The International Journal of Biochemistry & Cell Biology 64 (2015) 265–276

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

# Downregulation of microRNA-451 in non-alcoholic steatohepatitis inhibits fatty acid-induced proinflammatory cytokine production through the AMPK/AKT pathway

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ARTICLE INFO

*Article history:*
Received 12 January 2015
Received in revised form 16 April 2015
Accepted 28 April 2015
Available online 7 May 2015

*Keywords:*
Non-alcoholic fatty liver
Non-alcoholic steatohepatitis
MicroRNAs
MicroRNA-451
Cab39/MO25
Inflammation

ABSTRACT

Mechanisms associated with the progression of non-alcoholic fatty liver disease (NAFLD) remain unclear. We attempted to identify the pattern of altered gene expression at different time points in a high fat diet (HFD)-induced NAFLD mouse model. The early up-regulated genes are mainly involved in the innate immune responses, while the late up-regulated genes represent the inflammation processes. Although recent studies have shown that microRNAs play important roles in hepatic metabolic functions, the pivotal role of microRNAs in the progression of NAFLD is not fully understood. We investigated the functions of miR-451, which was identified as a target gene in the inflammatory process in NAFLD. miR-451 expression was significantly decreased in the palmitate (PA)-exposed HepG2 cells and in liver tissues of HFD-induced non-alcoholic steatohepatitis (NASH) mice. Its decreased expressions were also observed in liver specimens of NASH patients. *In vitro* analysis of the effect of miR-451 on proinflammatory cytokine provided evidence for negative regulation of PA-induced interleukin (IL)-8 and tumor necrosis factor-alpha (TNF-α) production. Furthermore, miR-451 over-expression inhibited translocation of the PA-induced NF-κB p65 subunit into the nucleus. Our result showed that Cab39 is a direct target of miRNA-451 in steatotic cells. Further study showed that AMPK activated through Cab39 inhibits NF-κB transactivation induced in steatotic HepG2 cells. miR-451 over-expression in steatotic cells significantly suppressed PA-induced inflammatory cytokine. These results provide new insights into the negative regulation of miR-451 in fatty acid-induced inflammation *via* the AMPK/AKT pathway and demonstrate potential therapeutic applications for miR-451 in preventing the progression from simple steatosis to severely advanced liver disease.



*Abbreviations:* AMPK, adenosine monophosphate activated protein kinase; DEGs, differentially expressed genes; GEO, gene expression omnibus; GOBPs, gene ontology biological processes; IL, interleukin; miRNAs, microRNAs; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PA, palmitate; qRT-PCR, quantitative real-time reverse transcriptase-polymerase chain reaction; TNF-α, tumor necrosis factor-alpha.

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http://dx.doi.org/10.1016/j.biocel.2015.04.016

## 1. Introduction

Non-alcoholic fatty liver disease (NAFLD) with accumulated fat in the liver is a common cause of chronic liver disease in Western countries (Lewis and Mohanty, 2010; Ogawa et al., 2014). NAFLD comprises a diverse spectrum of manifestations, ranging from simple steatosis to steatohepatitis, fibrosis and cirrhosis (Nguyen and Sanyal, 2012). A subset of individuals with NAFLD progress to decompensated liver diseases and liver cancer (Pais et al., 2013). The recent increasing prevalence of NAFLD is considered to be related to the epidemic of obesity and type 2 diabetes in the USA and other developed countries (Bellentani and Marino, 2009). In addition, NAFLD is reported to be strongly associated with metabolic syndrome including hypertension and cardiovascular disease and it is therefore regarded as a clinical manifestation of complex metabolic disease spectrum in the liver (Targher et al., 2010). For this reason, it is necessary to actively investigate the underlying disease mechanism to manage the individuals with NAFLD more effectively.

A "two-hit hypothesis" was initially proposed to explain the pathogenesis and progression of NAFLD (Day and James, 1998). However, recent investigations have provided evidence that 'multiple hits' of multiple factors are involved in the pathogenesis and progression of NAFLD (Tilg and Moschen, 2010). These hits execute the occurrence of parallel events of a complex interplay among host genetics, environmental risk factors and, the gut microbiota (Bechmann et al., 2012). Such interactions can cause isolated hepatic steatosis, innate immune activation, inflammation, apoptosis and/or progressive liver damage (Tilg and Moschen, 2010). Although considerable progress has been made recently in elucidating the pathogenesis of NAFLD, it remains unclear how the inflammatory mediators are involved in the progression of NAFLD. To investigate the molecular mechanisms underlying the progression of NAFLD, it is essential to develop appropriate *in vitro* and *in vivo* models. We previously established a high fat diet (HFD)-induced NAFLD mouse model that exhibits pathological features similar to those in humans (Kim et al., 2014). In addition, we developed an *in vitro* model of hepatocytic steatosis in a HepG2 cell line exposed to palmitate (PA). We also showed the preventive and therapeutic effects of oleuropein, a secoiridoid derived from olives (*Olea europaea*), on the progression of hepatic fibrosis from steatohepatitis in these experimental models (Hur et al., 2012; Kim et al., 2014; Park et al., 2011).

MicroRNAs (miRNAs) represent a class of small non-coding RNAs involved in various biological processes, including cell proliferation, development and differentiation (Shukla et al., 2011).

Recent studies have demonstrated that miRNAs play important roles in the control of energy, and hepatic metabolic functions regulating fatty acid (FA) and cholesterol metabolism in NAFLD (Alisi et al., 2011; Ceccarelli et al., 2013). Of the many differentially expressed miRNAs between steatosis and nonalcoholic steatohepatitis (NASH), we focused miRNA (miR)-451 and studied the role of miR-451 in the progression of NAFLD to NASH.

miR-451 is known to exert various biological functions in cancer, infectious disease, cardiovascular and metabolic diseases. Previous studies have revealed that the dysregulation of miR-451 expression is involved in carcinogenesis and in tumor progression by affecting cell proliferation, cell-cycle distribution, migration, and invasion (Li et al., 2013; Liu et al., 2013; Tian et al., 2012). Furthermore, a recent study showed that miR-451 regulates dendritic cell cytokines and suppresses neutrophil chemotaxis *via* down-regulation of p38 MAPK phosphorylation (Murata et al., 2014; Rosenberger et al., 2012). Specifically, the effects of miR-451 are mediated by the liver kinase B1 (LKB1)/5′-adenosine monophosphate activated protein kinase (AMPK) pathway, which controls key players in metabolic pathways and thus emerges as a major regulator of glucose and lipid metabolism with multiple beneficial roles in the target tissues (Godlewski et al., 2010). However, the expression and function of miR-451 in NAFLD are still unclear and the molecular mechanisms underlying the progression to NASH from simple steatosis have yet to be elucidated. Our study aimed to investigate the role of miR-451 in the molecular mechanisms of the pathogenic procession of NAFLD. We found that miR-451 expression was downregulated in the livers of HFD-induced NASH mice and that miR-451 regulates inflammatory cytokine secretion from steatotic cells through the AMPK, Akt and NF-κB singling pathways *via* direct targeting of Cab39.

## 2. Materials and methods

### 2.1. Animals and diets

Five-week-old male C57BL/6 mice were purchased from Orient Bio (Seoul, South Korea) and housed in a standard animal facility under a 12:12-h light–dark cycle with a constant room temperature. The mice were divided into four weight-matched groups. Mice were fed either a normal diet (ND) of 3.8 kcal/g (ND; 50.21% carbohydrate, 20.78% protein and 4.8% fat) or high fat diet (HFD: D12231, Research Diets, Inc., New Brunswick, NJ) of overall 5.2 kcal/g (25.5% carbohydrate, 16.4% protein and 58% fat) for 3 months or 9 months *ad libitum*. Food intake was measured by weighing the pellets once per week. All animals were cared for according to institutional guidelines, and all experiments were approved by the Institutional Animal Care and Use Committee at the Catholic University of Korea. In all experiments, each group consisted of at least 3–4 mice.

### 2.2. Hepatic pathological evaluation

Fresh liver tissue samples were fixed in 3.7% buffered formalin, and then embedded in paraffin wax. Liver pathology was assessed by hematoxylin-eosin (H&E) and Oil Red O staining of liver sections for evaluation of steatosis, fat droplets and inflammation. All stained slides were scored by two experienced pathologists and graded by a previously described classification (Jeen and Jin, 2009).

The degree of steatosis was graded on a four-point scale: grade 0, steatosis in <5% of hepatocytes; grade 1, steatosis in up to 33%; grade 2, steatosis in 33–66%; grade 3, steatosis in >66% of hepatocytes. Lobular inflammation was also graded on a four-point scale: grade 0, no foci; grade 1, fewer than two foci per 20× field; grade 2, two to four foci per 20× field; grade 3, more than four foci per 20× field. Hepatocyte ballooning was graded on a three-point scale: 0, none; 1, a few balloon cells; 2, many/prominent balloon cells. For the NAFLD activity score (NAS), features of steatosis, lobular inflammation, and hepatocyte ballooning were combined, and the range was scored from 0 to 8. Cases scoring ≥5.0 were diagnosed as NASH, while cases ≤2 were diagnosed as simple steatosis.

### 2.3. mRNA microarray experiments

Total RNAs were prepared independently from liver tissues of two different mice for each condition (3 month-HFD, 9 month-HFD and control) using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA integrity was assessed using an Agilent 2100 Bioanalyzer. RNA integrity numbers for all samples were above 8.5. RNA was then reverse-transcribed, amplified and hybridized to the Agilent SurePrint G3 mouse GE 8X60K microarray according to the Agilent's protocols. The probe intensities were obtained using the Agilent G2565BA microarray scanner and normalized using the quantile normalization method (Bolstad et al., 2003). The microarray data were deposited in the gene expression omnibus (GEO) database (accession ID: GSE59042).