NEDD4 Depletion Inhibits Hepatocellular Carcinoma Growth via Targeting PTEN

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Key Words
Hepatocellular carcinoma • Overall survival • NEDD4 • PTEN

Abstract

Background/Aims: Neural precursor cell-expressed developmentally down-regulated gene 4 (NEDD4) plays an important role in tumor cell growth, yet its role in hepatocellular carcinoma (HCC) remains unclear. This study is to establish NEDD4 as a prognostic biomarker by which the survival of HCC patients can be predicted and to reveal the role of NEDD4 in hepatocellular carcinoma cell growth. Methods: The expression of NEDD4 in 219 HCC specimens was assessed by immunohistochemistry. Postoperative overall survival and time to recurrence were evaluated by univariate and multivariate analyses. The roles of NEDD4 in hepatocellular carcinoma cell proliferation and invasion were determined. Results: The patients with low NEDD4 expression tumors had an average cumulative survival of 64.9 ± 6.5 months during follow-up while the patients with high NEDD4 expression tumors had an average cumulative survival of 20.3 ± 15.8 months. NEDD4 silencing inhibited Huh7 cell proliferation and altered cell cytoskeletal assembly, and NEDD4 depletion furthermore seemed to suppress cell migration and invasion. A possible molecular mechanism for the observed effects might be that NEDD4 silence led to an increase in PTEN (phosphatase and tensin homologue) expression, which in turn resulted in the inactivation of STAT3, AKT, and ERK1/2. Conclusion: Our findings indicate that NEDD4 may participate in the HCC progression and may therefore be a potential target for HCC therapy.

Introduction

Hepatocellular carcinoma (HCC) has recently become one of the leading causes of cancer death worldwide. Characteristics of HCC include rapid growth, vascular invasion, and high resistance to standard chemotherapy [1, 2]. Although there are several treatment options for
HCC, the effective treatment of HCC is challenging and the mortality rate for HCC remains high, and researchers worldwide are therefore interested in HCC [3]. There is mounting evidence indicating that ubiquitination plays an important role in HCC development by influencing cell proliferation, migration, invasion, and apoptosis [4-7]. A greater understanding of the role of ubiquitination molecules in HCC development is of great importance for the successful diagnosis and treatment of HCC.

Ubiquitination via the ubiquitin proteasome system (UPS) is a post-translational modification which regulates various cellular processes such as cell growth, migration, invasion, and cell cycle progression [6, 8, 9]. The UPS consists of three components: the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme, and the E3 ubiquitin ligase. Among these UPS components, the E3 ubiquitin ligase interacts directly with ubiquitin substrates and determines the specificity of target substrates [10]. Neural precursor cell-expressed developmentally down-regulated gene 4 (NEDD4) is a homologue to E6-AP C-terminus (HECT) family of the E3 ubiquitin ligase family, which is widely expressed in mammalian tissues [11]. It has been demonstrated that NEDD4 plays key roles in neuronal development in invertebrates, in protein trafficking, as well as in cellular processes such as those governing sodium homeostasis and antagonizing Notch signaling [12, 13]. Recently, the relationship between NEDD4 and phosphatase and tensin homologue (PTEN) has gained attention.

The PTEN phosphatase acts on both polypeptide and phosphoinositide substrates [14] and is one of the most important tumor suppressors that participates in many cell processes including cell survival, cell proliferation, cell motility and polarity, cell metabolism, and cellular senescence [15-20]. The expression of PTEN is regulated by various molecular mechanisms such as epigenetic silencing, transcriptional repression, microRNA regulation, and post-translational modifications [21-23]. The UPS can decrease PTEN levels in a post-translational manner, and previous investigations have shown that the relationship between NEDD4 and PTEN may be complex in terms of the role of NEDD4 in the degradation of PTEN differing between different cells [24-26].

Until now, the role of NEDD4 and the relationship between NEDD4 and PTEN in HCC has been unclear. The aim of this study was therefore to assess the role of NEDD4 in HCC.

Materials and Methods

Patients and specimens
A total of 219 formalin-fixed paraffin-embedded tissues from liver nodules were randomly selected retrospectively from HCC patients who underwent curative resection between 2003 and 2009 at the Changzheng Hospital and the Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China. The complete follow-up data for these patients were available. Another six HCC tissue samples taken from patients who underwent curative resection recently in Changzheng Hospital were frozen and stored in liquid nitrogen for future use. Written informed consent from each patient as well as approval from the Ethics Committee of the Changzheng Hospital and the Eastern Hepatobiliary Surgery Hospital were obtained prior to the use of these clinical materials for investigation.

Cell lines and reagents
Human hepatoma-7 (Huh7) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with penicillin and streptomycin. Antibodies to GAPDH, AKT, phosphor-AKT, ERK1/2, phosphor-ERK1/2, STAT3, and phosphor-STAT3 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies to NEDD4 and PTEN were purchased from Abcam (Cambridge, USA). Alexa Fluor 488 goat anti-mouse antibody, Alexa Fluor 555 rabbit anti-mouse antibody, Alexa Fluor 555 Phalloidin, HRP-conjugated secondary antibodies, DMEM, FBS, penicillin, streptomycin and lipofectamine 2000 were purchased from Invitrogen (Shanghai, China). siRNA targeting NEDD4 was purchased from Dharmaco (GE Healthcare Life Sciences, Piscataway, NJ) and Matrigel Matrix was obtained from BD Biosciences (San Jose, CA). Cell-light EdU DNA cell proliferation kit was purchased from RiboBio.
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Transwell chamber dishes and cell culture plates were purchased from Corning Inc (Corning, NY).

Tissue microarrays, immunohistochemistry, and scoring

Tissue microarrays (TMA) were constructed as reported previously [27]. For immunohistochemistry, slides were deparaffinized, rehydrated, and then subjected to heat-induced epitope retrieval in pH 6.0 citrate buffer. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and methanol, after which slides were incubated with NEDD4 polyclonal antibody (2μg/mL). All cases were reviewed independently by two pathologists who were blinded to the clinicopathological data. The immunostaining was evaluated as previously reported [28]. Briefly, a mean percentage (0–100) of NEDD4-positive tumor cells was determined in at least five randomly selected areas at ×400 magnification (50–250 cancer cells per area). Meanwhile, the immunostaining intensity in each case was scored as follows: weak, 1+; moderate, 2+; and intense, 3+.

To determine a weighted score for each case, the percentage of NEDD4-positive tumor cells and the staining intensity were multiplied, that resulted in scores ranging from 0 (0% of cells staining) to 300 (100% of the cells staining at 3+ intensity). Cut-off points were then determined based on these scores: negative, 0; weak, < 75; moderate, 75–150; and intense, >150. Scores <75 were considered indicative of low expression and scores >75 were considered indicative of high expression.

Cell proliferation assay

Cell proliferation was assessed using 5-ethynyl-2’deoxyuridine (EdU) according to the manufacturer’s instructions. After being treated with siRNA for 48 hours, cells were incubated in fresh medium containing EdU (50μM) for 2 hours. Cells were then fixed with 4% paraformaldehyde for 20 minutes, stained with Apollo fluorescent azide for 30 minutes at room temperature, and finally stained with Hoechst for 15 minutes at room temperature. Images were taken and analyzed using a digital microscope system (IX81, Olympus).

Wound healing assay

Thirty-six hours after transfection, cells were starved by incubation in culture medium containing 1% FBS for twelve hours to inactivate proliferation. Wounds were then created using pipette tips. After three washes with PBS, culture medium containing 1% FBS was added to the plate and images of the cells were acquired under an inverted microscope (IX81, Olympus) at 0, 36, and 72 hours. The width of the wound was measured by Image J software (NIH, http://imagej.nih.gov/ij/) and data were obtained from three independent assays.

Cell invasion assay

Cell invasion assays were performed as previously described [29, 30]. Prior to beginning the experiment, filters were precoated with Matrigel. Cells (5 × 10⁴) transfected with NEDD4 siRNA for 48 hours in culture media containing 0.5% FBS were added to the transwell chamber inserts, which were then placed into the lower chamber filled with culture media containing 5% FBS. Cells were allowed to migrate for 24 hours, after which cells remaining in the upper inserts were removed using cotton swabs. The migrated cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Images were captured with an inverted microscope (IX81, Olympus) and the migrated cells were counted manually. The experiments were repeated three times.

Cell cycle assay

Cells treated with NEDD4 siRNA for 48 hours were harvested using trypsin and fixed in 70% ethanol for 16 hours. After two washes with PBS, the cells were suspended in PBS containing propidium iodide and RNase for 30 minutes at room temperature. Samples were analyzed using Cell Lab Quanta SC (Beckman Coulter; Indianapolis, IN) and data were analyzed using Modfit software (Verity Software House, USA).

Cell apoptosis assay

Cells treated with NEDD4 siRNA for 48 hours were harvested using trypsin, washed twice in PBS, and incubated with FITC Annexin V for 15 minutes at room temperature in the dark. The stained cells were then incubated with propidium iodide for 5 minutes at room temperature in the dark. Stained cells were analyzed using Cell Lab Quanta SC the resulting data were analyzed by FLOWJO v7.6 software (FlowJo, LLC, USA).
Immunofluorescence staining

Cells grown on glass slides were fixed with 4% paraformaldehyde and then permeabilized with 0.5% Triton X-100. The cells were washed with PBS, blocked with 5% bovine serum albumin (BSA) (Invitrogen, Shanghai, China) for 30 minutes at room temperature, and then washed with PBS three times. Cells were then incubated with primary antibodies diluted in 1% BSA for 1 hour at room temperature, after which they were again washed three times with PBS before being incubated with Alexa Fluor 488 and Alexa Fluor 555 for 30 minutes. The slides were then washed once with PBS and incubated with DAPI for 15 minutes. After a final wash with PBS, the cells were analyzed with a Zeiss LSM-710 fluorescence microscope.

Western blotting

Cells were lysed by RIPA lysis buffer containing 50mM Tris(pH 7.4), 150mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and proteinase inhibitor mixture (Beyotime Biotechnology, Shanghai, China). Fresh tissue samples were ground to powder in liquid nitrogen and then lysed. The protein extracted from cells and fresh tissues was subjected to SDS-PAGE before being transferred to polyvinylidene fluoride membrane. The membranes were then incubated with the indicated primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Finally, the blots were incubated with Super Signal West Pico (Thermo Fisher Scientific Inc, Shanghai, China) chemiluminescent substrate and visualized using the GenegGnome HR Image Capture System (Piscataway, NJ).

Statistical analysis

Statistical analyses were performed using SPSS 13.0 software (SPSS, Chicago, IL). For survival analyses, NEDD4 expression levels were categorized as either low or high. Univariate analysis was performed using the Kaplan-Meier method (log-rank test) and multivariate analysis was performed using Cox’s multivariate proportional hazards regression model in a stepwise manner (forward, conditional likelihood ratio). Data obtained from at least three independent experiments were expressed as mean ± SD. Statistical significance was computed using One-way ANOVA analysis and differences with \( p < 0.05 \) were considered significant.

Results

NEDD4 overexpression is associated with decreased overall survival in HCC patients

Among the 219 HCC patients included in this study, 100 patients died at a median of 20.3 months after surgery (range: 1 to 69 months) and 129 patients experienced recurrence at a median of 15.7 months (range: 1 to 65 months). Tissue microarray assays revealed that 50 of the 219 HCC tumors (22.8%) stained strongly with anti-NEDD4 and 169 tumor samples (77.2%) exhibited weak staining (Fig. 1A). Correlations between NEDD4 expression and the pathological characteristics of HCC are listed in Table 1. In terms of TNM stages (T: category, N: category, and M: remote metastasis), NEDD4 overexpression was observed in 12/31 (38.7%) stage III-IV patients with severe lymph node metastasis or distant site metastasis compared to 10/65 (15.4%) stage I patients and 28/123 (22.8%) stage II patients with no or only minor lymph node metastasis (Table 1).

To assess the role of NEDD4 in prognosis, the association of NEDD4 expression with the cumulative survival of HCC patients (219 cases) was analyzed statistically. As shown in the Kaplan-Meier survival graph (Fig. 1B), patients with low NEDD4 expression tumors had an average cumulative survival of 64.9 ± 6.5 months, while patients with high NEDD4 expression tumors had an average cumulative survival of only 20.3 ± 15.8 months (Fig. 1B). The difference between the cumulative survival rates in these two groups was determined by a log-rank test. As shown in Fig. 1B, the cumulative survival rate between these two groups differed dramatically, indicating that NEDD4 expression is inversely associated with postoperative survival in HCC patients.

Univariate analysis indicated that NEDD4 status, tumor size, tumor number, disease TNM stage, liver cirrhosis, and alpha fetoprotein (AFP) are significant predictors of tumor recurrence. Patients with high NEDD4 expression tumors experienced a shorter time to recurrence compared to patients with low NEDD4 expression tumors (\( p < 0.001 \), Table
2) Multivariate analysis revealed NEDD4 expression to be an independent predictor of tumor recurrence (Table 2). The association between NEDD4 expression and the time to recurrence in HCC patients was investigated, and the resulting data indicated that patients with low NEDD4 expression tumors had a more favorable time to recurrence (median time to recurrence: 17.9 months) than those with high NEDD4 expression tumors (median time to recurrence: 10.9 months) (Fig. 1B).

Table 1. Association of NEDD4 expression with pathological characteristics of HCC

<table>
<thead>
<tr>
<th>Pathological category</th>
<th>Case number</th>
<th>NEDD4 expression</th>
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<td>Low</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td>(\geq50)</td>
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<td></td>
<td></td>
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NEDD4 depletion inhibits cell proliferation and induces cell cytoskeletal change

Although NEDD4 was previously known to play important roles in the development of different tumors, the role of NEDD4 in HCC remained unknown [25, 26, 31]. In this study, siRNA was used to specifically silence the expression of NEDD4 in HuH7 cells. The siRNA mix used was found to significantly decrease the expression of NEDD4 (Fig. 2A) and NEDD4 siRNA-treated cells exhibited inhibited proliferation compared with the control groups (Fig. 2B, C). NEDD4 knockdown was furthermore found to result in significant changes in the...
actin cytoskeleton as determined by confocal microscopy (Fig. 2D), suggesting that NEDD4 depletion may affect the status and motion of the cell.

**NEDD4 depletion suppresses cell migration and invasion**

Malignant hallmarks of cancer cells include proliferation, migration, invasion, and metastasis [1]. The high mortality associated with HCC is largely attributed to the metastasis of cancer cells, which is highly dependent on cancer cell migration and invasion. In this study, migration and invasion assays were therefore conducted as a means of evaluating the role of NEDD4 in cancer cell metastasis in vitro. As shown in Fig. 3A and Fig. 3B, NEDD4 depletion significantly reduced the migration ability of Huh7 cells compared with the control group. The ability of Huh7 cells to invade Matrigel also seemed to decrease in NEDD4-depleted cells compared to control cells (Fig. 3C, D). Together, these data suggest that NEDD4 is required for Huh7 cell migration and invasion.

**NEDD4 depletion induces cell cycle arrest at S phase**

The cell cycle, which consists of four stages (G1 phase, S phase, G2 phase, and M phase), is a complicated process which cells undergo in order to proliferate [32, 33]. To evaluate whether the cell cycle was affected by NEDD4 siRNA, flow cytometry was performed at 48 hours after siRNA interference. Compared with control groups, NEDD4-depleted cells exhibited an increase in S phase and a decrease in G1 phase (Fig. 4A). The effects of NEDD4 silencing on cell apoptosis were assessed using flow cytometry, which revealed that NEDD4 depletion had no effect on Huh7 cell apoptosis (Fig. 4B). These data indicate that NEDD4 plays a role in the cell cycle process but not in cell apoptosis in Huh7 cells.

**NEDD4 depletion inhibits HCC growth by targeting PTEN**

The relationship between NEDD4 and PTEN was investigated as a possible molecular pathway by which NEDD4 exerts its role in HCC. Unlike in the case of colon cancer, where...
Fig. 2. NEDD4 silencing in Huh7 cells inhibits cell proliferation and induces cytoskeletal changes. (A) The transfection efficiency of NEDD4 siRNA was analyzed by Western blotting at 48 hours post transfection. The densitometry for the blots was shown as the ratio between NEDD4 and GAPDH separately. (B) Representative images of EdU-positive replicating cells captured using a fluorescent microscope, where red nuclei represent replicating cells. (C) Quantitative data of EdU proliferation assay showing a significant decrease in cell proliferation. (D) NEDD4 depletion results in reorganization of the actin cytoskeleton in Huh7 cells. Data represent the means ± SD of three separate samples (*, p < 0.05).

Fig. 3. NEDD4 depletion in Huh7 cells inhibits cell migration and invasion. (A) Huh7 cells treated with NEDD4 siRNA were wounded using a pipette tip and then cultured for a further 72 hours. Representative images of wound-healing at different time points are shown. (B) Quantitative data of wound width in the wound-healing assay. (C) Huh7 cells treated with siNEDD4 were seeded in transwell dishes covered with Matrigel and were cultured for 24 hours. Representative images of invading cells were shown. (D) Quantitative data of invasive cells in cell invasion assay. Data represent the means ± SD of three separate samples (*, p < 0.05, **, p < 0.01).
NEDD4 promotes colonic cell growth independently of PTEN. NEDD4 is known to participate in HCC cell growth mainly by affecting the function of PTEN [25]. Although NEDD4 and PTEN were not found to co-localize in HCT-15 and HT-29 cells, Huh7 cells did exhibit co-localization of NEDD4 and PTEN and NEDD4 silencing led to increased protein levels of PTEN (Fig. 5A). To better understand the role of NEDD4 in the PTEN pathway, the effects of NEDD4 depletion on the phosphorylation of AKT, ERK1/2, and STAT3 were investigated. The results of this investigation revealed that NEDD4 depletion increased PTEN protein levels and significantly decreased the activation of AKT, ERK1/2, and STAT3 (Fig. 5B). Taken together, these data indicate that NEDD4 interacts with PTEN directly, thereby affecting AKT, ERK1/2, and STAT3 pathway.

Discussion

Being a leading cause of cancer-related deaths worldwide, HCC has received much attention; however, success of the various treatment options for HCC is still limited. For this reason, there is a need for novel therapies to be identified and developed, and the molecular therapies are promising in this regard, since a large number of molecules has been identified to play important roles in the progress of HCC. Being a HECT-domain E3, NEDD4 plays a crucial role in ubiquitination, and hence regulates axon guidance, cell proliferation, and signaling pathways [11, 24, 34, 35]. It has been shown that NEDD4 may act as an oncoprotein.
in colorectal cancer, gastric carcinomas, bladder cancer, and glioma as it participates in the malignant phenotype of cancer cells and is overexpressed in the aberrant tissues [31, 36, 37]. There are also reports indicating that NEDD4 may participate in the progress of HCC, yet the role of NEDD4 in HCC has not yet been investigated [3, 38]. In this study, the expression levels of NEDD4 in 219 HCC tumors were assessed by immunohistochemical techniques, revealing correlations between NEDD4 expression and various clinical outcomes in HCC patients. Silencing of NEDD4 by siRNA was shown to significantly inhibit Huh7 cell proliferation, which is in agreement with recent studies demonstrating a role for NEDD4 in various cancer cells [25, 26, 37]. Our data indicated that NEDD4 plays a similar role in different cancers and may therefore serve as a novel target in the treatment of HCC.

Metastasis of HCC is known to be one of the major reasons for the high mortality associated with HCC. Assessments of cell migration and invasion, which are well-established methods of emulating cancer metastasis in vitro, revealed that NEDD4 depletion significantly suppressed Huh7 cell migration and invasion, suggesting an important role for NEDD4 in HCC metastasis. The cytoskeletal changes induced by NEDD4 depletion further proved an indispensable role for NEDD4 in HCC metastasis, since cytoskeletal alterations impair cell migration and invasion [39, 40]. In accordance with the role of NEDD4 in cell proliferation, NEDD4 silencing led to cell cycle arrest at S phase (DNA synthesis phase), indicating that NEDD4 may play a role in the regulation of cyclins [32]. Since the induction of cell apoptosis is considered a potential treatment strategy for cancer, the effect of NEDD4 depletion on cell apoptosis was investigated in this study; however, NEDD4 was found to have no effect on cell apoptosis despite the protein levels of NEDD4 decreasing during apoptosis [41].

To further investigate the mechanism by which siRNA-mediated NEDD4 depletion inhibited cell proliferation, migration, and invasion, the relationship between NEDD4 and PTEN was assessed. Various studies have shown that NEDD4 plays important roles in regulating the both the degradation and the nuclear localization of PTEN, and that elevated protein levels of PTEN inhibit the expression of NEDD4 [34, 36, 42, 43]. Other reports, however, have revealed that the role of NEDD4 in PTEN regulation may be context-
dependent and tissue-specific: in colorectal cancer, for example, NEDD4 has been shown to be overexpressed and is known to promote colonic cell growth independently of PTEN [25, 43-45]. The confocal microscopy was used to evaluate the localization of NEDD4 and PTEN in Huh7 cells, and revealed that NEDD4 and PTEN were co-localized in Huh7 cells. To further assess the effect of increased protein levels of PTEN due to NEDD4 depletion, the activation of AKT, ERK1/2, and STAT3, which are known to be affected by PTEN, was investigated [46-49]. The data from this investigation further strengthened the conclusion that NEDD4 depletion increases the protein levels of PTEN in HCC.

In this study, high levels of NEDD4 expression were found to be associated with adverse clinical outcomes in patients with HCC. Targeting NEDD4 resulted in inhibited cell proliferation, migration, and invasion and induced cell cycle arrest at S phase. A possible mechanism by which these effects are exerted may be the up-regulation of PTEN yielding inactivation of AKT, ERK1/2, and STAT3. The findings reported here strongly support an oncogenic role for NEDD4 in HCC and suggest that NEDD4 inhibition may be a future therapeutic target in the treatment of HCC.

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Disclosure Statement

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