Clinics and Research in Hepatology and Gastroenterology (2016) 40, 297—303

Available online at ScienceDirect www.sciencedirect.com

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ORIGINAL ARTICLE

ShRNA-mediated silencing of the Ndc80 gene suppress cell proliferation and affected hepatitis B virus-related hepatocellular carcinoma

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Available online 14 September 2015

Summary
Background: Hepatocellular carcinoma (HCC) is one of the most common and lethal malignancies in the world, and hepatitis B virus (HBV) has been well established to cause HCC. Ndc80 complex is a conserved mitotic regulator dedicated to ensuring faithful chromosome segregation and plays an important role in inducing tumor formation. However, its role in HCC caused by HBV infection remains unclear.
Methods: Immunohistochemistry (IHC), Western blot (WB), and real-time qRT-PCR were used to measure the expression of Ndc80 in HBV-related HCC tissues. Ndc80-specific short hairpin RNA (shRNA) was used to knock-down Ndc80 expression in the hepatoma cell line HeG2 and HepG2.2.15, which is stable transcribed with HBV genome. Furthermore, the effect of Ndc80 on cellular proliferation and growth were examined, respectively.
Results: The expression level of Ndc80 was remarkably up-regulated in HBV-related HCC tissues. Down-regulation of Ndc80 expression suppressed HBV replication. With cell counting and the MTS assay, cellular proliferation and growth of Ndc80 knocking-down cell line was shown to be effectively restrained.
Conclusion: This study suggests that Ndc80 may play an important role in the process of HBV-related HCC, and that it may be a potential biological treatment target in the future.

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http://dx.doi.org/10.1016/j.clinre.2015.08.002
2210-7401/

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent malignancies in the world [1], accounting for around 350,000 deaths worldwide annually [2,3]. Hepatitis B virus (HBV) has been well established to cause HCC [4—6]. Numerous hypotheses have been proposed to explain the mechanism of HBV contributing to tumor generation and/or progression, including chronic inflammation of the liver, HBV DNA insertional mutagenesis, and HBV oncoproteins-driven tumorigenesis [7—9]. HBV-associated HCC occurs not only late in HBV infection, but also potentially throughout the course of chronic HBV infection, and the majority of cases occur at the advanced stage or anti-HBe-positive phase with the peak incidence at sixth decade [10]. The development of HCC related to the inflammation and liver regeneration is likely due to the cytotoxic T cell immune injuries toward HBV antigen-expressing hepatocytes. Immune tolerance is broken in the immune reactive HBeAg-positive phase which may result in bridging hepatic necrosis and fibrosis [11—14]. In the anti-HBe-positive phase, the mutant viral oncoproteins may play an important or driving role in HCC development.

Genomic instability is generally considered as susceptibility to the development of tumors. The dysfunction of chromosome segregation during the mitosis process is one of the reasons that contribute to genomic instability. Ndc80 complex, a dumbbell-like heterotetramer, consisted of Hec1 (also called Ndc80), Nuf2, Spc24, and Spc25, is a conserved mitotic regulator dedicated to ensuring faithful chromosome segregation and genome instability [15—17]. Over-expression of Ndc80 was found to be responsible for a hyper-activating mitotic checkpoint and inducing tumor formation [18]. These characteristics make Ndc80 identifying as one of most important cancer testis antigens. Previous studies showed that knocking-down Ndc80 significantly inhibited tumor proliferation and increased apoptosis in cancer cells, suggesting its potential role in diagnosis and immunotherapy of human cancers [19]. Although, Ndc80 has been reported to be implicated in tumor genesis of various kinds of human cancer, its role in HBV-related HCC development remains unclear.

In this study, we found that Ndc80 was remarkably upregulated in HBV-related HCC tissues and hepatoblastoma cells. Further investigations focused on the effect of downregulating Ndc80 on HBV replication and HBV-related HCC in hepatoblastoma cells. Our findings provide a new insight into the role of Ndc80 in the process of HBV-related HCC.

Materials and methods

Cells

HepG2 and HepG2.2.15 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Scotland, UK), supplemented with 10% fetal calf serum (Biological industries, Beit Haemek, Israel), 100 IU/ml penicillin, 100 μg/ml streptomycin (Pen-Strep, Biological industries, Beit Haemek, Israel), and 2% L-glutamine (Biological industries, Beit Haemek, Israel) at 37 °C in a 5% CO_2 and humidified atmosphere.

Immunohistochemistry

Ten pairs of paraffin-embedded tissue blocks were collected from the normal liver tissue, HBV-related HCC tissue and non-HBV-related HCC tissue specimens. Every block was cut into 4 mm using microtome and pasted onto the slide. The tissue sections were dewaxed in xylene and rehydrated through graded alcohol concentrations according to standard procedures. The sections were subsequently submerged in EDTA (pH 8.0) and autoclaved at 121 °C for 5 min to retrieve the antigenicity. Followed by washing in phosphate-buffered saline (PBS) for 3 times, and endogenous peroxidase was halted by the addition of 3% (v/v) H_2O_2, and then incubated for 15 min at room temperature. After washing with PBS, the sections were then blocked with 3% bovine serum albumin (BSA) at room temperature for 5 minutes, followed by 4 °C overnight incubation with Ndc80 antibody (sc-135934) overnight at 4 °C (diluted at 1:100 in PBS, 0.5% BSA). The sections were washed and incubated with biotin-labeled antibody, followed by peroxidase-conjugated streptavidin for another 30 min. 3, 30-diaminobenzidine tetrahydrochloride (Dako) was added in order to visualize the reaction, followed by counterstaining with commercial hematoxylin, whereupon it was sequentially dehydrated in alcohol and xylene and mounted. Negative controls with PBS were obtained by omitting the primary antibodies.

Western blot analysis

The tissue samples were treated with detergent buffer (50 mM Tris, 500 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 10 mM $MgCl_2$) containing 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. Cells were harvested by scraping on ice into a lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, and 0.5% deoxycholate) containing 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, and 2 μg/ml leupeptin. Lysates were cleared by centrifugation (14,000 rpm) at 4 °C for 10 min. The supernatant protein concentration was determined using a Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Normalized lysates were boiled in electrophoresis SDS sample buffer, run on a 10% SDS-PAGE gel, and transferred to a polyvinylidine difluoride membrane (Millipore). Membranes were blocked for 1 h in Tris-buffered saline containing 5% skim milk with 0.05% Tween 20. The membrane was probed with NDC80 antibody (sc-135934) (1:1000) for 2.5 h and washed twice with Tris-buffered saline containing 0.05% Tween 20 for 10 min, then incubated with Tris-buffered saline containing peroxidase-conjugated goat antibody to mouse IgG (1:5000; Amersham Pharmacia Biotech) for 40 min. After being washed three times, the membrane was developed by an enhanced chemiluminescence system (ECL; Amersham Pharmacia Biotech).

RNA isolation and qRT-PCR

qRT-PCR was used to measure the mRNA expression levels of NDC80 in cells and tissues. Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's