Integrated Analysis of a Noncoding RNA-Associated Competing Endogenous RNA Network in Non-Alcoholic Fatty Liver Disease

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ABSTRACT Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide. Dysregulation of both coding and long noncoding RNAs (lncRNAs) plays crucial role in the pathogenesis and development of the disease. In this study, we identified a profile of dysregulated RNAs using datasets from a public database. By incorporating all interactions between different RNAs, a competing endogenous RNA network was established to further illustrate the regulatory roles of noncoding miRNAs and lncRNAs. Moreover, a functional analysis showed that NAFLD is more closely associated with certain biological processes and pathways. Altogether, these results provide a helpful perspective on NAFLD and may assist future diagnosis and treatment of the disease.

INDEX TERMS Non-alcoholic fatty liver disease, mRNA, miRNA, lncRNA, ceRNA network.

I. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a common type of chronic liver disease worldwide [1], [2]. NAFLD is characterized by the excessive accumulation of fat in hepatocytes and is associated with obesity, insulin resistance, type 2 diabetes mellitus, hypertension and other metabolic syndromes [3]. The disorder results in a spectrum of complex pathological changes, characterized by the progression of simplified hepatic steatosis to characterized steatohepatitis and ultimately to hepatocellular carcinoma [4]. A number of studies have focused on NAFLD during the past decades, yet its underlying mechanisms remain largely obscure [5].

The majority of NAFLD studies have investigated protein-encoding genes and messenger RNAs (mRNA), while nonprotein-encoding RNAs have always been ignored and perceived as “evolutionary junk”. However, emerging evidence has revealed the role of noncoding RNAs in the pathogenesis and progression of NAFLD [6], [7]. A microRNA is a small (miRNA, 20-25 nucleotides in length), single-stranded noncoding RNA that regulates gene expression either at the transcriptional level by targeting promoter or enhancer regions or at the posttranscriptional level by blocking or cleaving target RNAs [6], [8]. Recently, another subtype of noncoding RNA, the long noncoding RNA (lncRNA, over 200 nucleotides in length), has gained increasing attention. Recent studies have demonstrated that aberrant up- or down-regulation of lncRNAs may be associated with various human diseases, especially tumorigenesis and cancer metastasis [9]–[11].

The concept of a competing endogenous RNA (ceRNA) network was proposed to explain how both mRNAs and lncRNAs...
IncRNAs could be regulated by microRNA via similar microRNA response elements (MREs; [12]). Namely, the ceRNA network is a posttranscriptional regulatory network in which different types of noncoding RNAs engage in cross-talk and compete for binding with target genes [13]. Further analysis of ceRNA networks has the potential to deepen our knowledge of the mechanisms involved in the coordination between different subtypes of noncoding RNAs.

In this study, we intend to investigate the mRNA-miRNA-lncRNA-ceRNA network associated with NAFLD. First, we will compare the expression of mRNA, miRNA, and lncRNA between NAFLD patients and healthy donors. Then, we will separately identify the mRNA-miRNA interactions and lncRNA-miRNA interactions using public domain databases. These two types of interactions will be integrated to generate the ceRNA network. The results of this study will reveal novel or pivotal genes that have the potential to become future research or therapeutic targets.

II. MATERIALS AND METHODS

A. MICROARRAY EXPRESSION PROFILING OF THE GEO DATASETS

The Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) is an open repository that provides high-throughput microarray and next-generation sequence datasets submitted by researchers worldwide. A total of three microarray datasets (GSE33857, GSE89632, and GSE107231) were obtained from the GEO. The three datasets aimed to compare the expression levels of miRNA, mRNA and lncRNA in liver samples from NAFLD patients and healthy donors, respectively. The details of the three datasets are summarized as followed (Table 1).

| Reference | GSE   | GPL   | RNAs   | Sample   | Control | NAFLD |
|-----------|-------|-------|--------|----------|---------|-------|
| 14        | 33857 | 10665 | miRNA  | Liver    | 12      | 7     |
| 15        | 89632 | 14951 | miRNA  | Liver    | 19      | 24    |
| 16        | 107231| 20115 | lncRNA | Liver    | 5       | 5     |

B. IDENTIFICATION OF DIFFERENTIALLY EXPRESSED RNAs IN NAFLD PATIENTS COMPARED TO HEALTHY CONTROLS

Differentially expressed RNAs (DERs) were identified using R programming software equipped with the ggplot2, edgeR, and pheatmap packages (http://bioconductor.org/biocLite.R). Datasets were standardized after the conversion of formats, imputation of missing values, and data normalization and grouping. To identify DERs (mRNA, miRNA, and lncRNA) in this study, the expression levels of all genes within the datasets were subjected to analysis by the R program. The threshold was set as a P value <0.05 and a |log2FC| >1.

According to the criteria, the DERs were screened and identified for further analysis.

C. FUNCTIONAL ANNOTATION ANALYSIS

The Database of Annotation, Visualization and Integrated Discovery (DAVID, http://david.ncifcrf.gov) is a public database with comprehensive online tools for functional annotation. Gene ontology (GO) enrichment classification and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed with DAVID. A P value <0.01 was considered statistically significant.

D. CONSTRUCTION OF THE ceRNA (lncRNA-miRNA-mRNA) REGULATORY NETWORK

The prediction of miRNA-mRNA interactions was performed with the open-source platform Encyclopedia of RNA Interactomes (ENCORI, http://starbase.sysu.edu.cn; [17]). The unique algorithm of ENCORI ensures that all determined interactions are confirmed according to at least one other major website used for RNA-RNA prediction, such as miRanda, PicTar, or TargetScan. In addition to sequence matching, the prediction was confirmed by multidimensional sequencing data. All these features make ENCORI a reliable source for miRNA-mRNA prediction. Two other databases, miRcode (http://www.mircode.org) and DIANA-RNA (http://carolina.imis.athena-innovation.gr), were applied in this study for predicting miRNA-lncRNA interactions. Afterwards, all interactions were inputted into Cytoscape (version 3.7.2, http://cytoscape.org) to visualize the ceRNA regulatory networks.

E. STATISTICAL ANALYSIS

All statistical analysis in this study was performed using SPSS 22.0 software. P < 0.05 was considered statistically significant.

III. THE RESULTS

A. DIFFERENTIALLY EXPRESSED mRNAs, miRNAs, AND lncRNAs IN THE LIVERS OF NAFLD PATIENTS IN COMPARISON WITH HEALTHY CONTROLS

From the datasets acquired from the GEO, a total of 411 DE-mRNAs were identified as significantly changed in NAFLD samples compared to normal tissues, including 163 upregulated genes and 248 downregulated genes (Figure 1 A-B). Volcano plots were generated to visualize all the DE-mRNAs. Among them, the top 10 upregulated and top 10 downregulated genes based on the value of logFC were indicated by red or blue dots. In addition, the representative 20 DE-mRNAs with the lowest P-values were also visualized with a heat map. Each column in the map represents a sample and each row represents a gene. The color intensity indicates the relative expression levels of the genes.

Similarly, 103 differentially expressed miRNAs and 162 differentially expressed lncRNAs from the GEO datasets were identified. The representative DERs identified according to the same criteria are shown in the figures (Figure 2 A-B and Figure 3 A-B).

B. FUNCTIONS AND PATHWAYS OF DIFFERENTIALLY EXPRESSED mRNAs

For functional annotation and pathway enrichment analysis, 411 identified DE-mRNAs were inputted into DAVID.
FIGURE 1. Differentially expressed mRNAs in NAFLD. (A) Volcano plots of the mRNA levels between NAFLD patients and controls. Red dots represent upregulated mRNAs and blue dots represent downregulated mRNAs (based on the absolute logFC value). (B) Heat map of differentially expressed mRNAs (top 20) based on P-values.
FIGURE 2. Differentially expressed miRNAs in NAFLD. (A) Volcano plots of miRNA levels between NAFLD patients and controls. Red dots represent upregulated miRNAs and blue dots represent downregulated miRNAs (based on the absolute logFC value). (B) Heat map of differentially expressed miRNAs (top 20) based on P-values.

(http://david.ncifcrf.gov; [18], [19]). GO analysis revealed that these mRNAs were mostly strongly enriched in inflammatory responses (FDR = 6.79E-10), immune responses (FDR = 2.76E-08), and regulation of apoptotic
FIGURE 3. Differentially expressed lncRNAs in NAFLD. (A) Volcano plots of lncRNA levels between NAFLD patients and controls. Red dots represent upregulated lncRNAs and blue dots represent downregulated lncRNAs (based on the absolute logFC value). (B) Heat map of differentially expressed lncRNAs (top 20) based on P-values.

processes (FDR = 1.30E-07). The GO terms and their gene counts were also summarized (Figure 4a). The KEGG pathway analysis revealed that the pathways most associated with the DERs were the TNF signaling pathway (FDR = 8.38E-06) and pathways involving cytokine receptor interactions (FDR = 3.04E-04) (Figure 4b).
FIGURE 4. Significantly enriched GO terms and KEGG pathways associated with differentially expressed mRNAs in the ceRNA regulatory network. (A) BP, biological process. The lengths of the bars represent the number of genes associated with each GO term; (B) KEGG pathways. The size of the dots indicates the number of genes associated with the respective terms. The colors of the bars and dots reflect the extent of enrichment.
C. CONSTRUCTION OF THE COMPETING ENDOGENOUS RNA REGULATORY NETWORK

The ENCORI database was used to screen potential interactions between DERs identified in the GEO datasets. In total, 230 interactions between mRNAs and miRNAs and 442 interactions between lncRNAs and miRNAs were identified. The ceRNA network was generated using Cytoscape as previously discussed.

D. FUNCTIONAL ANNOTATION OF THE mRNAs INVOLVED IN THE ceRNA NETWORK

To understand the biological processes (BPs) and signaling pathways regulated by ceRNAs in NAFLD, all 411 identified DE-mRNAs involved in the ceRNA network were subjected to functional annotation by using the DAVID database. Processes related to inflammation, including inflammatory responses, immune responses and the negative/positive regulation of inflammatory responses were all significantly enriched. Similar enrichment was also observed for BPs associated with apoptosis, indicating the critical roles of these biological processes in the development of NAFLD. Thus, the mRNAs of genes involved in these BPs, together with the correlated miRNAs and lncRNAs, were selected from the whole ceRNA network to create the individual subnetwork.

Hepatic inflammation is a strong driving force in the progression of NAFLD, which ultimately leads to liver fibrosis and cirrhosis. Pathophysiologically, the triggers
of inflammation in NAFLD might be either external (adipocytes or the gut) or internal (lipotoxicity or immune responses; [20]) to the liver. Consistently in this study, several mRNAs that encode critical inflammatory molecules, such as
chemokines and cytokines, were dysregulated and identified as DE-mRNAs in the NAFLD group. Nevertheless, despite the observed enrichment of mRNAs in the GO analysis, the number of mRNAs that was involved in the ceRNA network is limited. However, several important genes involved in the regulation of hepatic inflammation were modulated by ceRNAs. The Fos protein is a member of Fos family, which includes AP-1 and the Jun protein family. It was reported that Fos acts as a master regulator of inflammation by suppressing the production of inflammatory factors such as IL-12 and NO [21], [22]. LncRNAs ENSG00000227195, ENSG00000266904, and ENSG00000228794 could act as ceRNAs to regulate Fos through microRNA-362. Thrombospondin-1 (THBS-1), a glycoprotein first discovered in activated platelets, is considered a key player in thrombosis. However, an increasing number of studies have discovered a link between THBS-1 and inflammation via partner genes such as CD36, CD47, NO and TGFB1 [23]. THBS-1 is predicted to be a target of miRNA-520, thereby demonstrating that it can be further modulated by multiple other lncRNAs.

Apoptosis is a major process of programmed cell death and is highly involved in the pathogenesis and progression of NAFLD [24]. Normally, the level of hepatic damage in NAFLD would be correlated with the extent of apoptotic activity. Therefore, it is expected that the enrichment of DE-mRNAs would be observed in apoptosis-associated biological processes and signaling pathways. Interferon regulatory factor-1 (IRF-1) is a transcription factor that is also known as an indispensable mediator of the apoptotic process [25]. MiRNA-423 and miRNA-520 target IRF-1 in a process mediated by ceRNAs such as the lncRNAs ENSG00000228794 and ENSG00000249859. Surprisingly, the number of apoptosis-associated mRNAs exceeds the number involved in other pathological processes in the ceRNA network, implying comprehensive and thorough participation of noncoding RNAs in the apoptotic process.

E. Hub GENES AND POTENTIAL DRUG TARGETS
Since ceRNA dysregulation can either induce or suppress NAFLD progression, targeting the central miRNAs has become an attractive therapeutic approach. To target miRNAs, miRNA mimics are synthesized as double-stranded small RNAs with corresponding miRNA sequences to restore the function of a specific miRNA. In contrast, molecules designed to inhibit miRNA, including antisense oligonucleotides (ASOs) and anti-miRs, have a complementary sequence that allows them to bind to the corresponding miRNA and block its function. With the cytoHubba package, we determined the hub miRNAs of the whole network for further evaluation (Table 2).

IV. DISCUSSION
NAFLD represents a spectrum of diseases including metabolic disorders, hepatic inflammation, fibrosis, and end-stage hepatocellular carcinoma. The lack of efficacious treatments for NAFLD leads to marginal therapeutic benefits in the clinic. The majority of current treatments focus on the treatment of obesity and metabolic dysregulation, which are associated with NAFLD progression. However, without reversing NAFLD-induced fat accumulation in the liver, it is still highly possible for the disease to progress. Another treatment option is a liver transplant, although this requires patients to wait for several years and to need lifelong immunosuppressive medication.

Recently, an increasing number of studies have revealed that NAFLD is closely correlated with the deregulation of noncoding RNAs, including miRNAs and lncRNAs, which has a huge impact on diagnosis and prediction of the outcome. However, the mutual coordination of RNAs remains complex and obscure. Using public datasets, we identified the differential expression profiles of mRNA, miRNA, and IncRNA in NAFLD. Moreover, we created a ceRNA network by determining the interactions between them. Further analysis identified crucial biological processes and signaling pathways that were significantly enriched in the disease state. Finally, the ceRNA network might lead us from classic NAFLD-related mRNAs to novel genes that contain miRNAs and lncRNAs that were previously unknown.

The human genome contains over 2,000 miRNAs that participate in numerous biological processes, many of which inhibit the expression of target genes [26]. NAFLD is characterized by the aberrant accumulation of lipids in hepatocytes. MiRNAs were found to be involved in different aspects of NAFLD, including de novo lipogenesis, export of lipid into blood, and lipid beta-oxidation. Central miRNA modulation regulates multiple pathways involved in NAFLD.

| Rank | Name     | Score |
|------|----------|-------|
| 1    | hsa-miR-429 | 40    |
| 2    | hsa-miR-520e | 28    |
| 3    | hsa-miR-24  | 27    |
| 4    | hsa-miR-520h | 25    |
| 5    | hsa-miR-362-3p | 22    |
| 6    | hsa-miR-27a  | 20    |
| 7    | hsa-miR-103  | 19    |
| 8    | hsa-miR-16   | 19    |
| 9    | hsa-miR-15b  | 19    |
| 10   | hsa-miR-15a  | 19    |
| 11   | hsa-miR-142-3p | 18    |
| 12   | hsa-miR-142-5p | 17    |
| 13   | hsa-miR-199  | 17    |
| 14   | hsa-miR-423  | 16    |
| 15   | hsa-miR-548  | 16    |
| 16   | hsa-miR-181a | 14    |
| 17   | hsa-miR-18b  | 14    |
| 18   | hsa-miR-106b | 14    |
| 19   | hsa-miR-93   | 14    |
| 20   | hsa-miR-140-5p | 13    |
progression, and the functions of miRNAs could be efficiently and specifically regulated by miRNA mimics and anti-miRs. Due to recent achievements in the clinical development of miRNA-targeting therapeutics, miRNA mimics of miR-34 have already reached phase II trials for the treatment of cancer. Moreover, another phase II trial for the treatment of hepatitis was launched with an anti-miR targeting miR-122.

Several studies have confirmed the role of miR-520, a central gene in our list, as a tumor suppressor in human cancer, including breast cancer, colorectal cancer, lung cancer, and liver cancer [27], [28]. However, to date, no study of the role of miR-520 in metabolic disorders such as NAFLD has been carried out. Interestingly, miR-520 suppresses the growth of liver cancer by inactivating the oncogenes ERK1/2 and NF-kB [29]. Moreover, miR-520 was reported as an intermediate regulator of glycolysis in hepatocellular carcinoma cell lines, suggesting its great potential to affect other metabolic disorders such as NAFLD [30].

With 19 target mRNAs and 3 target lncRNAs, miR-362 is among the microRNAs with the most interactions identified in this study. Several studies using in vitro experiments indicated the role of miR-362 in regulating different cancers, but they did not find a link to metabolic diseases [31]. In human breast cancer, the downregulation of miR-362 promotes tumor progression and proliferation [32]. In contrast, tumor growth and metastasis increased after miR-362 was upregulated [31], [33]. The transcription factor Kruppel-like factor 11 (KLF11), which is among the predicted targets of miR-362, is closely associated with lipid metabolism and regulation of NAFLD [34], [35]. Interestingly, mRNA targets of miR-362, including KLF11 and BCL6, are significantly enriched in the “apoptosis-related” category based on GO classification, as previously discussed. This finding suggests a potential link between miRNA-362 and the apoptotic process, which has been proven to be associated with a wide variety of liver diseases.

Pharmaceutical companies have noticed the lucrative opportunities provided by the anti-miR drug development market and have begun to compete for these opportunities. Few candidate drugs have made it to phase III trials. The main NAFLD-related targets currently in clinic trails include PPAR, FXR, apoptosis signaling-regulating kinase 1 (ASK1), glucagon-like peptide (GLP), CCR-5 coreceptor, and molecules involved in cholesterol, fatty acid and triglyceride homeostasis (Table 3).

Although various clinical trials for the treatment of NAFLD are ongoing, noncoding RNAs play only a trivial role in them. However, embracing noncoding RNA, especially miRNA, as a part of the solution has attracted increased interest. As a key mediator of lipid metabolism, members of the PPAR family, including PPARα and PPARδ, have been validated as targets of several clinical trial candidate drugs. Nonetheless, it has also been reported that miRNAs affect the development and prognosis of metabolic disorders by regulating PPAR molecules. MiR-199, a hub DE-miR member identified in our network, has been reported as a modulator of PPARδ and thus to affect fatty acid oxidation in a heart failure model (24011070). This miRNA also exhibits a suppressive effect on triglyceride accumulation and inhibits the development of NAFLD by suppressing specificity

| Drugs | Mechanism of action | Clinical trial ID | Related miRNA regulator |
|-------|---------------------|-------------------|------------------------|
| MGL-3196 (Resmotitrom) | Thyroid hormone analog used to increase cholesterol uptake into the liver and increase its metabolism | NCT03900429 | - |
| Aramchol | Novel fatty acid bile acid conjugate used to modulate SCD-1 | NCT04104321, NCT02278924 | let-7c; miR-125a |
| Elafibran | Dual agonist of PPARα and PPARδ | NCT02704403 | miR-142; miR-199; miR-362 |
| Obeticholic Acid | Semisynthetic bile acid analogue used to activate farnesoid X receptor (FXR) | NCT02548351, NCT03439254 | miR-21; miR-221 |
| MSDC-0602K | Second-generation insulin sensitizer used to selectively modulate mitochondrial pyruvate carrier (MPC) | NCT03970031 | - |
| SAMe (S-adenosyl-l-methionine) | Precursor for the synthesis of glutathione and a principal methyl donor | NCT01754714 | - |
| Omacor | Reduces the synthesis of triglycerides (TGs) in the liver | NCT01277237 | miR-21 |
| Oltipraz | Inhibits fatty acid synthesis through the AMPK-S6K1 pathway and the LXRα-SREBP-1c pathway in liver | NCT02068339, NCT04142749 | miR-19; miR-21; miR-25; miR-429 |
| Selonsertib | ASK1 inhibitor used to suppress inflammation and fibrosis | NCT03653050 | let-7a; miR-17; miR-20 |
| Exenatide | GLP-1 agonist used to increase insulin secretion and decrease glucagon secretion | NCT01650546 | miR-21 |
| Metformin | Decreases hepatic glucose production | NCT0063635, NCT0305537 | miR-671 |

TABLE 3. Currently ongoing clinical trials for NAFLD treatment and drug target-related miRNAs.
protein 1 (SP1) and nuclear receptor corepressor 1 (NCOR1)
(28469795). Similarly, another hub miRNA in the network,
miR-21, was reported to be increased in a NAFLD patient.
In vivo models have suggested that the genetic depletion of
miR-21 would lead to attenuation of NAFLD and suppression
of NAFLD-induced HCC by targeting PPARα expression
and modulating the HBP1-p53-Srebp1c pathway (26338827,
26282675). In summary, the evidence has shown that several
noncoding RNAs share similar target mRNAs and pathways
with drugs currently in clinical trials, indicating a bright
future for noncoding RNA as a therapeutic solution for
NAFLD.

Compared to mRNAs and miRNAs, little is known about
how lncRNAs participate in regulating NAFLD; however,
the ceRNA network identified in our work could provide
valuable information that might expand our knowledge of
how lncRNAs affect various liver disorders. In fact, several
lncRNAs were found to be significantly up- or downreg-
ulated in NAFLD patients compared to healthy controls,
implying a hidden relationship between lncRNA and liver
diseases. Unfortunately, the number of lncRNAs in our
ceRNA network is relatively small, which is partly due
to the lack of RNA-Chip data on lncRNAs in liver
diseases. Interestingly, some miRNAs target and thus
regulate multiple lncRNAs, and vice versa. miR-423, one
critical miRNA involved in heart diseases, is predicted to reg-
ulate four lncRNAs: XLOC_008583, ENSG00000249859,
ENSG00000228794, and ENSG00000227195. Similarly,
the lncRNAs ENSG00000266904, ENSG00000228794,
and ENSG00000227195 are predicted targets of miR-362.

There are some limitations in this study. The data were
obtained from liver biopsies to reveal the ceRNA network
in the liver. However, identifying ceRNAs may be an advan-
tageous tool to help provide biomarkers for patient diagno-
sis and prognosis prediction via noninvasive liquid biopsies.
Therefore, high-throughput RNA screening data from liq-
uid biopsies could provide more information, and valuable
biomarkers could be identified by comparing this information
with our ceRNA network.

V. CONCLUSION
In this work, we first identified differentially expressed
mRNAs, miRNAs and lncRNAs by comparing NAFLD
patients and healthy donors. Afterward, the ceRNA network
presented in this work demonstrated the links between the
DERs. Hub genes and central miRNAs were identified to
provide novel insights into the pathogenesis of NAFLD and
potential therapeutic targets for the future study of this com-
mon liver disorder.

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AUTHOR CONTRIBUTIONS
H. F. and S. J collected and consolidated the data and
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H. F., M. D and G. X. designed the study. H. F. wrote the draft
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CONFLICT OF INTEREST STATEMENT

The authors disclose no conflict of interests.

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