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Integrated analysis of noncoding RNAs and mRNAs reveals their potential roles in the biological activities of the growth hormone receptor

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ABSTRACT

Accumulating evidence has indicated that noncoding RNAs (ncRNAs) have important regulatory potential in various biological processes. The molecular mechanisms by which growth hormone receptor (GHR) deficiency protects against age-related pathologies, reduces the incidence and delays the occurrence of fatal neoplasms are unclear. The aim of this study was to investigate miRNA, lncRNA and mRNA expression profiles and the potential functional roles of these RNA molecules in GHR knockout (GHR-KO) mice. Microarray expression profiles of miRNAs, lncRNAs and mRNAs were determined in wild type control mice and in GHR-KO mice. Differential expression, pathway and gene network analyses were developed to identify the possible biological roles of functional RNA molecules. Compared to wild type control mice, 1695 lncRNAs, 914 mRNAs and 9 miRNAs were upregulated and 1747 lncRNAs, 786 mRNAs and 21 miRNAs were downregulated in female GHR-KO mice. Moreover, 1265 lncRNAs, 724 mRNAs and 41 miRNAs were upregulated and 1377 lncRNAs, 765 mRNAs and 16 miRNAs were downregulated in male GHR-KO mice compared to wild type mice. Co-expression analysis of mRNAs, lncRNAs, and miRNAs showed that mRNAs including Hemxi2, Ero1lb, 4933434i20RIK, Pde7a and Lgals1, lncRNAs including ASMM9PARTA014848, EL605414-P1, ASMM9PARTA051724, ASMM9PARTA045378 and ASMM9PARTA049185, and miRNAs including miR-188-3p, miR-690, miR-709 and miR-710 are situated at the core position of a three-dimensional lncRNA–mRNA–miRNA regulatory network. KEGG analysis showed that the most significantly regulated pathway was steroid hormone biosynthesis. We identified a set of lncRNAs, miRNAs and mRNAs that were aberrantly expressed in GHR-KO mice. Our results provide a foundation and an expansive view of the biological activities of the GHR.

1. Introduction

Growth hormone (GH) is a major regulator of body growth and metabolism, particularly in the liver and adipose tissue, as well as the immune, reproductive and cardiovascular systems and the brain [1]. GH exerts its actions by binding to the membranous GH receptor (GHR). This interaction consequently activates the receptor-associated tyrosine kinase JAK2, which subsequently phosphorylates diverse signaling mediators [2]. Individuals with GHR deficiency (GHRD) exhibit dwarfism in association with low serum IGF-1 levels despite elevated GH levels; thus, they are insensitive or resistant to GH. Despite their obese phenotype, mice with GHRD are extremely sensitive to insulin and display decreased levels of insulin and low to normal levels of glucose [3]. Typically, one associates obesity with insulin resistance. Remarkably, one of the most noteworthy traits of mice with GHRD is that they have greatly extended life spans. Furthermore, GHRD is associated with a major reduction in pro-aging signaling, cancer and diabetes in humans [4]. However, the molecular mechanisms responsible for lifespan extension and cancer and diabetes resistance in GHRD individuals are not fully understood [5].

Increasing evidence has indicated that noncoding RNAs (ncRNAs) have important regulatory potential in various biological processes [6]. The most studied ncRNAs are microRNAs (miRNAs), which are molecules typically ~22 nt in length that negatively regulate target messenger RNAs (mRNAs) by inducing mRNA degradation or translational inhibition [7]. Accumulating evidence shows that miRNAs regulate diverse biological processes, including cell differentiation, proliferation, and apoptosis, and that aberrant expression of miRNAs may lead to severe diseases [7,8]. Long ncRNAs (lncRNAs), which are defined as ncRNAs ranging from 200 nt to ~100 kb in length, have become an area of increased research focus [6]. lncRNAs may function as guides, scaffolds, and decoys; thus, lncRNAs have the potential to regulate gene expression and spatial localization within the cell [8]. lncRNAs have been shown to be involved in chromosome dosage compensation, imprinting, epigenetic regulation, cell cycle control, nuclear and cytoplasmic trafficking, transcription, translation, alternative splicing, and cell differentiation [9].

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Table 1
The mRNA and lncRNA specific primer sequences used in this study.

Item	Forward	Reverse
mRNA		
Hexim2	5′-TGAGGTGGAGAGGCTCAGAA-3′	5′-GTGGGTCTGAAATGGGGCTT-3′
Ero1lb	5′-TGGAGTTCTGGATGATTGCTT-3′	5′-TCTTCTGCCCAGAAAGGACA-3′
Cyp3a16	5′-GCACCGCGTGGACTTTATTT-3′	5′-CAGTTATGACTGCATCCCGTG-3′
Pde7a	5′-GAGCCATCAGCTTCAGCTCC-3′	5′-TTCAAATCCTGCTCGGCTCC-3′
Lgals1	5′-AGCAACAACCTGTGCCTACA-3′	5′-TCTGGCAGCTTGATGGTCAG-3′
Akr1c18	5′-TCCCATCGTCCAGAGTTGGT-3′	5′-TCATTCCCTGGCTTCAGAGAC-3′
Cyp2c40	5′-TTGGGCAGTGCCTTACCAAT-3′	5′-CCACAATAGGCTGTGAGCCA-3′
Cyp2c68	5′-ATGGCCCTGTGTTCACTCTG-3′	5′-ATGGCCCTGTGTTCACTCTG-3′
Hsd17b2	5′-CACGCTTCTCTGCGGATGC-3′	5′-GCCATTCGCCCTGGGTAATA-3′
Hsd3b5	5′-TCAGCGCGATCTAGGCTATC-3′	5′-ACTTTTTGTGTAGTGTCTCCCTG-3′
Ugt2b38	5′-GCGCCACAAAAGGGCTAAG-3′	5′-AACACAAGAGAGTAGGAAGCCG-3′
Cyp2b10	5′-TTAGCCAGGGGACACCCAAA-3′	5′-ACCAGAGCCTCCCTTATGGT-3′
Cyp2c50	5′-ATGGAAAAACACAAGGCGCTTC-3′	5′-GCAACCAAGGGGTGCTCATT-3′
lncRNA		
ASMM9PARTA014848	5′-CCTCGGAAGCACAGACAGAA-3′	5′-TCACAGCATGCGAGGGTTG-3′
EL605414_P1	5′-GGTCAAAGCCGCAGGTAAGA-3′	5′-GTGGGTATGAACACGTTGCC-3′
ASMM9PARTA051724	5′-GAGGGGAGCTGCAGAGATTC-3′	5′-TCAGAGTGATGGTGCTTCGC-3′
ASMM9PARTA045378	5′-TACATGGCTGCGAGGGAGAA-3′	5′-ATTAGGACCCACTGCTGGGA-3′
ASMM9PARTA049185	5′-TCAGAGCACACCTCCATTGC-3′	5′-CAGGCTCTCCAGTGGAAACA-3′

Given the genetic complexity of GHR function, it is possible that aberrantly expressed ncRNAs may be partially responsible for GHRD-mediated lifespan extension and cancer resistance. Therefore, in this study, we performed microarray-based genome-wide analysis of the expression of lncRNAs, miRNAs and mRNAs in mice with GHRD. Then, we developed a correlation matrix-based integrative analysis approach to analyze lncRNA, miRNA and mRNA co-expression. We further performed functional analysis of a series of miRNA–mRNA–lncRNA co-expression networks to determine the potential role of these networks in mice with GHRD. Our findings suggest that ncRNAs may play an important role in GHR function.

Table 2
The miRNA specific primer sequences used in this study.

miRNA	loop primer	Forward
mmu-miR-188-3p	5′-GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACTGCAAACC-3′	5′-TGCGCCTCCCACATGCAGGGT-3′
mmu-miR-690	5′-GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACTTTGGTTG-3′	5′-TGCGCAAAGGCTAGGCTCACAA-3′
mmu-miR-709	5′-GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACTCCTCCTG-3′	5′-TGCGCGGAGGCAGAGGCAG-3′
mmu-miR-710	5′-GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACCTCAACTC-3′	5′-TGCGCCCAAGTCTTGGGGAGAG-3′

2. Materials and methods

2.1. Ethics statement

All experiments were performed in accordance with the Chinese and institutional guidelines for the care and use of laboratory animals. This study was approved by the Institutional Ethical Review Committee in Zhongnan Hospital of Wuhan University.

2.2. Animal model

GHR-KO mice 8–10 weeks old and control littermates [C57/BL6, wild type (WT)] were kindly provided by Professor Wan Yu of the Medical School of Wuhan University. The GHR-KO mice are homozygous for a gene deletion of the receptor gene created by insertion of a neo cassette into exon four. Background is C57Bl/b5 [10]. The mice were maintained on a 12-h light, 12-h dark cycle with free access to food and water. Three male or female mice were used in each group (total $n = 6$ for GHR-KO; $n = 6$ for WT). All experiments were conducted in accordance with the Chinese and institutional guidelines for the care and use of laboratory animals. Livers were harvested in liquid nitrogen for RNA preparations.

2.3. Microarray analysis

Three pairs of homozygotic GHR-KO and WT mice were sacrificed. Their livers were immediately frozen in liquid nitrogen, and then, these samples were transported to KangChen Bio-tech (P.R. China) for lncRNA, miRNA and mRNA microarray analysis. Subsequently, the results were transferred to OEbiotech (China) for primary and advanced analyses.

Liver tissues were collected 8 weeks after sexual maturation. To avoid the influence of cell populations on the expression levels of RNAs, infarcted and surrounding scarred myocardium were removed. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, California, USA) according to the manufacture's instructions. Total RNA from each sample was quantified using the NanoDrop ND-1000 bioanalyzer, and RNA integrity was assessed using standard denaturing agarose gel electrophoresis. For microarray analysis, an Agilent array platform was employed. Sample preparation and microarray hybridization were performed according to the manufacturer's standard protocols with minor modifications. Briefly, mRNA was purified from total RNA after removal of rRNA (mRNA-ONLY™ Eukaryotic mRNA Isolation Kit, Epicentre). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3′ bias utilizing a random priming method. The labeled cRNAs were hybridized to the Mouse LncRNA Array v2.0 (8 × 60K, ArrayStar). After washing the slides, the arrays were scanned using the Agilent G2505C Scanner.

Agilent Feature Extraction (version 11.0.1.1) software was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies). After quantile normalization of the raw data, lncRNAs and mRNAs that were flagged as Present or Marginal ("All Targets Value") in at least 1 out of 4 samples were chosen for further data analysis. Differentially expressed lncRNAs/mRNAs were identified via fold-change filtering (fold-change >= 2.0).

For miRNA analysis, the samples were labeled using the miRCURY™ Hy3™/Hy5™ Power labeling kit and were hybridized to the miRCURY™ LNA Array (v.18.0). After washing, the slides were scanned using the Axon GenePix 4000B microarray scanner. Then, the scanned images were imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. Replicated miRNAs were averaged, and miRNAs displaying an intensity>= 30 in all samples were chosen for calculation of the normalization factor. The expression data were normalized to the median expression level. After normalization, differentially expressed miRNAs were identified via fold-change filtering. Finally, hierarchical