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Hepatology Research 34 (2006) 256–265
HEPATOLOGY RESEARCH
http://www.elsevier.com/locate/hepres

Hepatic gene expression in hepatocyte-specific Pten deficient mice showing steatohepatitis without ethanol challenge

Wataru Sato [a], Yasuo Horie [a], Ei Kataoka [a], Shigetoshi Ohshima [a], Takahiro Dohmen [a], Masahiro Iizuka [a], Junko Sasaki [b], Takehiko Sasaki [b], Koichi Hamada [c], Hiroyuki Kishimoto [c], Akira Suzuki [c], Sumio Watanabe [a,*]

[a] *Division of Gastroenterology, Department of Internal Medicine, Akita University School of Medicine, Akita, Japan*
[b] *Division of Microbiology, Department of Pathology, Akita University School of Medicine, Akita, Japan*
[c] *Division of Molecular Biology, Department of Anatomy and Biochemistry, Akita University School of Medicine, Akita, Japan*

Received 26 December 2005; received in revised form 9 January 2006; accepted 14 January 2006
Available online 21 February 2006

Abstract

Hepatocyte-specific Pten deficient (Pten KO) mice possess almost the same hepatic lesions histologically as human NASH and are thought to represent some limited NASH patients. We analyzed a comprehensive gene expression of hepatocytes derived from 10- to 35-week-old Pten KO mice using the DNA microarray technology to find out the candidate genes related to development and aggravation of human NASH. Spp1, Vnn1, Itga6, Abcd2, Auh, Acox1, Pdk4, Cpt1a, Lcn2, Igfbp2, Gstm6, Socs3, Tgm2, and Aldh9a1 were regarded as the candidate genes related to inflammation. The candidate genes of fibrosis were Spp1, Ctgf, and Cyp2c39 and moreover Cidec and Spp1 were regarded as the candidate genes of carcinogenesis. To confirm that these genes contribute to the etiology of some human NASH, further investigations using human liver samples are needed.

Keywords: DNA microarray; Steatosis; Inflammation; Fibrosis; Carcinogenesis; Pten

1. Introduction

It was recognized that fatty liver was a reversible pathological condition and rarely progressed to liver cirrhosis or hepatocellular carcinoma (HCC). In 1980, however, Ludwig first reported nonalcoholic steatohepatitis (NASH), which initially developed as fatty liver but took the progressive course from steatohepatitis to liver cirrhosis and HCC [1]. In the developed countries, the number of fatty liver patients is increasing rapidly with the increase in people with obesity, diabetes, insulin resistance, and hyperlipidemia, and NASH is becoming a critical public health problem.

NASH is defined as a disease showing the histological findings similar to those of alcoholic steatohepatitis such as marked lipid deposits in hepatocytes, ballooning hepatocytes, inflammatory cells infiltration, presence of Mallory bodies, and liver fibrosis without history of excessive alcohol consumption. The most predominant mechanism of NASH is the two-hit hypothesis in which addition of certain stress to the preceding fatty liver promotes development and progression of steatohepatitis [2]. Lipid deposits in hepatocytes (first hit) are likely to be induced clinically by obesity, diabetes, insulin resistance, and hyperlipidemia. It is suggested that the same mechanism as alcoholic steatohepatitis contribute to inflammation (second hit). Accordingly, oxidative stress, lipid peroxidation, and endotoxin are probably involved in inflammation although the detailed mechanism remains unknown. The mechanism of liver fibrosis, cirrhosis and HCC secondary to inflammation also remains unclear. Therefore, the strategies for prevention and treatment of these condi-

* Corresponding author at: Division of Gastroenterology, Department of Internal Medicine, Akita University School of Medicine, Hondo 1-1-1 Akita, Akita 010-8543, Japan. Tel.: +81 18 884 6104; fax: +81 18 836 2611.
E-mail addresses: wataroo@doc.med.akita-u.ac.jp (W. Sato), sumio@doc.med.akita-u.ac.jp (S. Watanabe).

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doi:10.1016/j.hepres.2006.01.003

tions that determine the prognosis of NASH have not been established.

Generally, analysis of gene targeting mice with the phenotype similar to a certain human disease provides a very useful clue to clarify the etiology of the disease. Because the gene targeting mice exhibit the specific symptom in the predicted time course, we can expect the onset of specific symptoms in these mice. Therefore, samples before and after the onset of symptoms can be obtained, enabling the analysis not only of the pathological conditions in the backdrop of the specific symptom but also of the causative factor of the symptom.

Currently, the mechanism to develop steatosis in the liver is being clarified by analyzing various gene targeting mice with fatty liver. These gene targeting mice, however, rarely showed steatohepatitis, liver fibrosis, and HCC that were found in human NASH. Consequently, analysis of the mechanism of steatohepatitis, liver fibrosis, and carcinogenesis has rarely been undertaken on animal models. Against this backdrop, we developed the hepatocyte-specific Pten deficient mice (Pten KO mice) that suffered from fatty liver, steatohepatitis, liver fibrosis, and HCC. Although Pten KO mice livers have histological findings strikingly similar to those reported in NASH [3], these mice do not develop obesity, diabetes, insulin resistance, and hyperlipidemia, which are clinical characteristics of NASH, prior to fatty livers [3]. We know that there are a few NASH patients who do not show obesity, diabetes, insulin resistance, and hyperlipidemia. Therefore, we think that Pten KO mice are an animal model of such a few NASH patients but a precious model, which reproduce all disease stage of NASH including inflammation, fibrosis, and carcinogenesis.

We investigated the changes in gene expression involved in steatosis, inflammation, fibrosis, and carcinogenesis using DNA microarray analysis of hepatocytes derived from Pten KO mice at different weeks of age. The data obtained in this experiment represent gene expression profile of limited NASH patients. However, the gene expression profiles about inflammation, fibrosis, and carcinogenesis give us valuable information on a genetic level to develop and aggravate NASH although further investigations are needed to draw some original conclusions.

2. Materials and methods

2.1. Mice

Pten$^{flox/flox}$ mice (129Ola × C57BL6/J F_2), generated as previously described, were mated to *AlbCre* transgenic mice (C57BL6/J background; The Jackson Laboratory, Bar Harbor, ME), in which expression of Cre is controlled by the promoter of the hepatocyte-specific gene *Albumin* [4,5]. Offspring carrying *AlbCre* and two copies of the floxed *Pten* allele (*AlbCrePten*$^{flox/flox}$), *Albcre* plus one copy of the floxed *Pten* allele (*AlbCrePten*$^{flox/+}$), and *AlbCre* plus two copies of the wild-type *Pten* allele (*AlbCrePten*$^{+/+}$) were generated. The relative abundance of intrahepatic messenger RNA (mRNA) for a broad array of genes was measured in four groups (1) 10-week-old *AlbCrePten*$^{flox/flox}$ mice (10-week-old Pten KO mice, $n = 3$); (2) 10-week-old *AlbCrePten*$^{+/+}$ mice (10-week-old control mice, $n = 3$); (3) 35-week-old *AlbCrePten*$^{flox/flox}$ mice (35-week-old Pten KO mice, $n = 3$); and (4) 35-week-old *AlbCrePten*$^{+/+}$ mice (35-week-old control mice, $n = 3$). All mice were male. All animal experiments were approved by the Institutional Review Board of the Akita University School of Medicine.

2.2. Histology

To estimate the presence or absence of inflammation, fibrosis, and neoplasms in the livers of Pten KO and control mice at 10 and 35 weeks of age, formalin-fixed tissues were embedded in paraffin using standard procedures. Sections (4 μm thick) were cut and stained with either H&E for standard microscopy or Azan stain to show fibrosis.

2.3. Hepatocytes and RNA isolation

Hepatocytes were isolated from mice by modification of the procedure described by Seglen [6]. The isolated hepatocytes were filtered through a 70 μm cell strainer (BD Biosciences, Bedford, MA) and suspended in Ca^{2+}- and Mg^{2+}-free Krebs–Ringer-*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (KRH) buffer (pH 7.4) on ice for 10 min. The cell suspension was centrifuged for 2 min at 50 × *g*, and the pellet was resuspended on ice. The resuspended hepatocytes were washed with cold Ca^{2+}- and Ma^{2+}-free phosphate buffered saline three times to purify them. Total RNA was isolated from purified control and Pten KO hepatocytes using a RNeasy Mini Kit (QIAGEN, Hilden, Germany) and a RNeasy Lipid Tissue Midi Kit (QIAGEN, Hilden, Germany), respectively. Different RNA isolation kits were used because KO hepatocytes had more lipid deposits than control hepatocytes. Each three RNA samples derived from 10-week-old Pten KO mice, 10-week-old control mice, 35-week-old Pten KO mice, and 35-week-old control mice were equally mixed and served as templates for the DNA microarray analysis. The quality of these RNA samples was assessed using the Agilent 2100 Bioanalizer (Agilent Technologies Inc., Palo Alto, CA) to ensure their integrity before use.

2.4. Linear RNA amplification

Pten KO and control RNAs thus isolated were amplified, converted to complementary DNAs (cDNAs), and labeled with cyanin 3 (Cy3)-CTP and cyanin 5 (Cy5)-CTP using the LRIFLA kit (Agilent Technologies, Palo Alto, CA) following the protocol discussed in the user's manual [7], respectively. As a result, the amplified complementary RNA (cRNA) products derived from control and Pten KO mice were labeled with Cy3 and Cy5, respectively.