study were not detected here. The previous study that was undertaken differed in that all PCOS women fulfilled all of the PCOS criteria for diagnosis, were obese, insulin resistant and had features of the metabolic syndrome, in comparison with the non-obese PCOS patients here that only fulfilled 2 of the 3 criteria and did not have insulin resistance. This is in accord with the few phenotypes to date that have been associated with lncRNA expression, a number of which taken

together may represent metabolic syndrome due to their association with BMI, systolic blood pressure and dyslipidemia ((AP001999.1, IQCH-AS1), (AC093459.1, AC016831.6) and AC016831.6, respectively) (www.genecard.org). In addition, conditions that often reflect the metabolic syndrome such as type 2 diabetes (AC016831.6) and PCOS (LINC01828) are recognised (Sathishkumar et al. 2018)(www.genecard.org) that have been associated with insulin resistance (Sathishkumar et al. 2018). However, it should be noted that although the PCOS women were not insulin resistant, there was a trend to higher fasting blood glucose ($p=0.06$). This may be due to diminished pancreatic beta cell compensation that has been noted as an early defect in glucose homeostasis in peripubertal first degree relatives of PCOS women(Torchen et al. 2014).

It should be noted that the majority of work defining the function of lncRNA has been in cancer with few studies focusing on metabolic disorders (Marchese et al. 2017), and most lncRNA that we know are physiologically transcribed have unknown function (Mongelli et al. 2019). Consequently, the Ingenuity Pathway Assessment tool that maps the function to the differing genes was ineffective at functional pathway construction in this instance as too few pathways have been currently elucidated.

It has been reported that lncRNA may relate to androgen status (Jin et al. 2018) and that lncRNA were associated with insulin resistance in PCOS (Lin et al. 2018), with lncRNA RP11-151A6.4 being associated with increased BMI and insulin resistance (Zhao et al. 2019). Here, we show that when normal weight PCOS subjects were compared to matched normal controls that these correlations with BMI, FAI, HOMA-IR and AMH were not seen, suggesting that BMI/obesity may be major contributing factors in their expression; indeed, in this study, only AC095350.1 correlated with age. This emphasises that weight specific studies with matched

cohorts need to be undertaken to account for the changes that may be purely attributable to obesity, that was highlighted by the changes in lncRNA found in obese subjects with PCOS from the same ethnic population (Butler et al. 2019).

This pilot study was limited by the small number of subjects and therefore a type 2 error cannot be excluded, but the study clearly showed the differential expression of lncRNA between PCOS and normal control women, and that they differ from studies of obese PCOS women. However, whether these lncRNA are a specific feature of PCOS, metabolic dysregulation or insulin resistance needs to be determined.

In conclusion, 29 serum lncRNA differed between the normal weight PCOS and control subjects, and the pattern of expression differed from obese PCOS/normal subjects from the same population. This distinction may be due to obesity related metabolic features and insulin resistance differences that need to be accounted for in future studies.

Declarations

Competing interests/Conflict of interest: No authors have any conflict of interest to declare.

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