Expression of Notch family is altered in non-alcoholic fatty liver disease

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Received November 16, 2019; Accepted May 15, 2020

DOI: 10.3892/mmr.2020.11249

Abstract. The aim of the present study was to explore the dynamic relationship between Notch and non-alcoholic fatty liver disease (NAFLD), both in vitro and in vivo. The LX2, Huh7 and MIHA hepatic cell lines were used to establish a cell steatosis model induced by palmitic acid (PA) at different concentrations (0.1, 0.25 and 0.5 mM). Cell proliferation and migration were assessed using a 5-bromo-2'-deoxyuridine kit and a wound healing assay. The dosage of 0.25 mM PA for 36-48 h treatment was chosen for subsequent experiments. Steatotic cells were identified by Oil Red O staining. Feeding mice a methionine-choline-deficient (MCD) diet is known induce a model of NAFLD, compared with a methionine-choline-sufficient (MCS) diet. Therefore, Notch family mRNA expression was evaluated in the liver of MCD-fed mice at varying time points (days 5, 10, 21 and 70) using reverse transcription-quantitative PCR. Notch expression levels were also assessed in cell lines at 12, 24, 36 and 48 h after PA treatment. Notch signaling molecules changed in the PA or MCD model over time. In vitro, the mRNA levels of Notch1, -2 and -4 increased in all cell lines after 12-h PA treatment. At 24 h, these genes were upregulated only in LX2 cells, while showing a ‘down-up’ pattern in MIHA cells (i.e. these genes were downregulated at 24 h but upregulated at 36 h). However, expression of Notch1, -2, -3 and -4 mRNA rose significantly in the early stage (day 10) of NAFLD. At week 3, the levels of Notch1 and -2 were higher in the MCD group than in the MCS group, while the reverse was observed for Notch3 and -4. Expression of these four genes increased again in the late stage (day 70) of NAFLD. Therefore, these results indicated that Notch family members Notch1-4 were involved in the development of NAFLD and played an important role in steatosis in this model.

Introduction

Non-alcoholic fatty liver disease (NAFLD) refers to a spectrum of liver pathologies with hepatic fat accumulation, which are collectively the leading cause of chronic liver disease (1-3). The pathogenesis of NAFLD is not fully understood. The widely accepted ‘multiple hits’ hypothesis suggests that excessive oxidative metabolites and lipid peroxidation occur as a result of insulin resistance (IR). This causes oxidative stress (OS) and continuously damages the mitochondria within hepatocytes, while producing inflammatory mediators and cytokines (1,3). Hepatocytic inflammation and necrosis with steatosis is referred to as non-alcoholic steatohepatitis (NASH). Without intervention, NASH can exacerbate IR and inflammation, causing fatty liver accumulation and increasing the risk of liver cirrhosis and cancer (1,3). Currently, effective NAFLD treatment is still lacking (4).

The Notch family was initially discovered in Drosophila and is expressed in a number of species. Notch molecules serve an important role in the embryonic development of organisms, as well as the occurrence of malignant tumors (5,6). Previous studies predominantly focused on the role of Notch genes in the regulation of cell differentiation, proliferation and apoptosis (7,8). However, it has also been demonstrated that Notch genes are involved in cell metabolism. Indeed, changes in Notch gene expression leads to dysfunction in glucose and lipid metabolism, which can lead to IR, lipid deposition and obesity (9,10). Therefore, the relationship between Notch genes and metabolic diseases has gradually become a research focus.

NAFLD is the hepatic manifestation of metabolic syndrome. With recent changes in living standards and lifestyle, the incidence of NAFLD is rising rapidly, along with those of type 2 diabetes mellitus (T2DM) and overweight/obesity (11,12). Nevertheless, the association of the Notch family with NAFLD is rarely reported (13). Therefore, the aim of the present study

Key words: Notch signaling genes, palmitic acid, non-alcoholic fatty liver disease, mice
was to examine the dynamic association between Notch and NAFLD, both in vitro and in vivo. The time course of Notch gene expression was assessed in liver cell lines treated with palmitic acid (PA), as well as a murine NAFLD model induced by a methionine-choline-deficient (MCD) diet.

Materials and methods

Cell culture and treatments. The following hepatic cell lines were obtained from The Beijing Union Medical College Resource Center and cultured in DMEM: i) Hepatic stellate cell line LX2; ii) hepatocellular carcinoma (HCC) cell line Huh7; and iii) human immortalized hepatocytes MIHA. MIHA, LX2 and Huh7 cell lines were chosen so that different stages of NAFLD (hepatocytes steatosis, fibrosis and carcinoma, respectively), would be represented. PA (MedChemExpress) was dissolved in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 1% BSA (Sigma-Aldrich; Merck KGaA) and filtered through a 0.22-μm filter, then added to the cells and incubated at 37°C to 80% confluency in DMEM with 10% FBS. Filter-sterilized complete DMEM with 1% BSA without PA was used as a control. The concentrations of PA used were 0.1, 0.25 or 0.5 mM, and the incubation times were 12-72 h.

Cell proliferation and migration assay. Cell proliferation under different PA concentrations was assessed using a 5-bromo-2′-deoxyuridine assay kit (Sigma-Aldrich; Merck KGaA), following the manufacturer’s instructions. Cell migration was evaluated using a wound healing assay. A total of three cell lines were treated with or without PA. A thick black line was drawn under each plate as a base line, and shown in every field under a Leica DC 300F optical microscope (magnification, x400). The plate was scratched with the head of a pipette to disrupt the cellular growth, creating a break in the cells that simulates an injury. The scratch is vertical to the black line under the plate. The wound width (WW) was measured in three microscope fields. For every condition, the lower the ratio, the faster the cell migration. ImageJ software version 1.44p (National Institutes of Health) was used for imaging.

Oil Red O (ORO) staining. All three cell lines were counted and seeded into 24-well tissue culture plates in DMEM with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37°C to 80% confluency, then exposed to 0.25 mM PA. Cells were observed 12-72 h after PA treatment. Cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min, washed by 60% isopropanol for 1 min, and stained with fresh ORO dissolved in 60% isopropanol (Sigma-Aldrich; Merck KGaA) for 30 min, as previously described (14). Then, cells were washed with ddH2O, counterstained with hematoxylin for 1 min, and mounted by Permount solution (Thermo Fisher Scientific, Inc.). Finally, the cells were examined under a light microscope (magnification, x400) (Leica Microsystems GmbH) to observe lipid droplets and identify steatotic cells.

Expression of Notch signaling pathway genes. Total RNA was extracted from cell lines and primary mouse liver tissues using an RNeasy Mini kit (Qiagen GmbH). Primers and probes for qRT-PCR were purchased from Applied Biosystems (Thermo Fisher Scientific, Inc.). Sequences of primers are listed in Table S1. RNAase inhibitor, dNTP and Oligo (dT) were from Toyobo Life Science. RNA was then used for cDNA synthesis (42°C for 60 min, 70°C for 15 min, and 4°C) by reverse transcription according to the manufacturer’s instructions. Reverse transcription-quantitative PCR (RT-qPCR) was performed using a SYBR Green kit (Takara Biotechnology Co., Ltd.) in an ABI 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The standard conditions for qPCR were: 50°C for 10 min and 95°C for 15 and 45 sec annealing/elongation at 58°C or 60°C. GAPDH was used as an internal control. Relative gene expressions were calculated using the 2−ΔΔCq method. RT-qPCR experiments were repeated three times (15).

Animals and in vivo assay. A total of 80, 4-6-week-old, male C57BL/6 nude mice (weight, 13-20 g) were obtained from The Institute of Laboratory Animal Science (Chinese Academy of Medical Sciences). All animals were housed in a controlled room with 22.0±2.0°C temperature and 65±5% humidity under a 12-h dark-light cycle. Mice were randomized into 2 groups (n=40 in each group) receiving different diets. The MCD group was used as the NAFLD model, and the methionine-choline-sufficient (MCS) group served as a negative control. Both groups were examined daily, then divided into four subgroups (n=10 in each subgroup) euthanized at different time points (days 5, 10, 21 and 70) (16,17). All mice were subjected to overnight fasting, anesthetized by intraperitoneal injection of 400 mg/kg chloral hydrate, then euthanized by exsanguination performed by cardiac puncture. The liver tissues were harvested only after the mice were confirmed to lose the vital signs. No animal died accidentally during the experiment. All experiments were approved by the Ethics Committee of Xin Hua Hospital (Shanghai Jiao Tong University School of Medicine).

Harvested livers were frozen at -70°C for subsequent examination of the expression levels of Notch1-4, hairy and enhancer of split-1 (Hes1) and Hes-related family bHLH transcription factor with YRPW motif 1 (Hey1) using RT-qPCR.

Statistical analysis. A total of three independent experiments were performed. Data are presented as the mean ± SD. Multi-group comparisons were carried out using one-way ANOVA followed by Tukey’s post hoc test. P<0.05 was considered to indicate a statistically significant difference. All data were analyzed with SPSS 13.0 statistical package (SPSS, Inc.).

Results

Effect of PA on proliferation and migration of hepatic cells. To determine the effects of PA exposure on proliferation and migration of hepatic cells, Huh7, MIHA and LX2 cells were treated with 0.10, 0.25 or 0.50 mM PA for different incubation times. After 12 h of exposure to PA, cell proliferation increased in Huh7, MIHA and LX2 cells, compared with their respective untreated controls. Proliferation reached a maximal value at 36 h for LX2 and MIHA, and at 48 h Huh7 cells. Peak proliferation was observed with 0.1 mM PA in the Huh7 and MIHA cell line, as well as 0.25 mM for LX2. MIHA
Figure 1. LX2, Huh7 and MIHA cell lines were treated with filter-sterilized complete DMEM with 1% BSA without PA (CTL) or various concentrations of PA (0.1, 0.25 or 0.5 mM) over time. (A) Proliferation rates of hepatic cell lines following PA treatment (ratio to CTL). (B) Microscopic images of cell migration in hepatic cell lines following PA treatment (magnification, x400). (C) Quantification of cell migration. Data are presented as the mean ± SD. *P<0.05. PA, palmitic acid; CTL, control.
proliferation ratio seemed close at certain timepoint whatever PA concentrations (Fig. 1A).

In addition, a wound healing assay indicated more suspended (dead) LX2 cells in the medium at PA 0.25 mM after 48 h (WW, 0.70±0.03), and 0.5 mM from 24 h (WW, 0.78±0.03) onward (Fig. 1B; Table SII). Notable growth was observed between 12 and 72 h following treatment with 0.25 mM PA (P<0.05). The Huh7 cell line displayed increased growth rates between 12 and 72 h at all concentrations of PA (Fig. 1B and C). The WW at 0.1 and 0.25 mM PA were lower than that at 0.5 mM (Table SII). However, PA did not affect the MiHa growth rate at any concentration (P>0.05). Therefore, a dosage of 0.25 mM PA was used for subsequent experiments. It should be noted that the shape of the suspended LX2 cells was
different from that of the adherent cells, and were excluded when WW was calculated (Table SII).

**PA induces steatosis in hepatic cells.** ORO stains intracellular neutral lipids (18). Hepatic cells were stained with ORO in the presence or absence of PA (Fig. 2). Following exposure to PA, notably more lipid droplets were observed in three microscope fields, compared with the control. In addition, a time-dependent accumulation of lipid appeared in LX2, Huh7 and MIHa cells treated with 0.25 mM PA. These results suggested that PA could enhance lipid synthesis and steatosis in hepatic cell lines.

**Notch family gene expression in vitro.** RT-qPCR assays were carried out to examine the expression of Notch pathway genes in LX2, Huh7 and MIHa cells following 0.25 mM PA exposure. Notch pathway members Notch1, -2, -3, -4, Jagged1, Jagged2, Δ-like canonical Notch ligand (DLL) 1 and DLL3, Hey1 and Hes1 were affected by PA exposure. The expression of these signaling molecules followed varying trends over the course of the experiment (Fig. 3). After 12 h PA treatment, the expression of Notch4 was significantly upregulated in Huh7 lines, compared with control. The expression of Notch3 was similar in LX2 and Huh7, but significantly downregulated in MIHA cells at 12 h. At the 24-h time point, Notch2, -3 and -4 genes were upregulated only in the hepatic stellate cell line LX2, compared with the control. At this time, point, Notch1 and -4 were significantly downregulated in MIHA cells, while, Notch3 was upregulated in Huh7 cells. However, the expression of these four Notch genes partly recovered slowly with continuous PA exposure at the endpoint of the experiment [CTL vs. 36 h: Notch1 (Huh7), Notch2 (MIHA), Notch3 (LX2), Notch4 (LX2), P<0.05] [CTL vs. 48 h: Notch3 (Huh7, MIHA), Notch4 (LX2, Huh7), P<0.05]. For Jagged1/2, DLL1/3, Hey1 and Hes1, there were no regular trend changes found in the three cell lines, although their expression was statistically different at some time points.

**Abnormal expression of Notch signaling pathway genes during NAFLD in vivo.** C57BL/6 mice were fed an MCD diet (Fig. 4). The MCD model is mainly characterized by steatosis and inflammatory responses of hepatocytes at the third week, while liver fibrosis begins at week 8 (16,17). Notch1, -2, -3 and -4 mRNA decreased significantly in MCD-fed mice at the early stage of NAFLD, compared with MCS mice (day 5; P<0.05). This trend changed from day 10, at which point, the expression levels of Notch1 and -3 were significantly upregulated (P<0.05). At the final time point, the expression of all Notch genes was significantly higher in MCD-fed mice, compared with MCS controls (day 70; P<0.05).

**Discussion**

NAFLD is the main cause of chronic liver disease and cirrhosis worldwide, irrespective of age (19). The spectrum of NAFLD includes steatohepatitis, NASH and associated cirrhosis, and HCC (1,2). Although steatosis presents minimal clinical manifestations, NASH with lobular inflammation is regarded as a driving force in the progression of NAFLD. The ‘multi-hit’ theory is the most widely accepted theory accounting for the complex pathophysiology of NAFLD (12,13,16). The onset of NAFLD is characterized by the production of reactive oxygen species, reduced levels of β-oxidation and increased lipogenesis, followed by lipid accumulation in hepatocytes along with cellular inflammation (20). In addition, adaptive immunity, dysfunction of Notch signaling, vitamin D deficiency and
sleep deprivation have been reported as additional factors promoting liver inflammation or injury during NAFLD process (11,13,21-23). In the present study, an NAFLD cell line was established and verified using ORO staining. In order to examine the PA dosage and treatment duration required in this model, cell proliferation and migration were assessed in liver cells treated with PA over time. Peak proliferation was observed at 36 h (LX2, MIHA) or 48 h (Huh7) with 0.1 and 0.25 mM PA, respectively. In addition, increased cell death was observed in LX2 cells when PA was 0.25 mM (≥48 h) and 0.5 mM (from 24 h onward). Thus, a concentration of 0.25 mM PA was used in subsequent experiments, with a 36 to 48-h treatment time.

The Notch signaling pathway regulates downstream genes and plays important roles in the physiological and pathological processes of cell differentiation, proliferation, apoptosis, embryonic development and tumor formation (24,25). Previous studies suggested that the effects of Notch signaling vary between different types of tumor, or within the same tumor during different periods. In the liver, the Notch family is associated with the onset and development of a number of liver diseases (26,27). Aimaiti et al (28) demonstrated that LY450139, an inhibitor of the notch pathway, could decrease hepatic cell proliferation and apoptosis in patients with NAFLD (20). Further experiments suggested that the effects of notch signaling vary between different types of tumor, or within the same tumor during different periods. In the liver, the Notch family is associated with the onset and development of a number of liver diseases (26,27). Aimaiti et al (28) demonstrated that LY450139, an inhibitor of the notch pathway, could decrease hepatic cell proliferation and apoptosis in patients with NAFLD (20). Future experiments should focus on clinical samples or HFD animal models to verify the conclusion that Notch expression levels change dynamically in NAFLD and are differentially distributed across disease stages. Moreover, while the same dynamic between Notch genes and NAFLD was observed both in vitro and in vivo, the underlying mechanism and potential associations between different Notch molecules require extensive in-depth studies. Future studies should focus on these aspects.

In conclusion, the present study demonstrated that PA or MCD regulated the levels of Notch signaling genes during NAFLD. The Notch family participated in the development of NAFLD. These findings might provide a direction for molecular-targeted therapy and early detection of NAFLD. However, additional studies are needed to assess these possibilities.

Acknowledgements
Not applicable.

Funding
The present study was supported by grants from The National Natural Science Foundation of China (grant nos. 81400610, 81400799 and 81470840), the Cross-Institute Research Fund of Shanghai Jiao Tong University (grant no. YG2016MS72), The Project Sponsored by the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry (grant no. 20144802) and The Shanghai Municipal Commission of Health and Family Planning for Youth (grant no. 2013Y043).
manuscript. WJW generated and revised the figures. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were approved by the Ethics Committee of Xin Hua Hospital affiliated to Shanghai Jiao Tong University School of Medicine.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References