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Correlations of Genomic Localization of Pituitary-Hypothalamic-Gonadal Axis Genes with Reproductive Phenotype Manifestation in Mammalians

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Introduction

Mammals exhibit great variety in morphology, physiology, and reproduction. Validated variations in qualitative and quantitative criteria of reproductive phenotypic traits were observed within Mammalia class. The quantity of follicles ovulating in one reproductive cycle is considered to be one of the most essential reproductive characteristics [1]. Moreover, in each of the 4800 species comprising Mammalia class, rate of oocyte maturation and litter size are evolutionary conservative traits. For example, *Proboscidea* and Primates normally give birth to no more than 1-2 offspring, while the mean litter size of *Rodentia* is at least nine [1,2]. Another phenotypic reproductive characteristics for which variable values were observed in different mammalian species include female sexual maturity (days), male sexual maturity (days), gestational period (days), *mother feeding period* (days), number of litters per year, interval between births (days) and newborns birth weight. Nevertheless folliculogenesis and reproductive cycles are still the most important and well-studied of all these traits. These two properties are known to be regulated by complex genetic programs with an extensive pool of genes. Global transcriptome profiling and gene expression dynamics of oocytes during folliculogenesis showed that 10 genes are mainly responsible for folliculogenesis and reproductive cycles regulation in humans [1]. Nucleotide sequences of these protein-coding genes are mostly evolutionarily conserved within Mammalia classes. This can be explained from the evolutionary point of view by the fact that chromosomal location of a gene is less conservative than coding sequence of orthologous genes related with systemic regulation [3]. Accordingly, regulatory genes are conservative in all mammalian species, the observed phenotypic variability in reproductive traits is predicted to be a result of independent coding genome regulatory mechanisms and factors. On the other hand, gene expression is not only regulated by epigenetic factors [4,5], but it is also affected by the positions of their genes on the chromosome [4]. Gene proximity to regulatory elements (promoters) is known to be a genetic expression regulatory factor, chromatin loops between regulatory elements and the gene being regulatory mechanism [6]. However, the regulatory effect of gene proximity to telomeric elements has not been discovered until recent years (telomere position effect (TPE). It has been shown that gene proximity to the closest telomere affects genetic expression and, consequently, the related phenotype [7,1]. Positional effect was studied for genetic distances between some of somatotropic axis genes, and their surrounding conservative regulatory elements [8].These distances were shown to be significantly correlated with mammals body mass and lifespan. Moreover, genomic distances to the nearest telomeres for somatotropic axis genes *GHRH* and *SST* along with *PLAG1* were also shown to be correlated with age of maturity and lifespan of mammals. In accordance with somatotropic axis genes, genes that regulate folliculogenesis and reproductive cycles in mammals could be suggested to have this mechanism of genotype-phenotype regulation.

Investigating possible associations of distances to telomeric elements for genes that regulate folliculogenesis and reproductive cycles with genetic expression and phenotype looks very promising especially because recent studies, searching evolutionarily pathways regulating genetic programs showed the effect of genomic characteristics (length of neural genes) on life expectancy and physiological time (duration of certain physiological processes) [9].

Since previous studies confirmed significant associations of genomic distances to regulatory sequences with phenotypic reproductive traits (gestational period, mother feeding period and puberty age in males and females), this study aims to investigate the possible correlations between genomic distances from the gene to the nearest telomere and those traits, find the

possible correlations of all targeted genes distances to telomere and next (according to the resulted significant correlations) analyze the chromosomal position of correlated genes in different species.

Material and Methods

Selection of targeted genes and species

The next 10 genes regulating folliculogenesis, ovulation and early embryogenesis in human were selected for the study: *Cga*, *Fshb*, *Fshr*, *Gfra1*, *Igf2*, *Lhcgr*, *Nrtk2*, *Prkca*, *Pten* and *Tsc1* (Table1). Species representing Mammalia class were obtained from AnAge database [10].

Genes orthologs of all targeted genes were taken from NCBI Gene, gene data being available for 71 Mammalia species (Appendix table A_1).

Bioinformatic analysis of databases for relative genomic distances calculation

Python 3.8 was used to calculate the genomic distance of every targeted gene to the nearest telomere with the help of the following libraries: Biopython, pandas, Matplotlib and NumPy.

Gene data were obtained with the help of NCBI Entrez API Then for each targeted gene, the following characteristics were determined: reading frame start and end points, chromosomal position, orientation of the nucleotide sequence, chromosome length, and the chromosomal ID. To standardize all the studied sequences, genetic orientation was adjusted: (+) - oriented sequences were left unchanged, while (-) - oriented were reoriented to match (+) - orientation.

Gene length was detected in base pairs. To obtain universal values and facilitate data processing we aimed to detect relative genomic distance to the closest telomere through dividing each absolute distance by the corresponding to gene and species chromosome length. Relative chromosomal position was calculated according to the following formula:

$$Absolute genomic distance the closest chromosomal end (bases) \frac{\square}{Total chromosomal length (ba}$$

Species genetic data were taken from AnAge database. The next characteristics were investigated: female and male average maturity (days), gestation period (days), mother feeding period (days), average litter size, number of litters per year, birth interval (days) and newborns birth weight.

Statistical analysis

Selected species were divided into two subgroups according to maturing follicles number as follows. First group included 43 species with no more than 2 dominant mature follicles in every reproductive cycle (average litter size ≤ 2 springs). Species in which average litter size (and, therefore, number of mature follicles in each reproductive cycle) was strictly more than 2 were included in the second group consisting of 28 species. Correlation analysis was carried out in each of the studied groups to search for dependencies between the relative distances from the gene to the telomere and reproductive phenotypic traits (phenotype-genotype correlations) and between the relative genomic distances of the selected genes (genotype-genotype correlations).

Statistical analysis was conducted with the help of the Python Matplotlib library. This library was also used to render correlation plots and chromosomal loci. This study considered correlation significant only if $p<0.05$ after Bonferroni correction of multiple comparisons.

Due to samples' distribution not passing normality criteria, Spearman's rank correlation coefficient (ρ) was used instead of Pearson's correlation coefficient [11]. Correlation was considered weak if ρ=0.1-0.3, average if ρ=0.4-0.6 and strong if ρ≥0.7. Significant correlations with ρ values less than 0.4 (weak correlations) were not analyzed during this study.

Excluding artifacts

To exclude artifacts such as the possibility of one chromosomal pattern for all studied genes and/or other unconsidered directional factors that may affect figured correlations, same bioinformatic analysis of this study was also conducted on a random pool of control genes unrelated to reproductive regulation including: *E2f3, Ezh2, Gpc3, Mdk, Mycn, Peg3, Plagl1, Sirt6 and Smo* [8]. These genes relative distances to telomeres were analyzed for investigating their correlation with reproductive phenotypic traits.

Exploratory data analysis was applied to all control genes. Then, for those correlations where Pearson's correlation coefficient was over 0.4, a refinement analysis was performed for reliability parameters (significance coefficient *p*).

Results

Selected targeted genes with their role in folliculogenesis regulation are presented in Table 1. Relative genomic distance to the nearest telomere correlation with phenotypic reproductive traits was investigated in two studied groups. It should be mentioned that exploratory data analysis of all studied characteristics was carried out; therefore, we investigated both phenotype-genotype and genotype-genotype correlations. For each gene, we calculated relative distance to the closest telomere, then obtained data was used in correlation analysis.

Correlations with phenotypic reproductive traits of relative genomic distance to the nearest telomere (phenotype-genotype correlations)

Calculated relative distances for all target genes to the closest telomere are listed in Appendix Table $A_{1.}$ The relative distance to chromosomal end of *Prkca* showed a significant correlation with two reproductive traits, but only in the first comparison group (43 species, ≤2 dominant follicles). According to our data, *Prkca* relative genomic distance has a significant negative correlation with gestational period and newborns birth weight with statistical values (ρ = -0.52; *p* = 0.677 * 10-4) (Figure 1a) (ρ = -0.55; $p = 0.435 * 10^{-4}$) (Figure 1b), respectively.

Correlation of relative genomic distances to the nearest telomere between genes (genotype-genotype correlations)

Pairwise comparison of genome distances from the genes to the nearest telomere revealed the next significant correlations (see Figure 2): *Fshr* and *Lhcgr* values are strongly correlated (ρ = 0.98; $p = 1.545 * 10^{-30}$); *Cga* and *Lhcgr* values are moderately correlated (ρ = 0.55; $p = 1.54 * 10^{-5}$), as well as *Cga* and *Gfra1* (ρ = - 0.58; $p = 4.54 * 10^{-5}$). *Cga* and *Fshr* (ρ = 0.55; $p = 1.27 * 10^{-4}$). It should be noted that correlations of *Cga* to *Lhcgr* and *Cga* to *Fshr* relative telomere distance values were observed only in the first studied group (43 species, 2 or less dominant follicles).

Correlation of *Fshr/Lhcgr* relative genomic distances to the nearest telomere

We believe that three factors give *Fshr* and *Lhcgr* correlation a special importance. First, this correlation was observed in all the studied groups. Second, it is a positive strong correlation, and third important factor is that both genes are localized in the short arm of chromosome 2 in humans [12]. Therefore, visualization of both *Fshr* and *Lhcgr* chromosomal location was plotted in Python 3.8 for more detailed investigation of their correlation. Chromosomal lengths in some of the targeted species is shown in figure 3, while figure 4 represents the relative chromosomal position of *Fshr* and *Lhcgr*.

No significant correlations were found in control genes which excludes possible artifacts

Exploratory data analysis did not show significant correlations between any of the control genes and reproductive traits (see Figure 5 for correlation matrix). A positive correlation between *Mdk* position and litters per year was observed, but then refinement analysis showed that this correlation has no significance ($\rho = 0.41$; $p > 0.05$). This excludes possible presence of artifacts affecting study results.

Discussion

The regulatory effect of genetic chromosomal position is with high importance since it affects genetic expression and thus different phenotypic traits. Chromosomal clusters, telomeres loop structures [13] and chromosomal architecture (three-dimensional position of genes) have also revealed this regulatory effect [14]. This study represents a bioinformatic analysis investigating significant correlations between genetic distances to the nearest telomere and reproductive phenotypic traits. We have found significant strong correlations, at genotype- phenotype level and genotype-genotype level, which can be explained by previously suggested mechanisms of telomeric regulation effect though the exact mechanism is not fully understood. For example, genomic distance to telomere and telomeric length itself can change the intensity of gene expression and in some cases cause gene silencing. Furthermore, telomeres recently were observed in a form of loops, complementary to inner genomic regions of some genes located several mega bases far from chromosomal ends. Thus, telomeric loops can change gene expression, probably affecting phenotype in some cases [7,15].

These results are consistent with our earlier data, in which chromosomal localization of genes, related to regulation of mammalian reproductive cycles, showed a significant effect on reproductive processes regulation [8]. To our knowledge, this is the first study investigating association of genes chromosomal position to telomeres with reproductive traits. However, structural features of chromatin, as well as the presence of chromatin loops and chromosomal architecture, were not considered during data analysis.

Of our 10 targeted genes, *Prkca* relative genomic distances to the nearest telomere proved a significant but moderate correlation with both gestational period ($\rho = -0.52$; $p = 6.77 * 10^{-5}$) and newborn weight ($\rho = -0.55$; $p = 4,35 * 10^{-5}$).

Prkca encodes threonine selective protein kinase alpha which plays a regulatory role in ovulation, embryo implantation and embryogenesis. Previous studies have found several single-nucleotide polymorphisms (SNPs) in this gene, which are associated with spontaneous premature birth and miscarriage [16]. In addition, this gene is known to be involved in chromatin modification, carries out post-translational modifications and plays a significant role in the

function of methyltransferase 1 [17]. Thus, it could be suggested that correlation of *Prkca* value with gestational period and birth weight is a result of its epigenetic intervention.

Analysis *Prkca* genetic characteristics confirmed that this locus is pleiotropic and thus associated with several traits and diseases such as body mass index, asthma, multiple sclerosis, schizophrenia, and cancer. *Prkca* demonstrates a complex variant of alternative splicing and micro-RNA expression regulation. It forms two stable alternative transcripts and miR-634 is capable of specifically suppressing shorter alternative-polyadenylated isoforms [18]. Recently, another micro-RNA, let-7g-5p, was shown to inhibit *Prkca* genetic expression and thus inhibits mammary epithelial cells differentiation [19]. Therefore, considering our data and all these characteristics, additional clinical trials are necessary to obtain a more detailed understanding of the chromatin state of *Prkca* and the closest telomeric region.

Analysis also concerned correlation between targeted genes relative distances to the nearest telomere. The strongest positive correlation observed in all studied species was found between the relative genomic distances from *Lhcgr* and *Fshr* genes to the nearest telomere ($\rho = 0.96$; $p < 0.05$).

Lhcgr and *Fshr* encode the receptors of Luteinizing Hormone (LH) and Follicles Stimulating Hormone (FSH), respectively and thus play a key role in follicular maturation, embryogenesis, and childbirth [17]. Those genes encode similar peptides, qualitatively and quantitatively. In addition, they both have conservative coding sequences located on the same chromosome in all the representatives of each mammalian class with a proved linkage disequilibrium of them [20-22].

Plunkett et al. [23] hypothesized that the fetal size problem in humans is evolutionarily solved by gestation period reduction compared to other mammals. Accelerated evolution of coding and regulatory regions of genes involved in birth regulation along phylogenetic lines of humans and / or higher primates causes gestation period reduction and facilitates smaller fetus delivery that will more easily pass through the birth canal, which minimizes maternal and fetal delivery associated complications.

Regulatory of reproductive processes genes, which showed higher degree of divergence in humans than primates included *Fshr* and *Lhcgr*. Accordingly, multiple SNPs in this gene were found to be associated with gestational period regulation and such pathologies as preterm labor [24, 25]. More than 88% stillbirth-associated pregnancies showed very low *Lhcgr* levels (≤ 5 pmol /ml) compared to the control group [26].

We can conclude that *Fshr* and *Lhcgr* genomic distances are correlated in all studied species, and in the most studied species these genes are closely located on a mutual chromosome, but their distances to the nearest telomere were not found to be correlated to reproductive traits. However, expression levels of these genes have significant association with gestational period and birth weight.

Another significant correlation revealed in this study is between *Cga* and telomere, which encodes Glycoprotein Hormones alpha subunit, and *Lhcgr*, *Fshr*, *Gfra1* genes. Such correlations are understandable, since chorionic gonadotropin (CG), FSH and LH have the same alpha subunit. Both CG and LH bind to common receptors encoded by the *Lhcgr* gene [27]. Because *Cga* and *Lhcgr* encode a receptor and its alpha subunit, respectively, positive correlation *Cga* /

Lhcgr ($\rho = 0.55$; $p = 1.54 * 10^{-5}$) found in this study can be considered to be a functional marker of these receptors. Same explanation can be used for *Cga* / *Fshr* ($\rho = 0.55$; $p = 1.27 * 10^{-4}$) correlation since LH/CG receptors belong to the glycoprotein-G-protein coupled receptors family (GPCRs). In addition, the alpha subunit of chorionic gonadotropin is structurally similar to the respective subunit in follicle-stimulating hormone [28].

On the other hand, we found significant negative correlation *Cga / Gfra1* ($\rho = - 0.58$; $p = 4.54 * 10^{-5}$) which is hard to explain. GFRA1 protein belongs to the neurotrophic factor (NTF) family including glial cell line neurotrophic factor (GDNF) ligand family. These NTF members serve as key regulators of neuronal ontogenesis, proliferation of neural cells, their differentiation, migration, and plasticity of synapses [29,30]. In oogenesis, GDNF protein is involved in follicular proliferation regulation [1], but so far there were no specific mechanisms clarifying the figured correlation.

Conclusion

This study has investigated the correlation of genomic distances to the nearest telomere with different reproductive traits for 10 genes regulating folliculogenesis. Significant correlations were found between *Prkca* relative distance to telomere and gestational period / newborn weight in mammals. Moreover, We have found a strong positive correlation between the distances of two genes *Lhcgr* and *Fshr* to the nearest telomere. These two loci are located on the same chromosome and are in linkage disequilibrium in all species of each mammalian class, including Homo sapiens.

The three previously mentioned genes (*Prkca*, *Lhcgr* and *Fshr*) play a critical role in gestational age regulation. Therefore, further studies of both coding and non-coding sequences that regulate their expression are necessary to determine their functional role in reproduction physiological regulation and/or pathologies (premature birth, stillbirth and fetal growth restriction, for example). Our data will help to better understand folliculogenesis and reproductive cycles regulation mechanisms in different representatives of Mammalia, including humans.

Declaration of Conflicting Interests

The authors have no competing interests to declare that are relevant to the content of this article.

Ethics approval

This is an observational study. The institutional research committee has confirmed that no ethical approval is required.

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