Research Article

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p53/p66Shc-mediated signaling contributes to the progression of non-alcoholic steatohepatitis in humans and mice

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Background & Aims: The tumor suppressor p53 is a primary sensor of stressful stimuli, controlling a number of biologic processes. The aim of our study was to examine the roles of p53 in non-alcoholic steatohepatitis (NASH).

Methods: Male wild type and p53-deficient mice were fed a methionine- and choline-deficient diet for 8 weeks to induce nutritional steatohepatitis. mRNA expression profiles in normal liver samples and liver samples from patients with non-alcoholic liver disease (NAFLD) were also evaluated.

Results: Hepatic p53 and p66Shc signaling was enhanced in the mouse NASH model. p53 deficiency suppressed the enhanced p66Shc signaling, decreased hepatic lipid peroxidation and the number of apoptotic hepatocytes, and ameliorated progression of nutritional steatohepatitis. In primary cultured hepatocytes, transforming growth factor (TGF)-β treatment increased p53 and p66Shc signaling, leading to exaggerated reactive oxygen species (ROS) accumulation and apoptosis. Deficient p53 signaling inhibited TGF-β-induced p66Shc signaling, ROS accumulation, and hepatocyte apoptosis. Furthermore, expression levels of p53, p21, and p66Shc were significantly elevated in human NAFLD liver samples, compared with results obtained with normal liver samples. Among NAFLD patients, those with NASH had significantly higher hepatic expression levels of p53, p21, and p66Shc compared with the group with simple steatosis. A significant correlation between expression levels of p53 and p66Shc was observed.

Conclusions: p53 in hepatocytes regulates steatohepatitis progression by controlling p66Shc signaling, ROS levels, and apoptosis, all of which may be regulated by TGF-β. Moreover, p53/p66Shc signaling in the liver appears to be a promising target for the treatment of NASH.

Keywords: P53; Non-alcoholic steatohepatitis; P66Shc; Reactive oxygen species; Transforming growth factor-β.

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Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; ROS, reactive oxygen species; MCD, methionine-deficient and choline-deficient; ALT, alanine aminotransferase; HE, haematoxylin-eosin; HNE, hydroxynonenal; TUNEL, terminal deoxy-nucleotidyl transferase-mediated nick end-labeling; PCR, polymerase chain reaction; TGF-β, transforming growth factor-β; PFT, pifithrin; Col11, 1 (I) collagen; Col12, 2 (I) collagen; SMA, smooth muscle actin; MDA, malondialdehyde.

Introduction

Non-alcoholic fatty liver disease (NAFLD) afflicts as much as 20% of the US adult population [1]. Non-alcoholic steatohepatitis (NASH)—part of the spectrum of NAFLD—is the most prevalent liver disease in the US, affecting approximately 3–4% of the population [1].

NAFLD and NASH are often co-morbid with disorders characterized by insulin resistance, such as diabetes and obesity. Thus, these liver diseases can be considered hepatic manifestations of metabolic syndrome. Given the growing number of patients with metabolic syndrome, the incidences of NAFLD and NASH are expected to increase further, particularly in North America, Europe, Asia, and countries in the Western Pacific.

NASH is a progressive disease. In a study that followed NASH patients for ten years, the disease progressed to cirrhosis in 20% of the patients and led to fatal liver disease in 8% of the cases [2]. A population-based cohort study demonstrated that

Table 1. Histological characteristics of patients.

	NASH (n = 57)	Simple steatosis (n = 13)
Steatosis		
1	31	10
2	18	3
3	8	0
Inflammatory activity		
0	0	11
1	9	2
2	43	0
3	5	0
Fibrosis stage		
0	0	13
1	35	0
2	14	0
3	8	0
4	0	0

approximately 3% of the patients diagnosed with NAFLD developed cirrhosis or a liver-related complication [3]. The progressive nature and serious consequences of NASH highlight the need for effective therapies. The pathologic mechanisms underlying NASH, however, have not yet been clarified.

Recently, a number of diagnostic tests that incorporate clinical markers, including age, have been reported for NAFLD. Indeed, advanced age is a major risk factor for the progression of NASH [4]. On the other hand, the tumor suppressor p53—a master sensor of stressful conditions—controls many biological processes, including aging [5]. Reactive oxygen species (ROS), which are thought to make major contributions to aging, stimulate p53 stabilization and subsequent induction of apoptosis via a feed-forward regulatory loop [6]. Hepatic p53 expression is elevated in patients with NASH [7]. A recent report has also shown that hepatic p53 expression and hepatocyte apoptosis significantly increase in a mouse model of NASH [8]. These results suggest that p53 plays a role in the pathophysiology of NASH.

In the present study, we examined the role of p53 signaling during NASH using p53-deficient mice and a mouse model of NASH. We found that hepatic p53 signaling markedly contributes to the pathogenesis of NASH. Our findings suggest hepatic p53 signaling as a promising target for new modalities in the treatment of NASH.

Materials and methods

Please refer to the Supplementary Materials and methods section for more detailed descriptions.

Animal studies

Eight-week-old male C57BL/6J mice were purchased from CLEA Japan Inc. $p53^{+/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). $p53^{+/+}$ and $p53^{-/-}$ mouse littermates were obtained from crosses of $p53^{+/-}$ mice with the C57BL/6J background.

In experiments for nutritional steatohepatitis, 8-week-old male $p53^{+/+}$ mice or $p53^{-/-}$ mouse littermates were fed a methionine- and choline-deficient (MCD) diet (cat No. 960439; ICN, Aurora, Ohio) or a standard chow (CE-2; CLEA Japan Inc.) for 8 weeks. In order to make a time-course analysis of nutritional steatohepatitis, 8-week-old male C57BL/6J mice were fed an MCD diet for 3 or 8 weeks.

All animals received humane care in compliance with the National Research Council's criteria outlined in the "Guide for the Care and Use of Laboratory Animals", prepared by the US National Academy of Sciences and published by the US National Institutes of Health.

Human liver tissue samples

Liver tissues were obtained from 70 patients undergoing ultrasound-guided liver biopsy for suspected NASH and from 10 patients undergoing surgical operation at the Keio University School of Medicine and National Defense Medical College. Patient characteristics are shown in Table 1. Written informed consent was obtained from all patients. This study protocol was approved by the Ethical Committee of the Keio University Hospital and National Defense Medical College Hospital, and followed the ethical guidelines of the Declaration of Helsinki.

Statistical analysis

Data are expressed as means (SEM) or median and interquartile range. Statistical analyses were performed using unpaired Student's *t* test, one-way analysis of variance (ANOVA), or Mann–Whitney's U test for univariate comparison, as appropriate. Correlations were assessed using the Pearson product-moment correlation coefficient. Differences were considered statistically significant at p values less than 0.05. All of the computations were performed with a commercial statistical package (SPSS version 12, Chicago, USA).

Results

p53 deficiency ameliorates the progression of nutritional steatohepatitis in mice

Following administration of an MCD diet, mice rapidly and consistently develop a severe form of steatohepatitis with the characteristic pathology of steatosis, mixed cell inflammatory infiltrate, hepatocyte death, and pericellular fibrosis, which resembles human NASH [9]. Therefore, to examine the effects of p53 signaling on the progression of NASH, we fed wild type and p53-deficient mice an MCD diet for 8 weeks. After 8 weeks of MCD diet, the p53-deficient mice exhibited significantly fewer lipid droplets in their hepatocytes, and reduced infiltration of inflammatory cells into their livers, compared with those observed in the wild type group (Fig. 1A). The MCD diet also produced a higher increase of serum alanine aminotransferase (ALT) levels in wild type mice than in p53-deficient mice (Fig. 1B). After 8 weeks of MCD diet, the livers of wild type mice exhibited a significant increase in lipid droplets in hepatocytes and elevated hepatic TG concentrations compared with p53-deficient mice (Fig. 1C).

Moreover, the livers of wild type mice showed increased collagen deposition, whereas fibrosis was markedly reduced in the livers of p53-deficient mice (Fig. 1A). Quantification of Masson trichrome staining and liver hydroxyproline levels confirmed the histologic results (Fig. 1D). Those levels were significantly lower in p53-deficient mice compared with wild type mice (Fig. 1D). Real-time PCR analyses of whole liver homogenates from mice fed the MCD diet revealed significantly increased mRNA levels of collagen type I α1 (*Col1α1*), collagen type I α2 (*Col1α2*), and transforming growth factor *(TGF)-β*, compared with those of mice fed the control diet. Compared with the wild type