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Assessment of the expression levels of two long non-coding RNAs, lnc-CYP11A1-1 and RP11573D15.8, in human aneuploid and euploid embryos

Benedict Marshall[1], Hakan Aytacoglu[1], Onder Coban[2] and Pinar Tulay[1,3,4]

[1]Near East University, Faculty of Medicine, Department of Medical Genetics, Nicosia, Cyprus; [2]British Cyprus IVF Hospital, Embryology Lab, Nicosia, Cyprus; [3]Near East University, DESAM Research Institute, Nicosia, Cyprus and [4]Near East University, Center of Excellence, Genetics and Cancer Diagnosis-Research Center, Nicosia, Cyprus

Research Article

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Corresponding author:
Pinar Tulay; Email: pinar.tulay@neu.edu.tr

Summary

Introduction: Long non-coding RNAs (lncRNAs) are a subset of RNA molecules that have been shown to be involved in gene regulation. A lot of different pathways are involved during gametogenesis and any disturbance to these pathways may have a derogatory impact on producing a haploid gamete and thus a euploid embryo. Steroidogenesis pathway plays a crucial role in gametogenesis. The purpose of this work was to quantify the levels of lnc-CYP11A1-1 and RP11573D15.8 expression levels in aneuploid and euploid embryos. Materials and methods: A total of 20 surplus human embryos, of which 10 euploid and ten aneuploid embryos, were collected from an IVF centre. The expression levels of two lncRNAs, which have been hypothesized to regulate expression of *CYP11A1,* were evaluated in these embryos. RNA was extracted and used to synthesize cDNA for the experiments. Real-time polymerase chain reaction was performed to evaluate the expression levels of each lncRNA in aneuploid and euploid embryos, respectively. Results and discussion: This study shows that lnc-CYP11A1-1 was more expressed in aneuploid than in euploid embryos. RP11-573D15.8 is expressed more in aneuploid embryos than in euploid ones. The results for RP11-573D15.8 were statistically significant with a *p*-value of 0.02 (less than the standard threshold of *p* 0.05), whereas the results for lnc-CYP11A1-1 were not statistically significant with a *p*-value of 0.07 (greater than the standard threshold of *p* 0.05). Thus, the result of this study demonstrates that lncRNAs may have a role in gametogenesis and formation of aneuploid gametes.

Introduction

Long non-coding RNAs (lncRNAs) are a subset of RNA molecules that are distinct from those that code for proteins because of their length (usually greater than 200 nucleotides). The extraordinary properties of lncRNAs stem from their capacity to interact with other RNA, proteins and DNA. As a result, they can alter chromatin organizations, chromatin function and the transcription of nearby and far-off genes. RNA splicing, translation and stability are all a part of this. LncRNAs are involved in other physiological processes, such as satiety modulation (Alessio *et al.*, 2020). LncRNAs are produced by the transcription process of RNA polymerase 2 and have a 3′ poly A tail and a 5′ methyl-cytosine cap (Beaudoin *et al.*, 1997). Where they were first created in the genome affects the characteristics that classify them as sense, anti-sense, bi-directional, intronic, or intergenic (Dong *et al.*, 2019). These lncRNAs are sub-divided into three distinct groups based on their cellular location; nucleus, cytoplasm and mitochondria. Others may have special characteristics, such as being capped or polyadenylated because they were produced in a different way, such as by splicing. Ribonuclease P can snip off their 3′ ends, creating a circular shape that protects them from degradation (Gourvest *et al.*, 2019).

The expression level of the CYP11A1 gene can be affected by non-coding RNAs (ncRNAs) that target it if a similar sequence forms a competitive relationship with them (Lanzafame *et al.*, 2018). LncRNAs that are competitive have sequences that are highly similar to those of the genes they inhibit. By doing so, they can successfully counteract the regulatory effects of ncRNAs on the gene of interest by forming a connection with ncRNAs that target the gene in question (Lü *et al.*, 2015). Furthermore, lncRNAs play crucial roles in sperm production and the process of nuclear envelope maturation. NLC1-C (LINC00162) is a long non-coding RNA that has been identified as a potential gene for narcolepsy. This gene is expressed in both spermatogonia and maturing sperm. Reduced cytoplasmic expression and a greater accumulation in the central region of testicular cells have been associated with infertility. NLC1-C interacts with the microRNAs that are miR-320a and miR-383 in a physiological feedback loop that occurs during spermatogenesis. Hypothesized to bind specifically to nucleolin, a target of both miR-320a and

Table 1. List of primer sequences

Gene ID (Gene name)	Transcript	Forward primer	Reverse primer
ENSG00000277749 (lnc-CYP11A1-1; RP11-60L3.6	ENST00000607453	CATGACTCCTTGGTATTGG	AGAGTGGTGTTGTGAATGAC
ENSG00000197099 (RP11573D15.8)	ENST00000627551	TGATCATCCAGGAAGCCAACC	GAAGCCACTAAGACGGTGAGT

miR-383, NLC1-C regulates miRNA production in spermatozoa and primary spermatocyte nuclei. This interaction leads to an increase in germ cell proliferation, which is one factor in infertility among men (Seim *et al.*, 2007).

The purpose of this work was to quantify the levels of CYP11A1 and RP11573D15.8 expression levels in aneuploid and euploid embryos. Additionally, since CYP11A1 is a gene involved in the steroidogenesis pathway, the ultimate goal is to determine if these lncRNAs are involved in CYP11A1 regulation in an aneuploidy situation.

Materials and methods

Ethical approval was granted by Near East University Institutional Review Board (YDU/2021/96-1432) and the samples were collected from patients who consented to research. The surplus human embryos from patients at British IVF Center in the Turkish Republic of Northern Cyprus were collected. Twenty human embryos were collected. One sample had to be excluded from the final analysis, thus the aneuploid group included nine samples, while the euploid or control group included ten samples. Two distinct lncRNAs, lnc-CYP11A1-1 and RP11573D15.8, which may hold regulatory activities on the CYP11A1 gene, were studied by analysing the expression levels in the human embryos.

RNA extraction, cDNA synthesis and real-time polymerase chain reaction (PCR).

A total of 10 euploid embryos and nine aneuploid embryos were used in the study.

To isolate RNA, Total Nucleic Acid Extraction Kit (catalogue number MG-TNA-01-10; Hibrigen; Turkey) was used. ThermoFisher Professional High-capacity cDNA Reverse Transcription Kit (Catalogue No. 4368814) was used to synthesize cDNA after RNA isolation. For each lncRNA, real-time PCR with forward and reverse primer pairs designed specifically for that lncRNA was used for the amplification process. The individual lncRNA primer sequences have been compiled in Table 1. The primers were designed not to amplify off-target sequences, as confirmed by the primer-blast tool search. In particular, abundant mRNA present in the total RNA isolate was disconcerting, however, for the primer sets designed in Table 1, National Center for Biotechnology Information (NCBI) primer blast tool identified no amplicons for known Homo Sapiens cDNA sequences.

Real-time PCR analysis of cDNA samples was used to measure expression levels of target lncRNAs. The Hibrigen 2x the SYBR Green qPCR Master Mix (Hibrigen, Turkey; cat. no. MG-SYBR-01-400) was used for our real-time PCR experiments. Table 2 details the optimal PCR settings for lnc-CYP11A1-1, RP11573D15.8 and *ACTB*, which were used as a housekeeping gene, respectively.

Relative expression levels were calculated using the Ct values, which compared the activity of a target gene to that of a housekeeping gene *via* the delta-delta Ct method. Ct values were calculated using the Insta Q96TM Instantaneous Equipment.

Table 2. Optimized real-time polymerase chain reaction conditions for lncRNAs analyzed

lnc-CYP11A1-1	Temperature C°/time (second)	Cycles
Initial Denaturation	95/10 min	1
Denaturing	95/10 sec	40
Annealing	59/15 sec	
Elongation	72/30 sec	
High Resolution Melting analysis		1
RP11573D15.8	Temperature C°/time (second)	Cycles
Initial Denaturation	95/10 min	1
Denaturing	95/10 sec	40
Annealing	64/15 sec	
Elongation	72/30 sec	
High Resolution Melting analysis		1

GraphPad software was used to conduct a Student's *t*-test analysis, which was then used to establish statistical significance.

Results

Successful RNA extraction was obtained from all the samples that was followed by successful cDNA synthesis. From each cDNA sample, real-time PCR was performed to evaluate the level of gene expression that was run in duplicate. Table 2 shows the optimized PCR conditions. *ACTB* was used as a housekeeping gene and the normalization was performed according to *ACTB* gene. Each real-time PCR run was performed in the presence of no cDNA as a negative control.

Relative expression levels for lnc-CYP11A1-1 were found to be slightly elevated in aneuploid embryos than in euploid ones as can be observed in Fig. 1. However, the p-value for lnc-CYP11A1-1 was 0.07, higher than the cut-off ($p < 0.05$), indicating that the data were not statistically significant. In contrast, aneuploid embryos showed a significant elevation in relative RP11-573D15.8 lncRNA expression levels that can be observed in Fig. 2 ($p = 0.02$).

We also compared the levels of lnc-CYP11A1-1 and RP11573D15.8 expression among the same sample pool against each other. Averaged *ACTB* Ct values for lnc-CYP11A1-1 and RP11573D15.8 were similar, 31.15 for lnc-CYP11A1-1 and 31.2 for RP11573D15.8 respectively. On the other hand, Ct values for RP11573D15.8 spanned a greater range compared to lnc-CYP11A1-1. For lnc-CYP11A1-1, gene of interest Cts ranged between 29.10 and 31.60 whereas for RP11573D15.8 this margin was between 26.0 and 30.8. While Ct values for lnc-CYP11A1-1 showed a tighter grouping, $2^{-\Delta\Delta Ct}$ values presented a much higher

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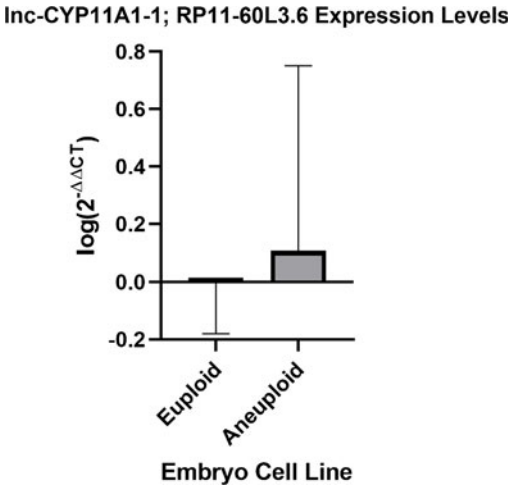

Figure 1. Relative expression levels for lnc-CYP11A1-1.
Aneuploid embryos show slightly increased levels of expression compared to their euploid counterparts, but this constitutes a statistically insignificant difference ($p < 0.05$).

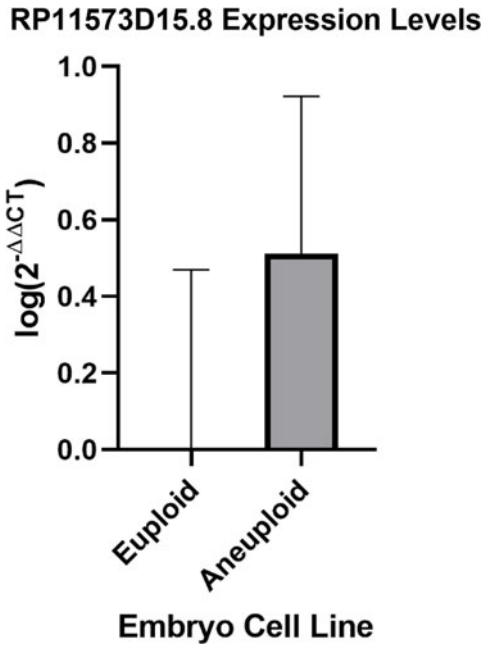

Figure 2. Relative expression levels for RP11573D15.8.
Aneuploid embryos show an obvious increase in RP11573D15.8 lncRNA expression compared to euploid embryos. The difference was found to be statistically significant in student's *t*-test analysis $p < 0.05$.

standard deviation compared to RP11573D15.8 (SD = 27.41 for lnc-CYP11A1-1 and SD = 1.67 for RP11573D15.8).

Discussion

LncRNAs serve a crucial function in genomic circuits and are expressed throughout embryonic development. There is a growing body of evidence in the disciplines of development, cell biology and illness that emphasizes the importance of studying lncRNAs. It has been increasingly apparent in recent years that the regulatory functions of lncRNAs in gene expression are critical to a vast array of biological processes, such as cardiovascular disease, cancer and neurodegenerative disorders. Therefore, lncRNAs may play a significant role in both aneuploid and euploid embryo development by controlling gene expression levels. Although the expression of lncRNAs has been studied in general, no specific investigations have been conducted on human embryos, especially comparing the aneuploid and euploid embryos. This is the first study on lncRNA expression in the context of aneuploidy and euploidy in the embryonic development.

In our previous study, we showed that CYP11A1 expression may vary in different settings, such as in oocytes obtained from patients with polycystic ovaries. Thus, we have hypothesized that it is a possibility that this gene may have different expression patterns in abnormal embryos too. Thus, *lnc-CYP11A1* and *RP11573D15.8*, which were shown to regulate CYP11A1 gene expression levels, are the two lncRNAs investigated in this study.

The results of this study showed that the expression levels in *RP11573D15.8* samples were statistically significant and showed a difference compared to the expression levels in *CYP11A1* samples. It is very thoughtful when pondering on the statistical difference of *CYP11A1* expression levels in aneuploid embryos and why it is non-significant statistically. A previously published study showed that CYP11A1 affects the genetic pathway of trophoblast differentiation when up-regulated (Xiang *et al.*, 2020). Steroidogenesis can theoretically change and affect the behaviour of trophoblast metabolically as well as the mitochondrial function when the expression levels of CYP11A1 are up-regulated in the placental trophoblast. This also leads to the result of impaired pregnancy which is due to drugs, hormones, inflammation and toxins which form part of the intrauterine environment and are various factors that aid in the regulation of CYP11A1 expression in the cells of trophoblast (Zhang *et al.*, 2019). Furthermore, lncRNAs are known to have a significant effect on mRNA stability and gene expression, while their precise involvement in aneuploid-euploid embryos is still being explored.

One of the drawbacks of this study is the relatively small sample size. This can account for the large standard deviation observed in the lnc-CYP11A1-1 dataset, which in turn may have contributed to those results being statistically insignificant. However, obtaining human embryos proves to be difficult for research studies. Thus, to date, smaller sample sizes have been used for human oocyte and embryo studies.

To our knowledge, no studies have examined lncRNA expression in the setting of aneuploid and euploid human embryos. LncRNAs are known to have a significant effect on mRNA stability and gene expression, while their precise involvement in aneuploidy is still being explored. The findings provide preliminary evidence that lncRNAs may have a role in the occurrence of aneuploidy and as such, can form the basis for future studies.

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