Roles of microRNAs in immunopathogenesis of non-alcoholic fatty liver disease revealed by integrated analysis of microRNA and mRNA expression profiles

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BACKGROUND: The integrative analysis of microRNA and mRNA expression profiles can elucidate microRNA-targeted gene function. We used this technique to elucidate insights into the immunological pathology of non-alcoholic fatty liver disease (NAFLD).

METHODS: We analyzed differentially expressed microRNA and mRNA expression profiles of $CD4^+$ T lymphocytes from the liver and mesenteric lymph nodes (MLNs) of mice with NAFLD using microarrays and RNA sequencing. Normal mice were used as controls. The target genes of microRNAs were predicted by TargetScan. Integrative analysis showed that the mRNAs were overlapped with microRNAs. Furthermore, the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to predict the key genes and pathways. Then, 16 microRNAs and 10 mRNAs were validated by qRT-PCR.

RESULTS: Microarray analysis suggested that 170 microRNAs were significantly de-regulated in $CD4^+$ T lymphocytes from the liver between the two groups. Eighty mRNAs corresponded with microRNA targeted genes. KEGG analysis indicated that the MAPK pathway was consistently augmented in the liver of NAFLD mice. miR-23b, let-7e, miR-128 and miR-130b possibly played significant parts in the MAPK pathways. Furthermore, between the two groups, 237 microRNAs were significantly de-regulated in $CD4^+$ T lymphocytes from MLNs. 38 mRNAs coincided with microRNA target genes. The metabolic pathway was consistently enriched in the MLNs of NAFLD mice. miR-206-3p, miR-181a-5p, miR-29c-3p and miR-30d-5p likely play important roles in the regulation of metabolic pathways.

CONCLUSION: The results of this study presented a new perspective on the application of integrative analysis to identify complex regulation means involved in the immunological pathogenesis of NAFLD.

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KEY WORDS: microRNA-mRNA; gut; non-alcoholic fatty liver disease; $CD4^+$ T lymphocytes; immunopathogenesis

Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by a broad array of pathological lesions from simple liver steatosis to non-alcoholic steatohepatitis (NASH), liver cirrhosis, and hepatocellular carcinoma. The key features of NAFLD include insulin resistance and liver inflammation.[1] Tilg and Moschen[2] proposed the "multiple hits" NAFLD pathogenesis model, which suggested that gut-derived factors and adipose tissue could promote liver inflammation.

Immune cells derived from the gut and cytokines transported to the liver by the portal vein can regulate intrahepatic immune reactions. We found that CD4/CD8 ratios in the peripheral blood mononuclear cells and Peyer's patches of high fat diet rats were augmented at the 4th week and decreased at the 8th week. However, on the 12th week, the CD4/CD8 ratio within the Peyer's patches increased to levels observed in the 4th week.[3] We also found that the activated $CD4^+$ T cells

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were increased in the mesenteric lymph nodes (MLNs) of NAFLD mice. The gut-derived lymphocytes from NAFLD have a tendency to travel to the liver and induce liver injury, and fatty liver stimulates the relocation of gut-derived lymphocytes.[4]

The liver itself demonstrates immune properties that can be considered as an "immunological organ".[5-7] The immune responses in NAFLD involve $CD4^+$ T-helper cells. Recent data recommended that its pathogenesis may be predisposed by an imbalance between the surplus of proinflammatory T-helper1 cytokines and the deficit of anti-inflammatory IL-4 and IL-10 cytokines.[8]

Recent studies[9, 10] have specified that the microRNAs hepatic expression profile is considerably diverse in subjects with NASH versus controls with regular liver histology. microRNAs comprise an imperative class of highly preserved non-coding RNAs that hinder the protein expression of their target genes through the process variation in the mRNA translation rate and/or stability.[11] The developing role of microRNAs as a significant player in adipocyte differentiation, hepatic metabolic functions, insulin resistance and NAFLD pathogenesis advocates some specific microRNAs as potential diagnostic and prognostic markers.[12, 13] There is an alteration in the microRNA signature that may underlie the metabolic changes, resulting in the initial formation of hepatic steatosis and the subsequent development of NASH. In this study, we aimed to apply an integrative molecular and bioinformatics approach by concurrently profiling both microRNA and mRNA for $CD4^+$ T lymphocytes of the liver and MLNs in NAFLD mice to examine the immunological pathogenesis of NAFLD.

Methods

Animal experiments

Wild-type C57BL/6 male mice (7 weeks old) were purchased from the Chinese Academy of Military Medical Sciences. The NAFLD model was induced by feeding mice with a high-fat diet (Diet#MD 45% fat, 35% carbohydrate, 20% protein, Medicience Ltd.) for 12 weeks; mice fed with a normal-fat diet (Diet#MD 10% fat, 70% carbohydrate, 20% protein, Medicience Ltd.) were used as controls.[14] Their weight was measured every week. The mice were sacrificed by isoflurane overdose. After perfusion with phosphate-buffered saline (PBS), the liver and MLNs were aseptically removed and placed in cold PBS. Part of the liver was embedded with paraffin as previously described.[15] Paraffin sections were stained with hematoxylin-eosin (HE). The animal experiments were approved by the Institutional Animal Care and Use Committee of Peking University People's Hospital.

Cell isolation

The hepatic mononuclear cells (HMNCs) were isolated by the method described previously.[16] Briefly, after perfusion with PBS, mouse liver was pressed through a 70-μm cell strainer (BD Falcon). Cell suspensions were washed twice with PBS, and HMNCs were separated from parenchymal cells by Percoll (GE Healthcare) sedimentation. Liver cells were pelleted and re-suspended in 4 mL of 40% Percoll and then layered onto 4 mL of 80% Percoll and centrifuged for 25 minutes at 750 g. HMNCs at the interface were collected and washed twice with PBS. The MLNs were isolated, pressed through 70-μm cell strainers and then washed twice. After that the cell suspensions were filtered twice using 40-μm cell strainers and MLN cells were collected and washed. HMNCs and MLN cells were sequestered and tarnished with fluorochrome-conjugated antibodies against CD4. After washing, $CD4^+$ T cells were arranged using the FACSAria II flow cytometer. Since the number of cells from one mouse is limited, we pooled the RNA of $CD4^+$ T cells from 6 mice equally into one pool to do further experiments.

microRNA microarray

Total RNA was isolated from two sets of $CD4^+$ T lymphocytes of liver from NAFLD mice and controls. Additionally, MLNs were harvested using TRIzol (Invitrogen) and then sterilized by means of the mirVana microRNA isolation kit (Agilent) according to the manufacturer's instructions. The concentration and purity of the total RNA were calculated by the NanoDrop ND 1000 at 260 and 280 nm (A260/280) and confirmed by gel electrophoresis. Each RNA sample was stamped using the microRNA Complete Labeling and Hyb Kit and crossbred on the Agilent mouse microRNA microarray, Release 18.0, 8×60K (Agilent), which contained probes for 1700 mature microRNAs. Imaging was performed with the Agilent microarray scanner. Agilent feature extraction (v10.7) was used to interpret the crude intensity of the image.

RNA profiling

Total RNA was harvested as described above. To identify the changes of mRNA transcription level among different groups, we performed RNA profiling by Illumina HiSeq 2000. The cDNA library of RNA profiling was constructed according to RNA Seq Library Preparation Kit for RNA Profiling (Gnomegen). Twenty to 30 nucleotide read length were sequenced (very 3' end close to polyA). A 36 nucleotides read could comfortably include an index read. The original data of sequencing was 50M per sample. Those with a greater than a 2-fold increase or a 2-fold decrease in expression in two samples were