Original Article

NVM-1 predicts prognosis and contributes to growth and metastasis in hepatocellular carcinoma

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Abstract: Novel metastasis-promoting gene 1 (NVM-1) has a significantly elevated protein level in a variety of tumor tissues and is involved in metastasis. However, its functions in hepatocellular carcinoma (HCC) are not clear. The current study aimed to investigate the functions of NVM-1 in cell proliferation, apoptosis, and epithelial-mesenchymal transition in HCC. NVM-1 protein expression in HCC was assessed by immunohistochemical staining. In vitro, cell proliferation, apoptosis, and aggressiveness were determined by CCK-8, fluorescence-assisted cell sorting, TdT-UTP nick-end labeling, and transwell assays, respectively. For in vivo studies, NVM-1 knockdown HCC cells were transplanted into BALB/c nude mice. NVM-1 was frequently upregulated in HCC tissues and positive NVM-1 expression was linked with poor prognosis. NVM-1 depletion significantly inhibited cell proliferation, migration, and invasion abilities in vitro and in vivo. Apoptosis was induced after NVM-1 knockdown. In conclusion, positive NVM-1 expression confers poor prognosis to HCC patients and the NVM-1 protein level correlates with HCC cell proliferation, apoptosis, and EMT.

Keywords: NVM-1, hepatocellular carcinoma, prognosis, proliferation, metastasis

Introduction

The prevalence of hepatocellular carcinoma (HCC) has progressively increased in recent years; it is currently the forth most common cancer and the second most common cause of cancer mortality in China [1]. The number of new cases diagnosed each year is growing, and it nearly equals the number of deaths from this cancer [2]. HCC usually remains undetected in the early stage, but it deteriorates rapidly once patients develop symptoms. For early HCC patients, radical resection and liver transplantation are still the most effective treatments; however, several recent clinical studies have indicated that the prognosis of these patients remains poor because of high recurrence and early metastasis rates after operation [3-5]. Generally, early diagnosis and treatment result in a better prognosis; therefore, early detection and diagnosis is particularly important in the management of HCC. The rapid development of biotechnology has allowed the identification of HCC prognostic biomarkers [6, 7]. However, as the molecular mechanisms involved in HCC progression are complex, the pathogenesis of HCC is still unclear. Thus, it is imperative to identify new biomarkers and explore their functions to provide clues for proper management of HCC.

Novel metastasis-promoting gene 1 (NVM-1; also known as METTL21D, C14orf138, or VCP-KMT), located on chromosome 14 (ORF138), was identified in 2011 as a gene functionally involved in tumor cell motility and metastasis. The protein has a molecular weight of 26-31 kDa and is endogenously expressed in a variety of tissues, with the highest expression observed in the ovaries, duodenum, and pancreas [8-10]. The protein level is significantly elevated in head and neck squamous cell, lung, esophageal, colorectal, thyroid papillary, endometrial, and cervical carcinomas [8]. However, the
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Table 1. Expression of NVM-1 in human HCC and its clinical significance

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cases</th>
<th>NVM-1 (negative)</th>
<th>NVM-1 (positive)</th>
<th>( \chi^2 )</th>
<th>( P )</th>
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<td>-</td>
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<tr>
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<td>BCLC stage</td>
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<tr>
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<tr>
<td>≥ 12</td>
<td>43</td>
<td>27 (62.8%)</td>
<td>16 (37.2%)</td>
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</tbody>
</table>

*Metastasis was defined as recurrence time < 6 months or cancer embolus (including portal vein tumor thrombus, biliary tract tumor thrombus, and hepatic vein tumor thrombus). #: Data are missing for 5 patients.

In the present study, we investigated NVM-1 function in HCC using in vitro assays for cell proliferation, migration, and invasion as well as in vivo studies using a xenograft mouse model.

Materials and methods

Ethics statement

The Southern Medical University Ethics Committee approved protocols according to the Helsinki Declaration (6th revision, 2008), and informed consent was signed by each patient enrolled in the study. Animal protocols were approved by the Institutional Animal Care and Use Committee of Southern Medical University.

Clinical samples

Ninety-two HCC samples and seven liver tissues with benign lesions were collected from patients who underwent surgical resection in the Department of Hepatobiliary Surgery at Nanfang Hospital of the Southern Medical University between November 2010 and October 2012. The inclusion criteria were as follows: (a) no other treatment before surgery; (b) curative surgical resection was performed; (c) resected specimens were available for pathological examination and examined for metastasis after the operation; (d) the cut edge was confirmed without residual carcinoma; (e) no hepatitis C diagnosis; and (f) a com-
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Cancerous tissues were defined as tissues within 1 cm from the tumor edge without necrosis, and noncancerous tissues as tissues exceeding the edge by more than 2 cm. The specimens were subjected to immunohistochemical staining, and the patients were monitored for prognostic analysis via outpatient examinations or telephone follow-up every six months. Demographic features and clinicopathologic data of the patients are shown in Table 1.

**Immunohistochemical staining**

Immunohistochemical staining was performed on paraformaldehyde-fixed paraffin sections. NVM-1 (kindly provided by Professor Wilko Thiele, Center of Biomedical and Medical Technology, Heidelberg University, Germany) (1:200) Ki-67, E-Cadherin, N-Cadherin and Vimentin antibody (Santa Cruz, CA, USA) were used in immunohistochemistry with the streptavidin peroxidase-conjugate method as reported [7]. Cells having brown nuclear granules were defined as positive cells, and positive tumor cells or hepatocytes were classified according to the following staining intensity criteria [11]: 0. no staining; 1. light staining; and 2. deep staining. Additionally, the percentage of cells showing NVM-1 expression was categorized as follows: 1. 1-25% positive cells; 2. 26-50% positive cells; 3. 51-75% positive cells; 4. > 76% positive cells. Finally, expression was evaluated semi-quantitatively based on the product of the color intensity and positive cell percentage scores: < 4, negative expression; ≥ 4, positive expression. Each specimen was evaluated independently by two professional pathologists.

**Cell lines**

HL-7702, HepG2, SMCC-7721, MHCC-97L, MHCC-97H, and HCCLM3 cells (Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences, China) were cultured in high-glucose DMEM (Gibco, CA, USA) supplemented with 10% fetal bovine serum FBS (Gibco) in a humidified atmosphere containing 5% CO₂. Cells in the exponential phase were harvested at approximately 80% confluence. The HL-7702 cell line was used as a control.

**Quantitative reverse transcription PCR (qRT-PCR)**

Total RNA of the tissue samples was extracted with RNAiso Plus reagent (Takara, Dalian, China). cDNA was prepared from total RNA using the PrimeScript™ First-Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). NVM-1 and GAPDH mRNA expression was measured by PCR using a SYBR green qPCR assay kit (Roche, Shanghai, China). Primer sequences were as follows: NVM-1 sense primer 5’-TGA GCT CCT TCA TCA AGA TTT GGA-3’ and antisense primer 5’-CAG GTT GTC TCA AGA GCC TTG GGT TTT-3’, and GAPDH sense primer 5’-AGC TGA ACG GGA AGC TCA CTG TTA-3’ and antisense primer 5’-TGC TTC AAA TTC GTC CTG G-3’. The data were interpreted using the 2^ΔΔCt method with GAPDH serving as a reference gene for normalization.

**Gene silencing**

For transient transfection, MHCC-97L, MHCC-97H, and HCCLM3 cells were plated in 6-well plates and transfected with small interfering RNA (siRNA) for 48 h using Lipofectamine 2000 (Invitrogen, CA, USA) following the manufacturer’s instruction. The sequences were as follows: S1 targeting 5’-GCA TCT TGT CAC TGG TTC TT-3’, S2 targeting 5’-AGA TAT CAG CGG ATT TGA AT-3’), negative control (NC) targeting 5’-TTC TCC GAA CGT GTC ACG TTT-3’. The transfected cells were used in further assays or for RNA/protein extraction. Lentivirus-mediated transfection of short hairpin RNA (shRNA) (GenePharma, Shanghai, China) was performed on HCCLM3 cells according to manufacturer’s protocol. The target and negative control sequences were 5’-GCA TCT TGT CAC TGG TTC TT-3’ and 5’-TTC TCC GAA CGT GTC ACG TTT-3’, respectively.

**Western blotting**

The following primary antibodies were used in immunoblotting assays: the NVM-1 antibody kindly provided by Professor Wilko Thiele, and apoptosis- and EMT-related antibodies (Bcl-2, Bax, Caspase-3, Caspase-8, p53, β-catenin, slug, MMP2, MMP9) purchased from Santa Cruz (CA, USA). Horseradish peroxidase-conjugated goat anti-mouse/rabbit secondary antibody (Abcam, CA, USA) was used at a 1:4000 dilution and detected using ECL substrate (Millipore, MA, USA) as described [12].

**Cell proliferation assay**

Cell growth was measured using the CCK-8 assay (KeyGen, Nanjing, China), as described previously [7].
Apoptosis assay

Cell apoptosis was measured by FACS analysis using the Annexin V-FITC/PI Apoptosis Detection Kit (KeyGen). TdT-UTP nick end-labeling (TUNEL) assays were performed with a one-step TUNEL assay kit (Roche, Switzerland), according to the manufacturer’s instructions, as described previously [7].

Transwell migration and invasion assays

Transfected cells were trypsinized and resuspended in serum-free medium. A total of 5 × 10⁵ cells (200 μl) were added to the upper chambers of transwell inserts with 8-μm pores in 24-well plates (Corning, CA, USA). Migration was induced with 20% FBS-supplemented medium added to the lower chambers. After 48 h, non-adherent cells in the chambers were removed and the inserts were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. Numbers of migrated cells were counted in three different fields under an inverted microscope (Nikon, Tokyo, Japan). For the invasion assay, all conditions were as described for the migration assay, except that cells were added to Matrigel-coated inserts.

In vivo experiments

HCCLM3 cells (5 × 10⁶) stably transfected with a Lentivirus encoding NVM-1 shRNA (sh-NVM-1)
or negative control shRNA (sh-NC) were subcu-
taneously injected into the flanks of BALB/c
nude mice as previously described [13]. The
tumor volume was determined by measuring
two of its dimensions and calculated as tumor
volume \((V) = \text{length}(a) \times \text{width}(b) \times \sqrt{a \times b \times \frac{2}{3}}\).
Tumor samples were subjected to further
detections.

Statistical analysis

Results are displayed as the mean ± SEM.
Data were analyzed with the SPSS statistical
package for Windows version 13 (SPSS, Chi-
go, IL, USA) and GraphPad Prism 5 (Graph-
Pad Software, Inc., San Diego, CA, USA). Means
were compared using the Pearson chi-squared
test, multi-variant Cox regression analysis, two-
tailed Student's t-tests, Kaplan-Meier plots,
log-rank tests, or ANOVA as appropriate.
Differences were considered significant at \(P <
0.05\).

Results

NVM-1 is frequently upregulated in HCC tis-
ues and positive NVM-1 expression confers
poor prognosis

To explore NVM-1 expression in HCC tissues,
we detected the protein expression in a retro-
spective cohort of 92 paired HCC samples by
immunohistochemical staining. NVM-1 protein
expression in HCC cancerous tissues was sig-
nificantly higher than that in matched noncan-
cerous tissues, while no staining was observed
in liver tissues with benign lesions (Figure 1).
NVM-1 mRNA expression was higher in various
HCC cell lines (HepG2, SMCC-7721, MHCC-
97L, MHCC-97H, and HCCLM3) than in the nor-
mal hepatocyte cell line, HL-7702 (\(P = 0.000\),
Figure 2A). Positive protein expression was
detected in 52.2% (48/92) of the HCC speci-
mens, whereas only 28.3% (26/92) of the
matched noncancerous tissues showed a posi-
tive signal (\(P < 0.05\), Figure 1A). As shown in
Table 1, positive expression was significantly associated with the degree of tumor differenti-
ation (\(P = 0.000\)), TNM stage (\(P = 0.016\)), BCLC
stage (\(P = 0.015\)), tumor metastasis (\(P =
0.000\)), recurrence (\(P = 0.001\)), and recurrence
time (\(P = 0.001\)). These results indicated that
elevated expression of NVM-1 is correlated with malignant clinicopathologic parameters of
HCC. Kaplan-Meier analysis of clinical survival
information from the patients indicated that
tumors with NVM-1 positive expression indeed
associated with a shorter relapse-free survival
time (\(P = 0.001\), Figure 1B and 1C), which sug-
gests that NVM-1 may act as a promising bio-
marker for predicting prognosis of HCC patients.

Successful NVM-1 knockdown in HCC cell lines

To identify the role of NVM-1 in HCC cells, we
chose MHCC-97L, MHCC-97H, and HCCLM3
cells for further functional studies in vitro as
they express a high level of NVM-1 mRNA and
have high metastatic potential. After transient
transfection of NVM-1 siRNA for 48 h, qRT-PCR
and western blot analyses showed that both
mRNA and protein expression were success-
fully knocked down in the three HCC cell lines
(\(P < 0.05\) in all cases, Figure 2B and 2C).

NVM-1 promotes tumor growth in vitro and in vivo

Limitless replicative potential is a hallmark of
cancer cells [14]. To better understand the
effects of NVM-1 silencing on cell viability, and
more specifically on cell growth, cell viability
after transfection was tested at different time
points using a CCK-8 assay. A marked reduc-
tion in cell viability was observed after NVM-1
depletion as compared with non-transfected
control cells in the three HCC cell lines (\(P <
0.05\) in all cases, Figure 2D). Next, we sought
to determine whether NVM-1 affects tumor
growth in a nude mouse xenograft model.
HCCLM3 cells stably transfected with a plas-
mid harboring sh-NC or sh-NVM-1 were implant-
ed into nude mice through subcutaneous injec-
tion. The results showed that subcutaneous
tumor volumes in mice of the sh-NVM-1-ex-
pressing group were obviously smaller than
those in the sh-NC-expressing group (Figure 2F).
The tumor growth curves revealed that
NVM-1 knockdown slowed down tumor growth
significantly (\(P < 0.05\), Figure 2E), which was
consistent with the in vitro data. Immunohisto-
chemical staining indicated that the expres-
sion of the proliferation marker Ki-67 in subcu-
taneous tumor was significantly lower in the sh-
NVM-1 group than in the sh-NC group (Figure
2F). Thus, we speculated that NVM-1 may act
as an oncogene by promoting the proliferative
ability of HCC cells.

NVM-1 silencing induces apoptosis in HCC
cells

Evasion of apoptosis is another hallmark of
cancer cells [14]. To explore the potential effect
The negative role of NVM-1 in HCC

Figure 2. NVM-1 knockdown by siRNA inhibits tumor cell proliferation in vitro and in vivo. A: Expression of NVM-1 mRNA in HL-7702 and five HCC cell lines. B: Efficiency of NVM-1 knockdown measured by RT-PCR. C: Efficiency of NVM-1 knockdown measured by western blotting. D: Cell proliferation after NVM-1 knockdown tested by CCK-8. E: Tumor volumes measured on the indicated days to assess the effects of NVM-1 on subcutaneous tumor growth in nude mice. F: Expression of Ki-67 protein in tumor nodules detected by immunohistochemical staining.
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Figure 3. NVM-1 silencing by siRNA induces apoptosis in HCC cells. A: Change in apoptotic cell fraction after NVM-1 knockdown analyzed by flow cytometry. B: Proportions of early and late apoptotic cells after NVM-1 silencing. C: Expression of apoptosis-related proteins detected by western blotting after NVM-1 knockdown. D: Apoptosis detected by TUNEL assay after NVM-1 depletion. E: Quantification of apoptotic cells detected by TUNEL after NVM-1 depletion.
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of NVM-1 on apoptosis, flow cytometry was used to detect changes in apoptosis in NVM-1-silenced versus non-silenced HCC cell lines. Remarkable changes were detected in NVM-1 depletion groups (> 17% late apoptotic cells) compared with the control groups (< 8% late apoptotic cells) in the three HCC cell lines ($P < 0.05$ in all cases, Figure 3A and 3B). Similar results were obtained by TUNEL assay (Figure 3D and 3E). Western blot analysis revealed increased p53, Bax, caspase-3, and caspase-8 expression, and decreased Bcl expression in the three HCC cell lines upon NVM-1 silencing (Figure 3C). Together, these results indicated that NVM-1 plays an important anti-apoptotic role and may be tightly associated with the levels of apoptosis-related proteins.

**NVM-1 promotes migration, invasion, and EMT progression in vitro and in vivo**

In the process of tumor formation, cancer cells tend to have stronger migration and invasion abilities, and epithelial-mesenchymal transition (EMT) progression is considered to be related to these phenomena [15]. To determine whether NVM-1 affects the HCC cell migration and invasion abilities, transwell migration and invasion assays were conducted after NVM-1 silencing. NVM-1 knockdown led to significantly
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lower migration and invasion ($P < 0.05$ for both, Figure 4). In addition, decreased N-cadherin, Slug, MMP2, MMP9, and β-catenin protein expression, and increased E-cadherin protein level were observed in NVM-1-depleted cells (Figure 5A). To further elucidate the effects of NVM-1 on HCC cell migration and invasion, HCCLM3 cells stably transfected with sh-NC or sh-NVM-1 were injected into the caudal vein in 4 pairs of nude mice. Livers and lungs were harvested after 2 months. Pathologic examination revealed no liver or lung metastases in the sh-NVM-1 group, while in the sh-NC group, all mice had liver metastases and 2 out of 4 mice had lung metastases in varying degrees (Figure 5B). Immunohistochemical staining indicated that N-cadherin and Vimentin expression in subcutaneous tumor was significantly lower in the sh-NVM-1 group than in the sh-NC group, while E-cadherin was upregulated (Figure 5C).

Discussion

Tumor metastasis and recurrence are thought to account for nearly 90% of cancer deaths [16]. Understanding the underlying molecular regulatory mechanism of metastasis formation and recurrence as a prerequisite for the development of novel therapies that improve survival. A previous study indicated a critical role for NVM-1 in tumor invasion and metastasis and suggested NVM-1 as a potential, novel target for anti-metastatic cancer therapy [8]. To our knowledge, the role of NVM-1 in metastasis or recurrence in HCC has not been thoroughly investigated to date.

To explore the role of NVM-1 in HCC, we initially detected NVM-1 protein expression in clinical tissue specimens. NVM-1 protein was observed in both the nucleus and the cytoplasm but was more abundant in the nucleus, and the NVM-1-positive rate was higher in cancereous tissues than in noncancereous tissues and benign hepatic lesions. Additionally, NVM-1 mRNA expression was higher in HCC cell lines than in normal hepatocytes, which is in line with a previous report [8]. We found that NVM-1 expression correlated with tumor stage, tumor
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recurrence, and metastasis, and NVM-1 was enhanced in poorly differentiated tumors, especially in patients displaying early recurrence (within 6 months). Furthermore, patients with high expression of NVM-1 had a significantly shorter relapse-free survival time. Altogether, these results suggest that NVM-1 expression is critical for prognosis determination in HCC patients, and the protein may have a role in tumor metastasis and recurrence.

In most HCC cases, hepatocarcinogenesis arises in the setting of chronic liver damage and inflammation, which leads to fibrosis and, ultimately, to cirrhosis [17]. In China, the leading risk factor for HCC is hepatitis B or C virus infection [18]. In our study, nearly 90% of HCC patients were diagnosed with hepatitis B; however, we found no competent evidence to confirm the correlation between expression of NVM-1 and hepatitis B infection. Moreover, we found no significant correlation between NVM-1 expression and alpha-1-fetoprotein (AFP) expression, tumor size, lymph node metastasis, etc. However, higher rates of NVM-1 positive expression were found in patients with high AFP level, larger tumors (> 3 cm), multiple tumors, or lymph node metastasis. The fact that we did not find significant correlations may be caused by an insufficient number of cases; further studies are required to explore the relationship between NVM-1 and HCC.

Tumorigenesis is a complex process involving a variety of factors, including uncontrolled cell proliferation [19], cell cycle disorders [20, 21], and apoptosis evasion [22]. Using siRNA, NVM-1 expression was successfully knocked down in the three HCC cell lines, MHCC-97L, MHCC-97H, and HCCLM3. The proliferation ability of the NVM-1-silenced cells was significantly weakened, apoptosis was induced, and apoptosis-related proteins changed accordingly. Thus, we speculate that the inhibition of cell proliferation induced by NVM-1 depletion may be achieved through promoting apoptosis.

In recent years, EMT has been considered to be related to tumor metastasis [23]. In the process of EMT, epithelial cells break-up their intercellular connections and cell–matrix junctions, reorganize their cytoskeleton, change shape, and reprogram gene expression and signaling pathways to increase their motility and acquire an invasive phenotype [24, 25]. In our study, cell migration and invasion were weakened after NVM-1 silencing. To verify the role of NVM-1 in HCC in vivo, we established a xenograft mouse model to assay tumor growth and metastasis. Silencing of NVM-1 significantly slowed down tumor growth and lowered the incidence of liver and lung metastases. Thus, NVM-1 may play an important role in tumor aggressiveness, which was corroborated by corresponding changes in EMT-related proteins in vitro and in vivo. This may be due to the function of NVM-1 protein as a methyltransferase; on the one hand, NVM-1 can change the methylation status of various proteins, promoting tumor cell proliferation and metastasis potential, and on the other hand, NVM-1 protein can appear in the nucleus and in the cytoplasm, but it functions in the nucleus to ultimately promote tumorigenesis [9]. In the light of these results, we believe that NVM-1 might exert a metastasis-promoting effect by activating EMT in HCC.

To our knowledge, this study for the first time showed that NVM-1 is upregulated in HCC tissues and negatively correlates with the prognosis of HCC. We found that NVM-1 knockdown significantly inhibits tumor cell proliferation, migration, invasion, and EMT progression. Thus, we expect NVM-1 to become a new, effective biomarker that will open up treatment options for HCC.

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Disclosure of conflict of interest

None.

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