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Long Non-Coding RNAs Induce Fatty Liver during Developmental Stages in Laying Hen

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ABSTRACT

Fatty Liver Hemorrhagic Syndrome (FLHS) is common in poultry. Long non-coding RNAs (lncRNAs) regulate gene expression in a variety of ways at epigenetic, chromatin remodeling, transcriptional, and translational levels. Chicken liver produces lipoproteins and most of the precursors to egg yolk with the help of RNA such as MicroRNAs (miRNAs) and lncRNAs. In order to analyze lncRNAs in liver, RNA-seq data of six samples were downloaded from National Center for Biotechnology Information (NCBI) (3 birds with fatty livers from the paternal group and 3 control birds).Then, using the DESeq2 package, the difference in expression of lncRNAs in the samples was analyzed. Functional enrichment analysis was established by STRING and the PPI network visualized by Cytoscape. Annotation of the data was carried out by DAVID 6.8. The biological pathways were searched in Kyoto Encyclopedia of Genes and Genomes (KEGG). The results of the analysis of Differentially Expressed Genes (DEGs) showed that there were 24356 annotated genes. Also, 101 lncRNAs were found. Gene Ontology (GO) term enrichment analysis suggested that DEGs significantly enriched in metallocarboxypeptidase activity, protein ubiquitination, etc. KEGG pathway analysis showed that DEGs related with biosynthesis of antibiotics and biosynthesis of amino acids ($P< 0.05$). Examination of gene loci revealed that the expression process of *GCGR, PDK3* and *PCK1* genes was in line with the expression of neighboring lncRNAs. Examination of this number of lncRNAs along with their target genes can help in selecting laying hen lines with less chance of developing fatty livers.

Keywords: Laying hen, Lipoproteins and gene, ncRNA, Poultry, RNA-Seq, Transcriptome.

INTRODUCTION

Globally, Fatty Liver Hemorrhagic Syndrome (FLHS) is a serious disease for chickens. FLHS is associated with genetic, endocrine, environmental, nutritional, and toxicological factors (Yang *et al*., 2017). Lipid homeostasis is closely dependent on some hepatic metabolic pathways, including lipid absorption, β-oxidation, lipoprotein transport, and lipid synthesis, but the disorder of these pathways seems to lead to the liver is the main organ responsible for lipid synthesis in chickens (Tan *et al*., 2020). FLHS is more common in laying chickens. Fatty liver hemorrhagic syndrome is a metabolic disorder of chickens. The presenting complaint is typically sudden death of birds fed high-energy diets with limited exercise. Diagnosis is based on finding liver hemorrhage and fat engorgement at necropsy. The disorder can be controlled by monitoring feed intake and body weight when birds are in a positive energy balance. Changing the balance of carbohydrates and fat in the diet and supplementing with selenium may also help. A bird's liver is responsible for most lipogenesis, and dietary fat is transported directly to the liver by its vein, unlike

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mammals. In general, birds are expected to have higher hepatic lipid concentrations and greater microvacuolization (Trott *et al*., 2014). Moreover, the epigenome comprising different mechanisms e.g. DNA methylation, remodeling, histone tail modifications, chromatin microRNAs and long non-coding RNAs, interact with environmental factors like nutrition, pathogens, climate to influence the expression profile of genes and the emergence of specific phenotypes (Barazandeh *et al*., 2016; Masoudzadeh *et al*., 2020). Multi-level interactions between the genome, epigenome and environmental factors might occur (Mohammadabadi, 2019). The information obtained from the analysis of biological data by bioinformatics helps in aligning sequences in information banks to find gene similarities and differences (Mohammadinejad *et al*., 2022). There is an increasing evidence that long non-coding RNAs (lncRNAs) have key functions in the regulation of various biological processes, such as imprinting control, cell differentiation, development, immune response, cell cycle, and apoptosis (Song *et al*., 2014). Today, RNA molecule's function goes beyond being a messenger. Only approximately 1-2% of the RNA present in a human cell is protein coding, with the rest being non-coding RNA (nc RNAs) (Karapetyan *et al*., 2013). A series of lipoproteins and most of the minor yolk precursors are synthesized by the liver of laying hens. This process of gene expression and regulation often involves complex and highly orchestrated molecular programming, including RNAs such as miRNAs and long noncoding RNAs (Ning *et al*., 2020). A variety of mechanisms are used by lncRNAs to perform their functions, including the following: acting as scaffolds, decoys, signals, and guides. There is ample evidence that most mRNAs can code for proteins. Likewise, a growing number of publications demonstrate that lncRNAs play an important role in gene regulation. LncRNAs can activate or repress epigenetic processes as well as act as transcription factors (Karapetyan *et al*., 2013). In liver-enriched lncRNA samples, Liver-Specific Triglyceride Regulator (lncLSTR) was identified as a possible regulator of plasma triglycerides. Knockdown of LncLSTR in the liver increases ApoC2 expression and LPL activities, increasing plasma TG clearance and decreasing plasma TG levels in a long time. Nonetheless, knockdown of lncLSTR in primary hepatocytes does not result in increased ApoC2 expression, indicating the existence of another mediator within the liver. LncRNAs are known to affect lipid homeostasis by influencing liver lipid metabolism and adipogenesis. By binding to proteins or by base-pairing with RNA and DNA, lncRNAs control lipid metabolism-related gene expression (Chen, 2016). Recent evidences of lncRNAs and liver disease, fibrosis, and Hepatocellular Carcinoma (HCC) suggest that lncRNAs play an important role in the pathogenesis of liver diseases. H19 was the first known long noncoding RNA identified related to liver disease. Many others have been identified to be associated with liver disease (Sulaiman *et al*., 2019). Some lncRNAs have been associated with adipogenesis and energy metabolism (Chen *et al*., 2015). The reference article (Zhang *et al*., 2018) does not discuss non-coding RNA and gene networks associated with them.

In this work, we extend the search for analysis of long non-coding RNAs associated with fatty liver in chicken. The present study also investigated the genes network associated with lncRNAS. The results would offer new insight to select better chickens for breeding lines.

MATERIALS AND METHODS

Data Acquisition

To investigate lncRNAs associated with fatty liver in chicken, we used noncoding information of Chinese chicken's liver tissues. The study (Zhang *et al*., 2018) showed that acquired fatty liver in cocks might be inherited. The study used 36-week-

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old male animals obtained from Jilin University in China. To determine the heredity of fatty liver, a paternal fatty liver group and a control group were formed. RNA-seq analysis was conducted on six samples obtained from the F1 generation of JXH chickens (3 birds with fatty livers from the paternal group and 3 control birds). The paternal group was fed a high-fat HF diet, whereas the control group was fed a basic diet without inducing fatty liver. The birds produced through the F1 breeding program were fed a basic diet without fatty liver induction. At the end of the 36th week, by slaughtering the birds, a piece of liver was stored for RNA extraction at -80°C. The libraries, generated by PCR amplification, were sequenced by HiSeq 2500 (Illumina, San Diego, CA, USA). A fragmentation buffer was used to fragment the enriched mRNA into short fragments of around 200 bp. Our study focused on lncRNAs associated with fatty liver in this strain of chickens. The data used for this study is accessible at the following URL: (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE111909).

Data Sequencing, Filtering and Alignment

Chinese raw sequence data were downloaded directly from the NCBI Sequence Read Archive (SRA) database (SRA project accession number: SRP135813; BioProject: PRJNA438519; GEO: GSE111909) and converted into FASTQC (Version 0.11.5) (FastQC aims to provide a simple way to do some quality control checks on raw sequence data coming from high throughput sequencing pipelines). (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) files using the SRA (Sequence Read Archive) Toolkit software (Version 2.9.2) (The SRA Toolkit provides tools for downloading data, converting different formats of data into SRA format, and vice versa, extracting SRA data in other different formats) (http://www.webgestalt.org/). Totally, 6 samples containing raw sequence read were cleaned by Trimmomatic (Version 0.39) (https://mybiosoftware.com/trimmomatic-0-30-flexible-read-trimming-tool-illumina-ngs-data.html) with the default settings to remove Illumina adaptor sequences, overrepresented sequences, PCR primers and low-quality reads. Finally, contaminants were removed, and another quality control was performed to ensure the sequence data was acceptable.

Differential Expression Analysis and Principal Component Analysis

For distinguishing the mRNAs and lncRNAs, GTF and fastA files were obtained from Ensembl (http://asia.ensembl.org). The remaining high-quality reads were used as input for STAR (version 2.5.3a) alignment to the GRCg6a reference genome (Ensembl release 102). The aligned reads were calculated by featureCounts (subread-2.0.1, http://subread.sourceforge.net/). For the subsequent Differential Expression (DE) analysis, we used DESeq2 v1.30.0 with R v4.0.4 (https://bioconductor.org/packages/release/bioc/html/DESeq2.html/). $P< 0.05$ was considered a threshold. We determined each gene's chromosomal location using bioDBnet (http://biodbnet.abcc.ncifcrf.gov (accessed on 6 September 2021).

Functional Enrichment Analysis

Gene Ontology (GO) and KEGG pathway enrichment analysis were performed. GO analysis was performed to explore the functional roles of the target genes by DAVID (Database for Annotation, Visualization, and Integrated Discovery). Protein–Protein Interaction (PPI) network was established with the STRING online search tool. PPI network was constructed by setting medium confidence at 0.400.

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Cytoscape v3.8.0 (http://www.cytoscape.org/) was used to visualize the PPI network. We used DAVID 6.8 Biological Processes (BPs), Cellular Components (CCs), and Molecular Functions (MFs) of the gene groups. The biological processes in Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) were searched for pathways at the significance level set (recommended P-value< 0.05).

RESULTS

Analysis of Differentially Expressed Genes

To identify Differentially Expressed (DE) genes between two groups of chickens, we utilized the DESeq2 package in R/Bioconductor. Using DElncRNAs identified in the livers of chickens, we investigated their distributions on chromosomes. More DElncRNAs were distributed on chromosome 1 (Figure 1).

PPIs Network Construction

For genes that were significantly differentially expressed between the groups, the dots appear blue, and non-significantly differentially expressed genes appear grey. We made a MAplot (Figure 2), which shows a highly significant correlation between control group and treatment group.

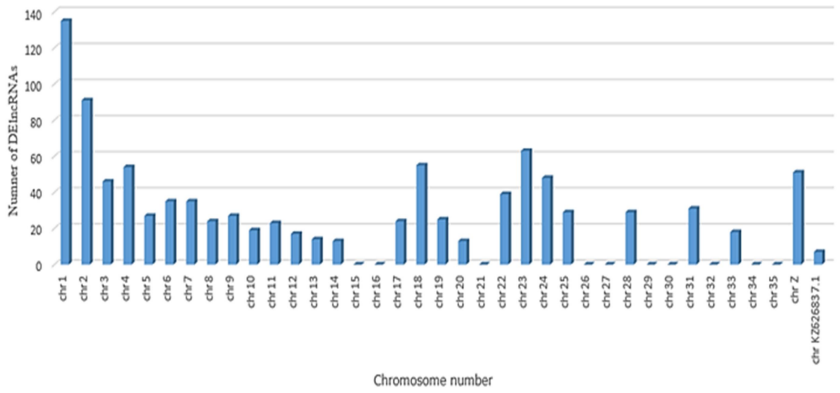

Figure 1. DElncRNAs and their locations on chicken chromosomes.

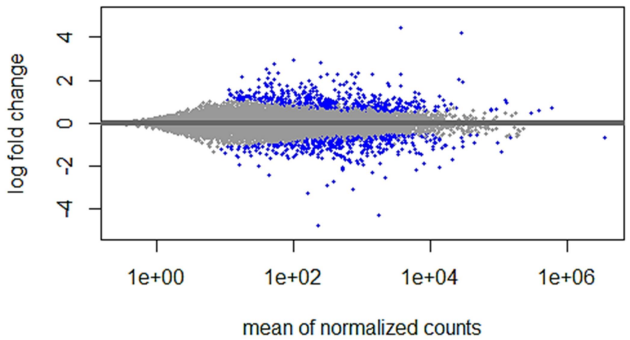

Figure 2. MAplot of DESeq2 (Version 1.30.0) output indicating genes that are significantly differentially expressed (blue circles), non-significantly differentially expressed (grey circles), and significantly expressed outside of the 4 to -4 log fold change limit.

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Analysis of lncRNAs Target Genes

Furthermore, 992 target genes were surveyed by using Biological DataBase Network (BioDBnet) software [http://biodbnet.abcc.ncifcrf.gov (accessed on 6 September 2021)]. The biological functions of the genes were analyzed using DAVID. Generally, 276 genes were identified by DAVID. Among these 276 genes, GCGR, PCK1 and PDK3 may be associated with fatty liver in chickens (Table. 1).

By bioinformatics tools, we explored 101 lncRNAs, which were differentially expressed between fatty liver tissues and adjacent non-fatty liver tissues. PPI network showed 291 nodes and 353 edges. Furthermore, the expression of the genes GCGR, PCK1 and POK3 associated with fatty liver induction was observed (Figure 3). Some gens had high degrees, such as PRKAG2 (Degree= 13) and NEK2 (Degree= 11). It is possible that these genes might play an important role in the development of diseases, based on their high degree of hub

Table 1. List of relevant long non-coding RNAs (lncRNA) in fatty livers with their target genes.

lncRNA ID	Target gene	Gene symbol	David gene name
ENSGALG00000019795	ENSGALG00000011219	GCGR	Glucagon Receptor
ENSGALG00000053815	ENSGALG00000007636	PCK1	Phosphoenolpyruvate Carboxykinase 1
ENSGALG00000033758	ENSGALG00000016323	PDK3	Pyruvate Dehydrogenase Kinase, isozyme 3
ENSGALG00000054206	ENSGALG00000002667	OLFML2B	Olfactomedin-Like 2B
ENSGALG00000037079	ENSGALG00000008736	PNPLA7	Patatin-like Phospholipase domain containing 7

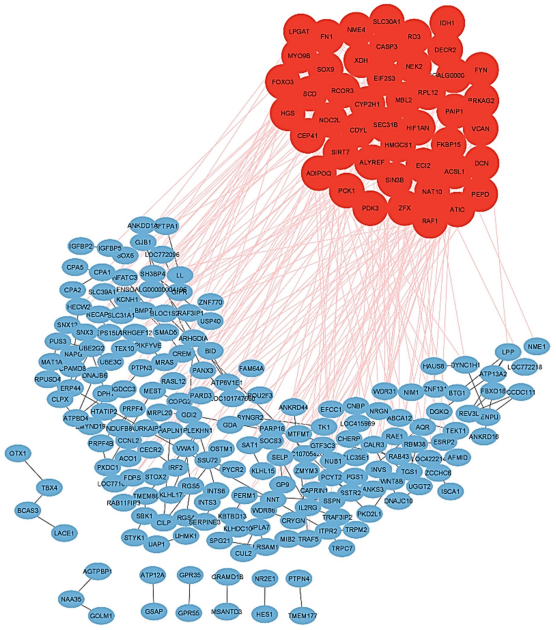

Figure 3. The red circle mean that the genes have a high degree and have more connections with other genes; conversely, the blue circles have less degree of the genes, and the less connection with other genes.

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gene activity (Table. 2).

Functional and Pathway Analysis of Differentially Expressed lncRNAs (DElncRNA) Targets

In our study, the GO analysis was performed at the BP, CC and MF levels (Figure 4). The top 7 GO functional annotations (–log2 fold change and P< 0.05) indicated that they had some roles like, metallocarboxypeptidase activity in the MF, cytosol in the CC, response to starvation, protein ubiquitination, extracellular matrix organization, negative regulation of protein catabolic process and protein localization to nucleus in the BP accounted for most of the proportion (Figure 4).

In the enrichment analysis of KEGG pathway, two pathways were significantly associated with the targeted mRNAs (Figure 5). KEGG pathway analysis also showed that pathways related with biosynthesis of antibiotics and biosynthesis of amino acids (–log2 fold change and P< 0.05).

DISCUSSION

For chickens and humans, the liver is the most important organ for de novo lipogenesis (Lin *et al*., 2021). FLHS is a serious disease, which causes liver lipid adiposity and hemorrhaged spots. There has been a reported prevalence of FLHS of 4% and even up to 16% in native chickens (Tan *et al*., 2021). Previous studies have shown that lncRNAs play a key role in both transcriptional and epigenetic regulation. Some lncRNAs influence Non-Alcoholic Fatty Liver Disease (NAFLD) susceptibility (Shabgah *et al*., 2021).

To analyze the lncRNA in fatty liver, we firstly performed the expression profiles of lncRNA by RNA-seq analysis. The average number of reads aligned to the Gallus genome was 92.44% in the samples. Statistical analysis revealed that, out of 24,356 transcripts included in analysis,

Table 2. Overview of top ten genes related to Nonalcoholic Fatty Liver Disease (NAFLD) in chickens using Cytoscape software.

Gene	Degree
PRKAG2	13
NEK2	11
ECI2	10
ATIC	9
SCD	9
CASP3	8
FN1	8
HGS	8
LPGAT1	8
NAT10	8

1,912 were differentially expressed gene (P< 0.05) in livers between control group and affected group. Nine hundred and ninety five (995) genes were up-regulated and 917 genes were down-regulated, as summarized in a MAPlot (Figure 2). An HFD-induced mouse model of NAFLD revealed that over 290 lncRNAs differed in their expression. In addition, another study identified 67 differentially expressed lncRNAs in chickens, only two of which were annotated (Tan *et al*., 2020).

There are 276 genes in DAVID that have been identified as being associated with chicken fatty liver, including GCGR, PCK1 and PDK3. It has been suggested that GCGR-s and GCGR-v1 in Table 1 play important roles in hepatic glucose metabolism in birds. The liver of chickens expresses the highest levels of GCGR compared to other tissues (Wang *et al*., 2008). Oxaloacetate is converted to phosphoenolpyruvate by PCK1, the rate-limiting step in hepatic and renal gluconeogenesis and adipose glyceroneogenesis, and is expressed at high levels in liver and adipose tissue (Duan *et al*., 2013). When PDK3 is overexpressed, glucose aerobic oxidation is increased, which has an important effect on liver disease (Tan *et al*., 2020). A large number of Hepatocellular Carcinoma (HCC) cases are linked to chronic liver

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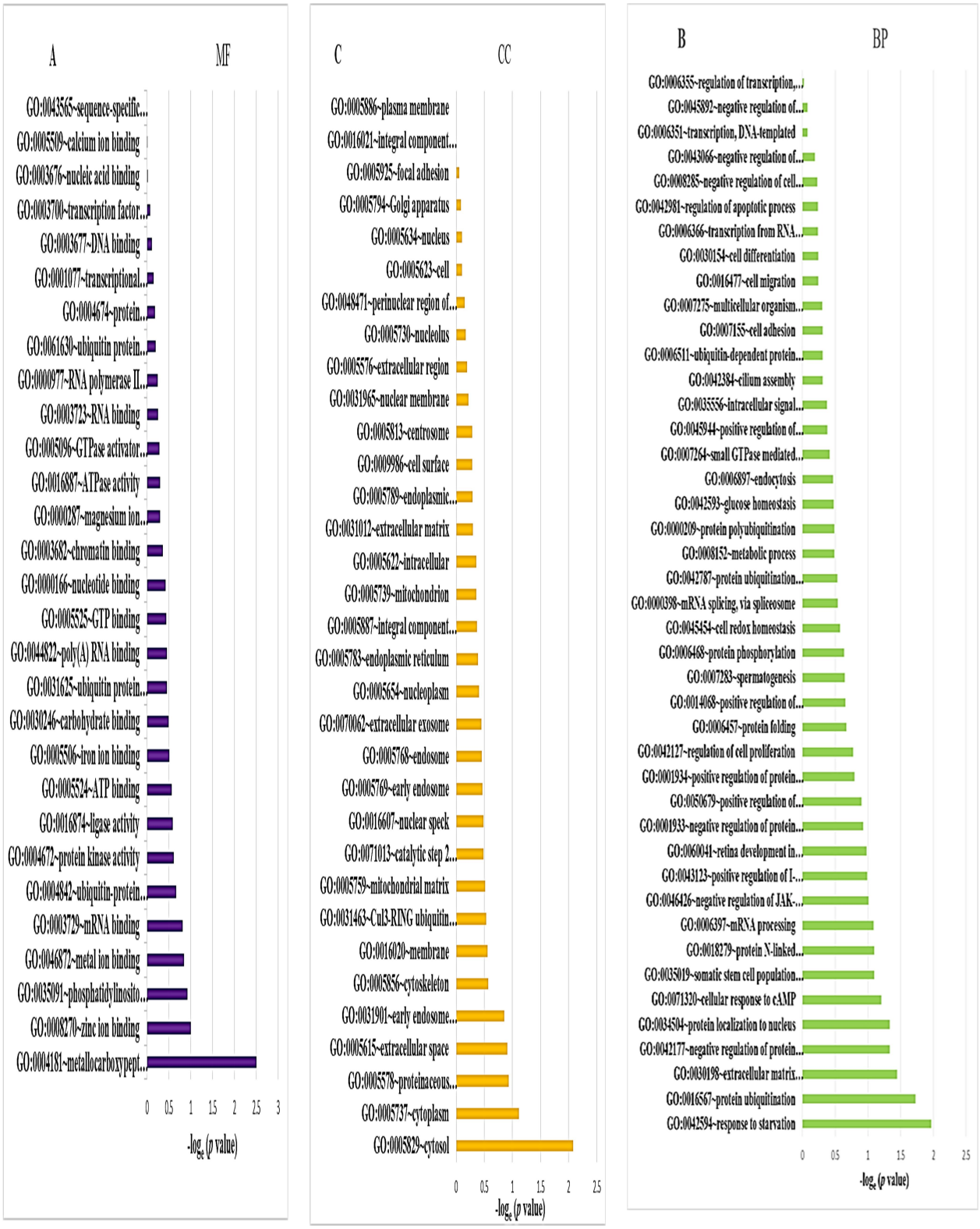

Figure 4. (A) GO Molecular Function (MF); (B) GO Biological Processes (BP), and (C) GO Cellular Component (CC).

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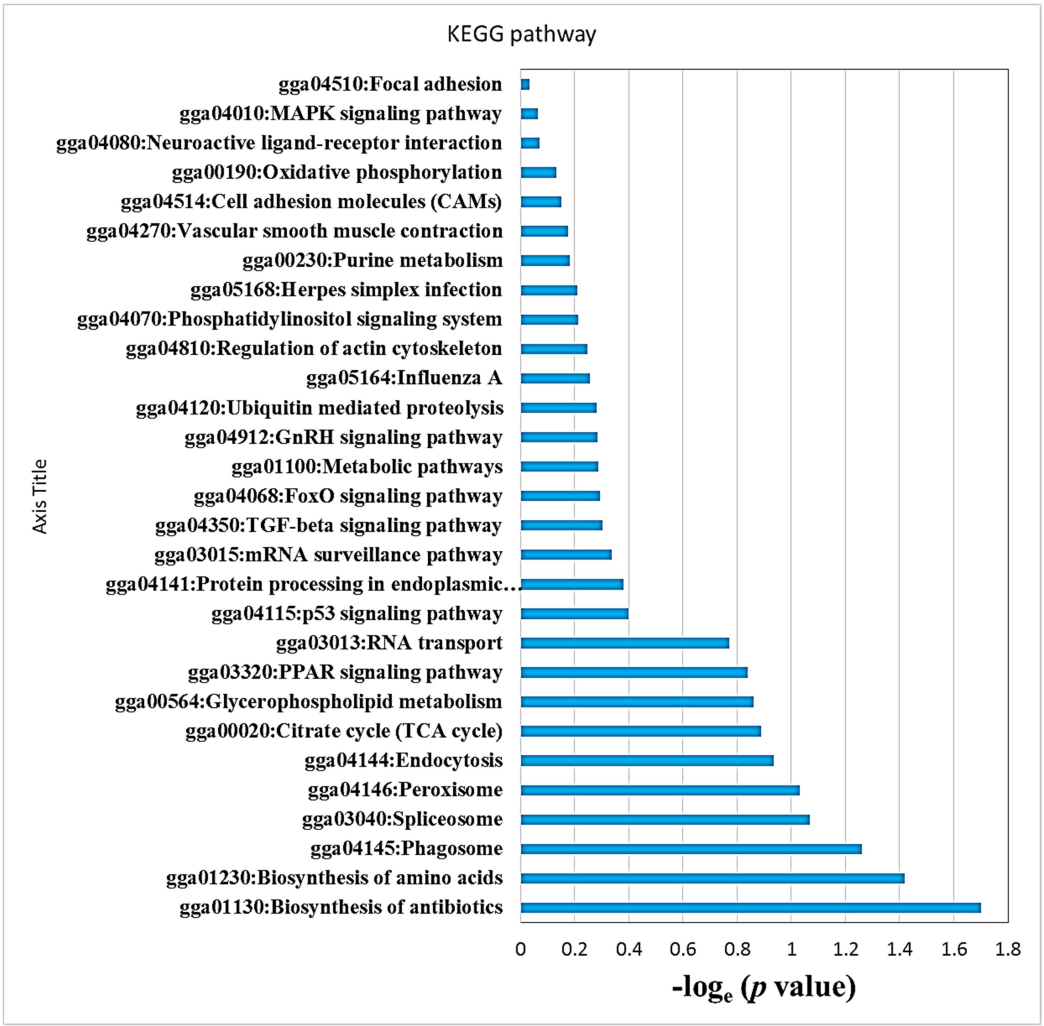

Figure 5. KEGG pathway enrichment analysis. KEGG, Kyoto Encyclopedia of Genes and Genomes.

diseases, including chronic viral hepatitis, Nonalcoholic Fatty Liver Disease (NAFLD), and alcoholic liver disease (Liu *et al*., 2020). THY1 has little effects on NAFLD (Zheng *et al*., 2021). OLFML2B associated with CD90 (THY1) in HCC tissues (Liu *et al*., 2020). PNPLA7 levels were dramatically down-regulated in both HCC cell lines and tissues in human (Zhang *et al*., 2016).

Top ten nodes including PRKAG2, NEK2, ECI2, ATIC, SCD, CASP3, FN1, HGS, LPGAT1 and NAT10 in the cytoscape networks were commonly distinguished in fatty liver (Table 2). Some significant hub genes were PCK1 and POK3 and were observed in DAVID, too (Table 1). Participates in the regulation of fatty acid biosynthesis (Prkag2) is involved in the glucagon signaling pathway and insulin signaling pathway (Guo *et al*., 2019). NKE2 increases the risk of HCC metastasis and is associated with recurrence, thus it may serve as a promising biomarker for high-risk HCC in human liver (Chang *et al*., 2018). Enoyl-CoA delta isomerase 2 (ECI2) helps the catabolism of unsaturated fatty acids by

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catalyzing an isomerization of cis-Δ3 and trans-Δ3-enoyl CoA esters into trans-Δ2-enoyl-CoA esters (Shaw *et al*., 2018). ATIC encodes the last enzyme in the de novo purine biosynthesis pathway. Cancer cells proliferation is associated with the purine synthesis pathway. In HCC, ATIC is an oncogene that promotes survival, proliferation, and migration by targeting AMPK-mTOR-S6K1 (Qin *et al*., 2021). A key role in cancer pathogenesis is altered lipid biosynthesis and metabolism. In liver cancer, Stearoyl-CoA Desaturase (SCD), an enzyme that regulates lipid homeostasis, is overexpressed. Down regulating SCD by pharmacological or genetic means can increase sensitivity to chemotherapy-induced cell death (Devan *et al*., 2021). Decreased TIP30 expression elevated fatty acid synthesis and increased the levels of lipogenic enzymes SCD and FASN in HCC cells (Yin *et al*., 2017). It appears that cell death, such as apoptosis, is important for NAFLD progression (Kanda *et al*., 2018). It is well established that CASP3 plays a crucial role during apoptosis, which in turn can cause inflammation, fibrosis and cirrhosis (Lin *et al*., 2021). FN1 receptor causes cirrhosis (Ogunwobi *et al*., 2019). In cell lines with CTNNB1 oncogenic mutations, HGS serves as a survival factor (Canal *et al*., 2015). Lysophosphatidylglycerol acyltransferase 1 (LPGAT1) is regulated by SND1, and LPGAT1 participates in the synthesis of phosphatidylcholine, phosphatidylserine and triacylglycerol (Chidambaranathan-Reghupaty *et al*., 2018). In the liver, LPGAT1 encodes lysophosphatidylglycerol acyltransferase 1, which may participate in triacylglycerol syntheses and secretions. Among mice lacking LPGAT1, oxidative stress, mitochondrial DNA depletion, and mitochondrial dysfunction were observed (Huang *et al*., 2021).

The recent evidence suggests that NAT10 plays a role in the development of cancers in humans. P53 plays a vital role in the processes of apoptosis, DNA repair, cell cycle control, and tumor suppression. NAT10 acetylates the tumor suppressor P53 and regulates its activity. NAT10 expression is correlated with P53 levels in HCC. NAT10 also increased mutant P53 levels in HCC cells and promoting proliferation in cells carrying P53 mutation. (Li *et al*., 2017). Indeed, through our differential expression analysis, we revealed that lncRNAs and target genes related to metabolism have differential expression in the liver. Meanwhile, some genes have not been proved to be included in the process of fatty liver or other liver disease, such as INVS, LACE, etc.

In summary, according to the research, there were 101 significant lncRNAs in the treatment and control groups of this study. Also, the significant expression of GCGR, PRKAG2, PDK3, NEK2 and PCK1 genes involved in fatty liver induction was determined. In order to select and produce laying chicken lines, it can be helpful to investigate this number of lncRNAs and their related target genes. As a result, a more suitable line can be produced, with less chance of fatty liver. Our study provides new insights into the discovery and annotation of lncRNAs associated with fatty liver in chickens. Further research is required to validate the functions of these lncRNAs and their targets in chicken.

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RNA های طولانی غیر کدکننده مسبب ایجاد کبد چرب در طی مراحل رشد در مرغ

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چکیده

سندرم کبد چرب هموراژیک (FLHS) در طیور شایع است. RNA های طولانی غیر کد کننده (lncRNAs) بیان ژن را به روش‌های مختلف در سطوح اپی ژنتیکی، بازسازی کروماتین، رونویسی و ترجمه تنظیم می‌کنند. کبد مرغ لیپوپروتئین‌ها و بیش‌تر پیش سازهای زرده تخم مرغ را با کمک RNA مانندMicroRNA (miRNAs) و lncRNA تولید می‌کند. به منظور تجزیه و تحلیل lncRNA ها در کبد، داده های RNA-Seq شش نمونه از مرکز ملی اطلاعات زیست‌فناوری (NCBI) (سه پرنده با کبد چرب از گروه پدری و سه پرنده‌ی شاهد)، دانلود شد. سپس با استفاده از پکیج DESeq2، آنالیز تفاوت در بیان lncRNA ها در نمونه‌ها مورد بررسی قرار گرفت. تجزیه و تحلیل غنی سازی عملکردی به‌وسیله‌ی STRING ایجاد شد و شبکه تعامل پروتئین-پروتئین PPI به‌وسیله‌ی Cytoscape تجسم شد. حاشیه نویسی داده‌ها به‌وسیله‌ی DAVID 6.8 انجام شد. مسیرهای بیولوژیکی در دانشنامه ژن و ژنوم کیوتو (KEGG) جستجو شدند. نتایج تجزیه و تحلیل ژن‌های بیان شده متفاوت (DEGs) نشان داد که ۲۴۳۵۶ ژن حاشیه‌نویسی شده وجود دارد. همچنین، ۱۰۱ lncRNA به صورت معنی دار پیدا شد. آنالیز غنی‌سازی اصطلاح هستی‌شناسی ژن (GO) نشان داد که DEG ها به طور قابل‌توجهی در فعالیت متالوکربوکسی پپتیداز، یوبی‌کوئیتیناسیون پروتئین و غیره غنی می‌شوند. بررسی جایگاه‌های ژنی نشان داد که روند بیان ژن‌های GCGR، PDK3 و PCK1 با بیان lncRNA های مجاور همخوانی دارد. بررسی این تعداد lncRNA به همراه ژن‌های هدف آن‌ها می‌تواند به انتخاب لاین‌های مرغ تخم‌گذار با شانس کمتر به ابتلا به کبد چرب کمک کند.

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