RESEARCH ARTICLE

Silencing of NUF2 Inhibits Tumor Growth and Induces Apoptosis in Human Hepatocellular Carcinomas

Qiang Liu1*, She-Jiao Dai2, Hong Li2, Lei Dong2, Yu-Ping Peng1

Abstract

Background: As an important component of the NDC80 kinetochore complex, NUF2 is essential for kinetochore-microtubule attachment and chromosome segregation. Previous studies also suggested its involvement in development of various kinds of human cancers, however, its expression and functions in human hepatocellular carcinoma (HCC) are still unclear. Materials and Methods: In the present study, we aimed to test the hypothesis that NUF2 is aberrant in human HCCs and associated with cell growth. Results: Our results showed significantly elevated expression of NUF2 in human HCC tissues compared to adjacent normal tissues, and high expression of NUF2 in HCC cell lines. Using lentivirus-mediated silencing of NUF2 in HepG2 human HCC cells, we found that NUF2 depletion markedly suppressed proliferation and colony formation capacity in vitro, and dramatically hampered tumor growth of xenografts in vivo. Moreover, NUF2 silencing could induce cell cycle arrest and trigger cell apoptosis. Additionally, altered levels of cell cycle and apoptosis related proteins including cyclin B1, Cdc25A, Cdc2, Bad and Bax were also observed. Conclusions: In conclusion, these results demonstrate that NUF2 plays a critical role in the regulation of HCC cell proliferation and apoptosis, indicating that NUF2 may serve as a potential molecular target for therapeutic approaches.

Keywords: Hepatocellular carcinoma - NUF2 - cell growth - apoptosis

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common and lethal cancer in the world (Jemal et al., 2011), and the second leading cause of cancer-related death in China, accounting for approximately half of all HCC deaths worldwide (Chen et al., 2013; Fan et al., 2013). What’s worse, the overall incidence of HCC has been steadily increasing and the prognosis remains poor. The most frequent risk factors for this malignancy include chronic viral hepatitis, chronic alcohol abuse and dietary exposure to aflatoxins (Evans et al., 2002; Block et al., 2003). Despite the identification of a number of molecular markers involved in the proliferation, invasion, differentiation and metastasis of HCC, the molecular pathogenesis of HCC still remains unclear (Thorgeirsson et al., 2002; Faivre et al., 2011).

Genomic instability is generally considered an important characteristic of cancer progression and dysfunction of chromosome segregation during the mitosis process is one of the reasons that contribute to chromosome instability. The spindle assembly checkpoint functions as an important safeguards by regulating chromosome segregation (Bharadwaj et al., 2004; Foley et al., 2013). The kinetochore is the protein assembly built on centromeres of chromatids where the spindle fibers attach during cell division to pull sister chromatids apart. In another word, the kinetochore is the bridge that connected the spindle and the centromere in mitotic cells (Santaguida et al., 2009). The microtubule-binding interface of the kinetochore, namely the outer kinetochore, is critical for accurate chromosome segregation. As a core component of the outer kinetochore, the NDC80 complex is evolutionarily conserved and essential for stabilization of the kinetochore-microtubule anchoring and supporting the centromeric tension implicated in the establishment of correct chromosome congression (McCleland et al., 2003). The NDC80 complex has four components: NDC80 (also called HEC1 or KNTC2), NUF2 (also called CDCA1), SPC24 and SPC25, which together form a dumbbell-like heterotrimer (Cheeseman et al., 2006).

NUF2, whose full name is NDC80 kinetochore complex component, was first identified and cloned as a component of kinetochore complex (Nabetani et al., 2001; Wigge et al., 2001). In particular, NUF2 was reported to stabilize microtubule attachment as part of a linker between the kinetochore and tubulin subunits of the spindle, and depletion of NUF2 could induce kinetochore attachment defects and spindle checkpoint activation, and finally deaths of mitotic cells (DeLuca et al., 2002). Further studies revealed that NUF2 binds to CENPE and is required for stabilizing CENPE in the kinetochore, and

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both proteins are essential for stable kinetochores and proper chromosome segregation during mitosis (Liu et al., 2007). As a tight subcomplex, NUF2-HEC1 was essential for organizing microtubule attachment sites, and recently more details concerning NUF2-HEC1 heterodimerization and their distinct contributions to kinetochore-microtubule attachment were studied (Ciferri et al., 2005; DeLuca et al., 2005).

Meanwhile, their implications in various kinds of human cancers have been widely investigated. NUF2 was reported to be overexpressed in various kinds of carcinomas including lung cancer, cholangiocellular cancer, urinary bladder cancer, renal cell cancer, colorectal cancer, gastric cancer, ovarian cancer and serous adenocarcinoma (Hayama et al., 2006; Harao et al., 2008; Kaneko et al., 2009; Sethi et al., 2012). Moreover, elevated expression of NUF2 was associated with poorer prognosis of non-small cell lung cancer patients (Hayama et al., 2006). Knockdown of NUF2 significantly caused cell growth delay and increased apoptosis in cell lines of non-small cell lung cancer, colorectal cancer, gastric cancer and ovarian cancer (Hayama et al., 2006; Kaneko et al., 2009; Sethi et al., 2012). Further study identified NUF2 as a potential cancer testis antigen, and its HLA-A2-restricted cytotoxic T lymphocyte (CTL) epitopes could induce tumor-reactive CTL, suggesting its potential role in diagnosis and immunotherapy of human cancers (Harao et al., 2008). Additionally, alternative splicing variants in NUF2 were also reported to have different expression between cancer cells and corresponding normal tissues (Ohtuma et al., 2009). These findings strongly suggested the potential role of NUF2 in tumorigenesis, however, its expression and functions in human HCC is not well characterized.

In the present study, we sought to examine the expression and functional role of NUF2 in human HCCs. We first assessed the expression of NUF2 in pairs of human HCC tissues and several HCC cell lines, and then studied the impact of NUF2 knockdown in HCC HepG2 cells via a lentivirus-delivered knockdown system. Our data for the first time revealed the involvement of NUF2 in human HCCs.

**Materials and Methods**

**Patients**

The ethics committee at the Second Affiliated Hospital, Medical School of Xi’an Jiaotong University approved this study, and written informed consent on the use of clinical specimens was obtained from all participants. Fresh-frozen tissues from 15 pairs of HCC tissues and adjacent normal tissues were collected between May 2011 and July 2013 for real time RT-PCR assay.

**RNA extraction, reverse transcription and real time PCR**

Total RNA was extracted from cells or tissues by Trizol (Invitrogen, USA) reagent, and 2 μg total RNA was reverse-transcribed into cDNA with random primers using a First Strand cDNA Synthesis Kit (Promega, USA). Real time quantitative PCR was performed using SYBR Green Mix (Takara, Japan), and the PCR conditions were as follows: 94°C for 5 min, 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 20 s. Relative expression levels of NUF2 mRNA were compared to the levels of GAPDH as an interval control by comparative cycle threshold method (Livak and Schmittgen, 2001). All samples were examined in triplicate. Sequences of PCR primers were as follows: GAPDH-F, 5’-GGCCGTCACTGGTGTCTGTC-3’, GAPDH-R, 5’-GTCCTTCCCATATGTTGAAGAC-3’; NUF2-F, 5’-GGCCGGACTGTGATTAC-3’, NUF2-R, 5’-GTCAAGACTTCAGGCTTT-3’. **Cell lines and culture**

Human HCC cell lines HepG2, Smmc-7721, Sk-hep-1 and human embryonic kidney cell line 293T were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, USA), penicillin (100 units/ml; Life Technologies, USA) and streptomycin (100 μg/ml; Life Technologies, USA) in an 37°C humidified incubator containing 5% CO₂.

**Lentivirus construction**

The lentivirus-mediated silencing system (Genechem, China) targeting NUF2 contained three vectors: pH-F-L shRNA vector and two packing vectors, pVSVG-I and pCMV R8.92. Briefly, small interfering RNA targeting NUF2 (5’-CCCCAAGAATGATCTTTATCCAA-3’) and negative control RNA sequence (5’-ATTCTGGAACGTGATCCAG-3’) were designed, and the corresponding stem-loop short hairpin RNA (shRNA) sequences were synthesized, annealed and cloned into the NheI-PacI sites of pFH-L lentiviral vector and two packing vectors, pVSVG-I and pCMV R8.92. Briefly, small interfering RNA targeting NUF2 (5’-CCCCAAGAATGATCTTTATCCAA-3’) and negative control RNA sequence (5’-ATTCTGGAACGTGATCCAG-3’) were designed, and the corresponding stem-loop short hairpin RNA (shRNA) sequences were synthesized, annealed and cloned into the NheI-PacI sites of pFH-L lentiviral vector, named as shNUF2 and shControl. To generate reconstructed lentiviruses, the pFH-L vectors were cotransfected with pVSVG-I and pCMV R8.92 vectors into 293T cells using a LipofectamineTM 2000 kit (Invitrogen, USA) according to the manufacturer’s instructions. Reconstructed lentiviruses were harvested by centrifugation after 72 hours and applied to HepG2 cells.

**Cell proliferation assay**

Cell proliferation was determined by Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Japan) following the manufacturer’s instructions. Cells were seeded into 96-well plates at a density of 2000 cells/well in sextuple. The CCK-8 reagent was added into each well at a volume of 10 μl and then incubated for 2 hours. Cells proliferation was determined by measuring the absorbance at 450 nm using a microplate reader (BIO-RAD Laboratories, USA).

**Colony formation assay**

HepG2 cells were seeded in 6-well plates at a density of 1000 cells/well, and allowed to form natural colonies for 14 days before Giemsa staining. Cells were washed and fixed by paraformaldehyde, treated with Giemsa solution (Sigma, USA) for 10 min, and then photographed with a digital camera. The number of colonies (defined as cell clusters consisting of at least 50 cells) were counted and
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Results

**NUF2 was elevated in human HCCs**

We first determined the expression levels of NUF2 in 15 pairs of fresh HCC and adjacent non-tumor samples by real time quantitative PCR assays. As illustrated in Figure 1A, the expression of NUF2 was significantly enhanced in human HCC tissues in comparison to non-tumor controls. Furthermore, we assessed the protein and mRNA levels of NUF2 in several cell lines, and observed relative high expression of NUF2 in HCC cell line HepG2, moderate NUF2 expression in another two HCC cell lines SMMC-7721 and SK-HEP-1, and low expression of NUF2 in a non-tumor cell line 293T (Figure 1B). These data showed that NUF2 was overexpressed in human HCC tissues and cell lines.

**NUF2 was silenced by lentivirus-delivered shRNA in HepG2 cells**

To examine the functional role of NUF2 in human HCC, we used lentivirus-delivered shRNA to knock down expression of NUF2. Due to high expression of NUF2 in HepG2 cells as mentioned above, we chose this cell line as the subject in our experiments. To monitor the infection efficiency, infected HepG2 cells were examined by fluorescence microscopy. As shown in Figure 2A, the expression of NUF2 was significantly enhanced in HCC cell line HepG2, moderate NUF2 expression in another two HCC cell lines SMMC-7721 and SK-HEP-1, and low expression of NUF2 in a non-tumor cell line 293T (Figure 1B). These data showed that NUF2 was overexpressed in human HCC tissues and cell lines.

**Figure 1. Elevated Expression of NUF2 in Human HCCs.** A). mRNA levels of NUF2 were determined by real-time quantitative PCR in 15 pairs of HCC tissues and adjacent non-cancer tissues. GAPDH was assessed as an interval control. Scatter dots represent relative mRNA expression levels and the horizontal lines represent mean with SD. Statistical significance: **p<0.01. B). Protein and mRNA levels of NUF2 were detected by western blot (upper panel) and realtime PCR (lower panel) in human HCC cell lines HepG2, SMMC-7721, SK-HEP-1 and non-tumor cell line 293T. GAPDH served as an interval control.
and fluorescence (Figure 2A, lower panel) microscope. In both shControl and shNUF2 groups, more than 80% of cells were successfully infected, while the uninfected parental cells showed no green fluorescence signals. Silencing effect of lentivirus-delivered shRNA was further confirmed by real-time PCR and western blot assays 48 hours after lentivirus infection. Consequently, more than 70% of NUF2 expression was significantly suppressed in HepG2 cells infected by lentiviruses carrying shRNAs against NUF2 compared to the parental and shControl cells, both mRNA (Figure 2B) and protein (Figure 2C) levels. Therefore, the suppression efficiency of NUF2 was verified in our lentivirus system.

**Suppression of NUF2 inhibited HepG2 cell growth both in vitro and in vivo**

To investigate the biological significance of NUF2 in the survival of HepG2 cells, we assessed the effect of NUF2 depletion on cell growth both in vitro and in vivo. Cell growth was first detected via CCK-8 assay in a 5-day period monitoring. As shown in Figure 3A, suppression of NUF2 significantly retarded HepG2 cell proliferation compared to control cells on the 4th and 5th day (p<0.0001). We further evaluated the impact of NUF2 on long-term cell growth via colony formation assay. The results showed that blockade of endogenous NUF2 expression dramatically inhibited the colony formation ability of HepG2 cells. As shown in Figure 3B-D, colonies number was markedly decreased and colony area was significantly reduced in NUF2 suppressed cells compared to control groups.

To further examine the role of NUF2 in tumorigenicity of human HepG2 cells in vivo, we employed a subcutaneous xenograft mouse model. NUF2 deleted HepG2 cells or control cells were injected into the right flank of nude mice. As shown in Figure 4, abrogation of NUF2 greatly retarded tumor growth, resulting in significantly smaller and lighter tumors than those the control group. These results indicated an essential role of NUF2 in optimal growth of human HCC cells both in vitro and in vivo.

**Reduction of NUF2 induced cell cycle arrest and apoptosis of HepG2 cells**

Since cell proliferation inhibition might be due to blocked cell cycle progression or enhanced apoptosis, we next analyzed the impact of NUF2 depletion on cell cycle and apoptosis. We first examined cell cycle distribution by flow cytometry. Compared with uninfected parental HepG2 cells and control shRNA infected cells, NUF2 silenced HepG2 cells showed a substantial increase in G0/G1 phase and decrease in S and G2/M phase populations (Figure 5A). Cell apoptosis was evaluated by both Hoechst staining and Annexin V/PI double staining followed by flow cytometry. The apoptotic morphologic changes were observed in NUF2 deleted HepG2 cells compared with both controls. In detail, neclei of parental and

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**Figure 2. Lentivirus-mediated silencing of NUF2.** A). Lentivirus infection of HepG2 cells was observed via fluorescence microscope under both bright and green fluorescence field at a magnification of 200x. Con, parental control HepG2 cells that were not infected; Lv-shCon, HepG2 cells infected by lentiviruses delivering shRNAs against control sequence; Lv-shNUF2, HepG2 cells infected by lentiviruses carrying shRNAs against NUF2. B-C). Determination of knockdown efficiency on both mRNA and protein levels of NUF2 via real-time PCR and western blot respectively. GAPDH served as interval controls. Statistical significance: **p<0.01

**Figure 3. Effects of NUF2 Depletion on in vitro Cell Growth of HepG2.** A). Cell proliferation of NUF2 deleted HepG2 cells or control groups of cells were determined by CCK-8 assay in a 5-day period. Cell numbers were expressed as fold of the first day. B). Long-term cell growth was assessed by colony formation assay. HepG2 cells of different groups were stained with Giemsa and observed by routine bright field microscopy. C). The number of colonies (> 50 cells) was counted. D). The average area of colonies was estimated. Statistical significance: *p<0.05, **p<0.01

**Figure 4. Effects of NUF2 Knockdown on in vivo Cell Growth of HepG2 Cells.** A). In vivo tumor growth was examined by subcutaneous injection of tumor cells of NUF2 silenced HepG2 cells or control cells into nude mice. Tumor sizes were monitored and tumor growth curves were determined. B). Tumor weights were assessed when animals were sacrificed. Statistical significance: *p<0.05
Degraded NUF2 caused alteration of cell cycle and apoptosis related proteins

To investigate the underlying molecular events in changes of cell cycle and apoptosis caused by silencing of NUF2, we further measured the expression levels of several important proteins involved in cell cycle and apoptosis by western blot assays. As shown in Figure 5D, compared with both controls, we observed significantly reduction of cell cycle related proteins CyclinB1, Cdc25A and Cdc2, and enhancement of apoptosis related proteins Bad and Bax.

Discussion

HCC is one of the most frequent and lethal malignancies worldwide (Jemal et al., 2011), and the identification of more molecular markers and therapeutic targets that contribute to the development and progression of HCC is obviously desirable to combat this deadly disease. Genomic instability and aneuploidy have been recognized as characteristics of cancer (Rajagopalan et al., 2004). A dysfunctional kinetochore represents a possible reason of chromosome instability and aneuploidy, due to defects in directing proper chromosome segregation (Yuen et al., 2005). Previous studies have suggested a correlation between mutation or overexpression of several kinetochore proteins and cancer (Cahill et al., 1998; Tomonaga et al., 2003; Kim et al., 2005; Tomonaga et al., 2005). As a core component of the NDC80 kinetochore complex, NUF2 has been reported to be implicated in tumorigenesis of various kinds of human cancer, but its role in HCC development remains unclear. Thus, in the present study, we aimed to investigate the expression and biological functions of NUF2 in the proliferation and apoptosis of HepG2 HCC cells via a lentivirus-mediated silencing system.

Expression profile analysis demonstrate that NUF2 was overexpressed in various types of cancers including lung cancer, cholangiocellular cancer, urinary bladder cancer, renal cell cancer, colorectal cancer, gastric cancer, ovarian cancer and serous adenocarcinoma (Hayama et al., 2006; Harao et al., 2008; Kaneko et al., 2009; Sethi et al., 2012). Moreover, upregulated NUF2 could predict worse prognosis for patients with non-small cell lung cancer (Hayama et al., 2006). In agreement with these findings, we found that expression of NUF2 was significantly enhanced in human HCC tissues relative to corresponding adjacent normal tissues. Additionally, it was also highly expressed in several kinds of HCC cell lines.

We further address whether NUF2 depletion would affect HCC cell growth, and efficient gene knockdown is the cornerstone of the whole study. Lentivirus-mediated shRNA provide an attractive approach to efficiently suppressed gene expression. In the present study, we monitored infection efficiency of lentivirus by fluorescence microscope, and confirmed knockdown of target gene by real-time PCR and western blot, which gave a basis for the continued observation of NUF2’s role in HepG2 cells.

It is well known that uncontrolled cell proliferation is one of hallmarks of cancer (Hanahan and Weinberg, 2011). In this study, we observed that suppression of NUF2 in HepG2 cells led to marked inhibition of cell growth, not only for short-term cell growth and long-term colony formation of cultured cells, but also for tumorigenicity of injected xenografts. The results indicated that some steps involving with cell proliferation might be established after NUF2 depletion, although the exact molecular mechanisms were far from elucidated. Therefore, NUF2 may play critical role in the process of cell proliferation in HCCs.

The suppression of HepG2 cell growth may partly be resulted from the dysregulation of cell cycle progression, which was also one of the hallmarks of cancer (Hanahan et al., 2011). Actually, we found that NUF2 depletion did cause alterations of cell cycle distribution in HepG2 cells. In particular, G0/G1 phase was arrested, while proportion of S and G2/M phases were decreased. We also explored expression of cell cycle related proteins, and found significant reduction of CYCLIN B1, CDC25A and CDC2. This observation was consistent with previous findings that CDC25A is essential for cell cycle progression from G1 to the S phase through activating the cyclin-dependent kinase CDC2 by removing two phosphate groups (Boutros et al., 2007; Santamaria et al., 2007). CYCLIN B1, another cell cycle associated protein directly activated by CDC25A, was reported to show markedly
various expression through the cell cycle, with its peak level in G2/M phase and lowest level in G1 phase, which was in agreement with our results (Maity et al., 1995). Our findings were also consistent with a previous study that identified NUF2 as a novel cell cycle gene named cell division cycle associated 1 (CDCA1), which was found to co-express with several known cell cycle genes including CDC2, CDC7, CDC23, cyclin, MCAK, mki67a and topoisomerase II (Walker et al., 2001).

In company with cell cycle arrest, we also discovered increased apoptosis in NUF2 suppressed HepG2 cancer cells, which represented another potential mechanism of cell proliferation delay caused by NUF2 depletion. Actually, NUF2 inhibition was reported to block stable kinetochore-microtubule attachment and induce mitotic cell death in HeLa cells, which for the first time suggested implication of NUF2 in cell survival (DeLuca et al., 2002). Recently, increasing evidence confirmed the apoptosis induction role of NUF2 suppression in lung cancer, colorectal cancer, gastric cancer and ovarian cancer cells (Hayama et al., 2006; Kaneko et al., 2009; Sethi et al., 2012). Our present results were in good agreement with these observations. Besides Hoechst staining and Annexin V/PI double staining, we also employed western blot to detect apoptosis related proteins. Consequently, two apoptotic activators of the BCL-2 family, BAD and BAX, were found to be upregulated in response to NUF2 depletion.

In summary, NUF2 is consistently overexpressed in human HCC tissues and cell lines. Sufficient expression of NUF2 is critical to ensure proper growth of HCC cells both in vitro and in vivo. Cell cycle arrest and increased apoptosis are triggered in NUF2 suppressed HCC cells. The substantial roles of NUF2 in cancer cell biology provoke its potential application as a molecular therapeutic target for HCC treatment.

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