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Original Article

Increased expression of Zinc finger protein 267 in non-alcoholic fatty liver disease

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Abstract: Hepatocellular lipid accumulation is a hallmark of non-alcoholic fatty liver disease (NAFLD), which encompasses a spectrum ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) and ultimately cirrhosis. Zinc finger protein 267 (ZNF267) belongs to the family of Kruppel-like transcription factors, which regulate diverse biological processes that include development, proliferation, and differentiation. We have previously demonstrated that ZNF267 expression is up-regulated in liver cirrhosis and is further increased in hepatocellular carcinoma (HCC). Here, we analyzed the expression of ZNF267 in tissue specimens of NAFLD patients and found a significant up-regulation compared to normal liver tissue. Noteworthy, ZNF267 mRNA was already significantly increased in steatotic liver tissue without inflammation. In line with this, incubation of primary human hepatocytes with palmitic acid induced a dose-dependent lipid accumulation and corresponding dose-dependent ZNF267 induction *in vitro*. Furthermore, hepatocellular lipid accumulation induced formation of reactive oxygen species (ROS), and also chemically induced ROS formation increased ZNF267 mRNA expression. In summary with previous findings, which revealed ZNF267 as pro-fibrogenic and pro-cancerogenic factor in chronic liver disease, the present study further suggests ZNF267 as promising therapeutic target particularly for NAFLD patients. In addition, it further indicates that hepatic steatosis *per se* has pathophysiological relevance and should not be considered as benign.

Keywords: Non-alcoholic fatty liver disease, Kruppel-like factor, ZNF267

Introduction

The Kruppel-like transcription factor (KLF) family is involved in the regulation of diverse aspects of cellular development, differentiation, growth, and activation [1]. KLFs contain multiple zinc fingers, which represent one of the most common DNA binding domains. A zinc finger contains two cysteine and two histidine residues that coordinate a single zinc ion and fold the domain into a finger-like projection [2]. Seventeen mammalian KLFs have been identified and some of them have been recognized to be involved in numerous disorders such as cancer, obesity, cardiovascular disease, and inflammatory conditions [1].

KLF proteins are also involved in the development and progression of different chronic liver disease. For example, Kruppel-like factor 15

activates hepatitis B virus gene expression and replication [3]. KLF8 is up-regulated in hepatocellular cancer (HCC), promotes tumour invasion and indicates a poor prognosis for patients with liver cancer [4]. In contrast, KLF6 (also named human zinc finger 9 (ZF9) or COPEB) is a tumor suppressor gene for HCC [5,6]. Furthermore, KLF6 is up-regulated in association with increased steatosis, inflammation, and fibrosis in non-alcoholic fatty liver disease (NAFLD) [7,8].

One further member of the Kruppel-like zinc finger family is zinc finger protein 267 (ZNF267; also named human zinc finger 2 or HZF2) [9]. It contains a conserved Kruppel associated box (KRAB) domain in the amino terminal part, which is separated through a linker region from a clustered zinc finger domain [9]. The first zinc finger is followed by three degenerated fingers,

and then continues with 13 zinc fingers at the carboxy terminus [9]. The function of ZNF267 is widely unknown; its mRNA is up-regulated upon nitric oxide treatment in venous endothelial cells [10], and previously, we have demonstrated that ZNF267 mRNA is up-regulated in liver cirrhosis compared to normal human liver tissue [11]. Furthermore, we found that ZNF267 expression is increased in human HCC-cells and tissue compared to primary human hepatocytes (PHH) and corresponding non-tumorous hepatic liver tissue, respectively [12].

Nonalcoholic fatty liver disease (NAFLD) is today recognized as the most common liver disease and one of the most frequent causes of liver cirrhosis [13,14]. Furthermore, NAFLD is a main risk factor for the development and progression of HCC [15]. NAFLD is characterized by lipid accumulation in the liver that may progress from simple hepatic steatosis towards inflammation (non-alcoholic steatohepatitis [NASH]) with progressive fibrosis [16,17,18].

NAFLD is strongly associated with the metabolic syndrome and its defining pathophysiological components obesity, diabetes and dyslipidemia. Thus, overeating delivers an excess of triglycerides to the liver. Further, peripheral lipolysis in obese and insulin resistant states leads to an increased hepatic influx of fatty acids (FA). In addition, hepatic FA synthesis is increased secondary to elevated glucose and insulin levels. Together, both uptake of exogenously derived FA and de novo hepatic synthesis of FA lead to an increase of hepatic lipid content, e.g. hepatic steatosis [19,20,21].

In the present study we aimed to assess the effect of hepatic steatosis on ZNF267 expression in an in vitro model of hepatocellular lipid accumulation [22] and in hepatic specimens of NAFLD patients.

Materials and methods

Cells and cell culture

Isolation and culture of primary human hepatocytes (PHH) were performed as described [23]. The HCC cell line HepG2 (ATCC HB-8065) was cultured as described previously [24,24]. Hepatocellular lipid accumulation was induced as described [25]. Briefly, a 10 mM palmitate/1% bovine serum albumin (BSA) stock solution was

obtained by complexing the appropriate amount of palmitate to BSA. PHH were grown in DMEM supplemented with penicillin (400 U/ml), streptomycin (50 mg/ml), L-glutamine (300 mg/ml), 0.2% FCS and palmitate for 24h in different concentrations as indicated. FFA-free-BSA-treated cells served as controls.

To induce ROS formation cells were incubated with 10 μ M arsenic trioxide (As₂O₃; Sigma Aldrich) as described [12].

Human tissue

Human liver specimens from patients were obtained during resection of metastasis of non-hepatic tumors. Tissue samples were immediately snap frozen after surgical resection and stored at -80°C until subsequent analysis. For this study, the following exclusion criteria were applied: 1. chronic alcohol abuse (more than 30 g/day for men and 20g/day for women), 2. medications known to cause hepatic steatosis (at present or within the last 2 years), 3. significant weight loss (more than 3 kg within the last 3 months), 4. hepatobiliary diseases, 5. ascites on ultrasound, 6. inflammatory bowel disease, 7. infection with the human immunodeficiency virus or Hepatitis B and C virus, and 8. chemotherapy prior to partial hepatectomy.

Hepatic steatosis and inflammation was scored applying a modified histological score according to the recommendations of the Nonalcoholic Steatohepatitis Clinical Research Network [26]. Briefly, four histological features were evaluated semi-quantitatively: steatosis (0-3), lobular inflammation (0-2), hepatocellular ballooning (0-2), and fibrosis (0-4). Simple steatosis was diagnosed in the presence of steatosis but the absence of inflammation, fibrosis and hepatocellular ballooning. Steatohepatitis was diagnosed when the sum of the scores was 4 or more.

Human liver tissue was obtained and experimental procedures were performed according to the guidelines of the charitable state controlled foundation HTCR, with the informed patient's consent, and the study was approved by the local ethics committee of the University Regensburg.

Expression analysis

Isolation of total cellular RNA from cultured cells

and tissue and reverse transcription were performed as described previously [27]. Quantitative real-time PCR was performed with primers specific for ZNF267 (forward: 5'- ATG GGA GCT GTG ATC TTG AGA; reverse: 5'-GCA ATG ATG AAT GAG TAA AGA CC), employing LightCycler technology (Roche, Mannheim, Germany) [28].

Detection of ROS

Cells were plated in 96-well-plates and cultured in DMEM without phenol red (PAN Biotech GmbH, Aidenbach, Germany). Three hours after treatment with As₂O₃ or palmitate cells were washed with PBS and were incubated for 30min at 37°C with the redox-sensitive dye 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA, Invitrogen, Darmstadt, Germany; 100µM). Subsequently, ROS formation was detected using a multiwell fluorescence plate reader (Spectra Fluor Plus, Tecan, Männedorf, Switzerland) with excitation and emission filters of 485 and 535nm, respectively.

Statistical analysis

Statistical analyses were performed using GraphPad Prism Software (GraphPad Software, Inc., San Diego, USA). Results are expressed as mean ± standard error. Comparisons between groups were made using the unpaired t-test. P-values ≤0.05 were considered statistically significant.

Results

ZNF267 expression in NAFLD

First, we assessed ZNF267 mRNA levels in liver specimens from patients with NAFLD and normal human liver tissue. Quantitative RT-PCR analysis revealed significantly increased ZNF267 expression in both simple steatosis and NASH compared to non-steatotic liver tissue (**Figure 1**). ZNF267 mRNA levels did not differ significantly between simple steatosis and NASH. These findings indicated that hepatocellular lipid accumulation rather than hepatic inflammation causes an upregulation of ZNF expression in NAFLD.

ZNF267 expression in an in vitro model of hepatocellular lipid accumulation

To follow up with this hypothesis, we next ana-

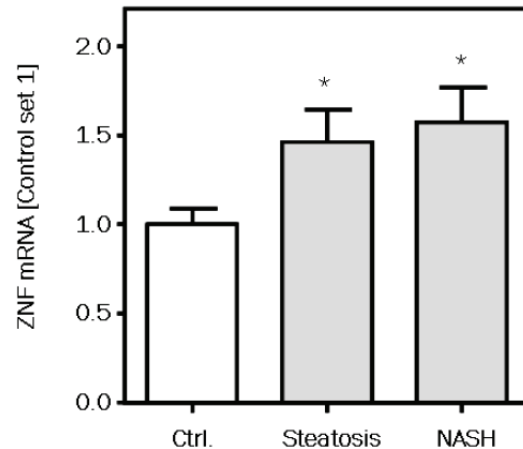


Figure 1. ZNF267 expression in NAFLD: Expression of ZNF267 mRNA in normal, non steatotic human liver tissue compared to steatotic liver tissue without inflammation and NASH specimens analyzed by quantitative real-time PCR (*:p<0.05 compared to normal liver tissue).

lyzed ZNF267 mRNA expression in an in vitro model of hepatocellular lipid accumulation which we have recently described [22]. In this model incubation of primary human hepatocytes (PHH) with palmitate leads to a dose dependent cellular lipid accumulation [22]. Palmitate (C16:0) is the most abundant saturated fatty acid in the human diet, and the major one synthesized de novo [29,30]. Increasing doses of palmitate caused a dose-dependent increase of ZNF267 expression in PHH (**Figure 2a**). Similarly, lipid accumulation can be induced in the hepatoma cell line HepG2 [22], and also here, steatosis caused increased ZNF267 expression (data not shown). In line with previous reports [31,32,33], we detected increased ROS formation in response to palmitate stimulation in HepG2 cells (**Figure 2b**). Arsenic acid is a chemical inducer of ROS formation [34] (**Figure 2b**), and stimulation of HepG2 cells with Arsenic acid leads to a marked increase of ZNF267 expression (**Figure 2c**). Together these findings indicate that ROS formation is at least one mechanism by which hepatocellular lipid accumulation increases ZNF267 expression.

Discussion

The aim of this study was to investigate the expression and function of ZNF267 in non-alcoholic fatty liver disease (NAFLD). Assess-

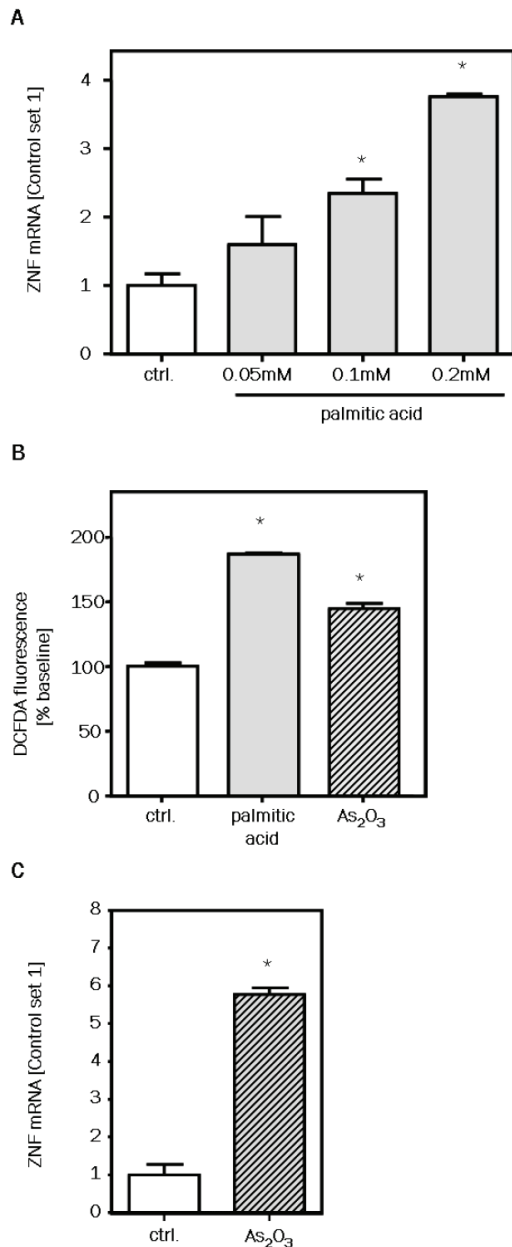


Figure 2. ZNF267 expression in an in vitro model of hepatocellular lipid accumulation: **(A)** Expression of ZNF267 mRNA in PHHs treated with different doses of palmitic acid. **(B)** Relative fluorescence measured in HepG2 cells treated with palmitic acid (0.2mM) and As₂O₃ (10µM) and stained with H₂DCFDA. **(C)** ZNF267 mRNA Expression in HepG2 cells with As₂O₃ (10µM) stimulation. (*:p<0.05 compared to control)

ment of human liver specimens revealed that ZNF267 mRNA levels were significantly higher in NAFLD compared to normal hepatic tissue.

Interestingly, ZNF267 mRNA levels did not differ significantly between simple steatosis and NASH. These findings indicate that hepatocellular lipid accumulation and not inflammation is the inducer of ZNF267 gene transcription in NAFLD. In accordance, incubation of primary human hepatocytes with palmitic acid induced a dose-dependent upregulation of ZNF267 expression. In line with previous studies [31,32,33] we observed considerable ROS formation in response to the hepatocellular lipid accumulation, and moreover, we demonstrated that also chemically induced ROS formation led to a marked upregulation of ZNF267 mRNA expression in hepatoma cells. Most recently, we have identified the transcription factor Ets-1 as key regulator of basal as well as induced ZNF267 expression in liver cancer cells [12], and the release of free radicals as ROS has been shown to induce the transcription of Ets-1 in tumor cells [35]. In summary, these findings suggest that ROS-induced Ets-1 activation is at least one loop by which hepatic steatosis induces ZNF267 expression. With regards to this, we recently demonstrated that ZNF267 promotes proliferation and migration of HCC cells [12], and further, that Ets-1 is strongly linked to HCC progression [36,37,38]. Moreover, we have recently shown that ZNF267 promotes tumorigenicity of HCC cells in vitro [12]. Together these findings indicate that enhanced ZNF267 expression may be at least part of the mechanisms by which Ets-1 exhibits its pro-cancerogenic effect in NAFLD. Furthermore, it has to be once more emphasized that ZNF267 expression is already increased in steatotic livers in the absence of inflammation. This further underscores that simple steatosis cannot be considered as benign but per se is a risk factor for HCC development and progression [15]. Furthermore, it has to be considered that ZNF267 is already up-regulated in human cirrhotic liver tissue and might promote liver fibrosis through alteration of matrix degradation [11]. Since cirrhosis is the main risk factor for HCC development, ZNF267 appears as promising target for both prevention as well as treatment of HCC in patients with chronic liver disease.

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Abbreviations: HSC: hepatic stellate cell; KLF: kruppel-like factor; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; PHH: primary human hepatocytes; ZNF: zinc finger protein.

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