Nucleolar protein 6 promotes cell proliferation and acts as a potential novel prognostic marker for hepatocellular carcinoma

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Abstract

Background: Nucleolar protein 6 (NOL6) is a nucleolar RNA-associated protein that is highly conserved between species. It has been proved to be associated with the prognosis of liver cancer. However, the underlying mechanism has not been fully established. This study aimed to assess the relationship between NOL6 and liver cancer prognosis.

Methods: We constructed an *NOL6*-short hairpin RNA (shRNA)-expressing lentivirus. Through viral transfection, cell growth assay and fluorescence-activated cell sorting, we evaluated the effect of shRNA-mediated *NOL6* knockdown on the proliferation, colony formation, and apoptosis of hepatocellular carcinoma (HCC) cells. The relationship between *NOL6* expression and HCC patient survival has been established through bioinformatics analysis. We also explored the downstream molecular regulatory network of *NOL6* in HCC by performing an Ingenuity Pathway Analysis in the database.

Results: Increased *NOL6* expression was detected in HCC cells compared to normal controls; HCC patients with high *NOL6* expression had poorer prognoses than those with low expression. *NOL6* knockdown inhibited HCC cell proliferation, apoptosis, and colony formation. Also, *MAPK8*, *CEBPA*, and *FOSL1* were selected as potential downstream genes of *NOL6*.

Conclusions: *NOL6* up-regulates HCC cell proliferation and affects downstream expression of related genes. Moreover, *NOL6* is considered to be associated with poor prognosis in HCC patients.

Keywords: *NOL6*; Hepatocellular carcinoma; Prognostic marker; Bioinformatics; MAPK8; CEBPA; FOSL1

Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver malignancy.[1-4] Treatment for early-stage HCC involves resection and/or liver transplantation. However, for those late-stage patients, only kinase inhibitors (sorafenib and regorafenib) and immune checkpoint inhibitors (pembrolizumab, nivolumab) have been approved.[5-7] The lack of diagnostic markers for early detection and the limited number of available treatment options increases the risk of lethality and poor prognosis.[8,9] It is necessary to conduct a more comprehensive analysis of HCC to develop an effective therapeutic strategy.

Nucleolar protein 6 (NOL6), also known as NRAP, utp22, and baa311h10.1, is encoded by the *NOL6* gene on human chromosome 9. The gene encodes proteins related to nucleolar RNA and is highly conserved among species. RNase treatment showed that its nucleolar localization is RNA dependent. The molecular functions of *NOL6* include the establishment of RNA binding and localization.[10] Further studies suggest that this protein is associated with ribosome biogenesis through interaction with pre-rRNA primary transcripts.[11] Alternative splicing has been found at this locus, and two splice variants encoding different subtypes have been identified. The Human Protein Atlas indicates that *NOL6* is related to HCC prognosis.[12] High *NOL6* expression protects against the occurrence of liver cancer. However, the specific mechanism of how *NOL6* affects the prognosis of liver cancer remains unclear.

In this study, we investigated the functions of *NOL6* in the human liver cancer cell lines, BEL-7404 and SMMC-7721. We then constructed a *NOL6*-shRNA-expressing lentivirus and evaluated the effects of shRNA-mediated *NOL6* knockdown on cell proliferation, colony formation, and apoptosis. Furthermore, we constructed a gene expression profile chip containing samples with *NOL6* knockdown, and we studied the potential downstream genes that may be altered by changes in the *NOL6* expression and also examined their functional pathways. The results revealed

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correlations between *NOL6* and HCC properties, and the molecular regulatory network of *NOL6* in HCC. We also explored the relationship between *NOL6* expression and HCC patient survival.

Methods

Cell culture

Human HCC cell lines, BEL-7404, BEL-7402, HepG2, and SMMC-7721, were obtained from Shanghai Genechem Co., Ltd. (China). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Corning, NY, USA) plus 10% fetal bovine serum (Bovogen, Melbourne, Australia) in a 5% CO2 incubator at 37.0°C.

Antibodies

Primary antibodies against NOL6 (Abcam, Cambridge, UK), CEBPA (CST, Boston, USA), MAP2K5 (Abcam), ID1 (Abcam), MAPK8 (CST), SNAI2 (CST), FOSL1 (Abcam), Flag (Sigma, St. Louis, MO, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz, CA, USA) were used.

Lentiviral NOL6-shRNA vector construction and packaging

Small interfering RNAs targeting the *NOL6* gene were designed, and the following sequence was selected as the interference target, 5′-TCGGATTGATGCCTTCCTA-3′. Lentiviral-mediated transduction was carried out using synthetic short hairpin RNA (shRNA) targeting *NOL6* and non-targeted (control) oligonucleotides, which were synthesized by Shanghai Genechem Co., Ltd. according to the manufacturer's instructions.

Infection of HCC cells with lentivirus

Well-growing BEL-7404 and SMMC-7721 cells were cultured. And based on the results of the preliminary experiment of lentivirus infection, the experimental conditions of each group were designed for formal infection. For fluorescently labeled lentivirus infection, the expression of green fluorescent protein (GFP) was observed under a fluorescence microscope concerning the infection time determined by the pre-experiment. The fluorescence rate was approximately 70% to 80%, and the cell confluence was approximately 80%. These cells were collected for subsequent experiments. In the case of infection with a lentivirus marked with resistance genes, after 48 to 72 h infection, cells were screened with antibiotics and well-grown cells were collected for downstream experiments.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted using TRIzol reagent (Shanghai Pufei Biotechnology, Shanghai, China) and reverse transcribed to complementary DNA with Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega Corp, Madison, WI, USA) following the manufacturer's instructions. The primers used were as follows: *NOL6*-F, 5′-TGAGGCACGGCTGTCTATGAT-3′ and *NOL6*-R, 5′-GGAGATGCAGGACATGGTC-3′. The *GAPDH* gene was amplified as internal control with the following primer sequences: GAPDH-F, 5′-TGACTTCAACAGCGACACCCA-3′ and GAPDH-R, 5′-CACCCTGTTGCTGTAGCCAAA-3′. qPCR was performed using SYBR Green PCR Master Mix (TAKARA, Kyoto, Japan) in 20 μL reactions.

Cell growth assay

After infection with *NOL6* shRNA lentivirus and scrambled shRNA lentivirus, cells in the logarithmic phase were digested, resuspended, counted, and inoculated in 96-well plates. The next day, cells with green fluorescence were photographed and counted by a Celigo Reader (Nexcelom, Boston, MA, USA) at the same time every day. Cell growth was observed continuously for 5 days, and then cell growth curves were drawn.

Colony formation assay

Cells at the logarithmic phase after infection were inoculated in six-well plates at a density of 800 cells/well. The culture medium was changed every 3 days. The cells were allowed to grow for 12 days to form colonies. When the number of cells in most single colonies was >50, the cells were washed with PBS once and fixed in paraformaldehyde (Sangon Biotech [Shanghai] Co., Ltd., Shanghai, China) for 30 min. The cells were then washed with PBS and stained with Giemsa dye (Sangon Biotech [Shanghai] Co., Ltd.) for 20 min. After washing with deionized H_2O (ddH2O) several times, the colonies were counted under a fluorescence microscope (Olympus, Tokyo, Japan).

Apoptosis by fluorescence-activated cell sorting (FACS)

Cell apoptosis was assayed by annexin V-APC staining and detected by FACS. The cells were harvested, washed once in 1 × D-Hanks, once in 1 × binding buffer, and then resuspended in staining buffer for analysis. Then, 10 μL annexin V-APC was added to the above cell suspension. After incubation, the cells were detected using a Millipore Guava cytometer (Millipore, Burlington, MA, USA). The data were performed with Millipore's InCyte™ Software (Millipore).

Microarray

An Affymetrix PrimeView Human Gene Expression Array was used in this study and was constructed by Shanghai Genechem Co., Ltd. Total RNA was extracted by the TRIzol method. Total RNA was detected by a Nanodrop 2000 (Thermo, MA, USA) and an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). A GeneChip Hybridization Oven 645 (Affymetrix, Santa Clara, CA, USA) was used in the hybridization process. A GeneChip Fluidics Station 450 (Affymetrix) was used for chip washing and dyeing. A GeneChip Scanner 3000 (Affymetrix) was used for chip scanning.

Data analysis

In the process of chip information analysis, the quality evaluation of raw chip data is usually carried out first.

After quality control, the data are filtered, and the remaining data that meet the filtering standards are subjected to further analysis, including significant difference analysis and functional analysis of differentially expressed genes.

Statistical analysis

The data shown are representative of at least three repeated experiments. The graphs represent the mean ± standard deviation. Student's *t* tests were used for significance analysis. SPSS 13.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. $P < 0.05$ was accepted as statistically significant.

The causal analytics tools "Upstream Regulator Analysis," "Mechanistic Networks," "Causal Network Analysis," and "Downstream Effects Analysis" are implemented and available within Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com). Differential expression was determined using filtering criteria of ≥1.2-fold change and analysis of variance (false discovery rate [FDR] <0.05) to determine significance.

Results

NOL6 was relatively highly expressed in HCC tissue and cells

We first validated the expression of *NOL6* in The Cancer Genome Atlas database (368 liver cancer tissues *vs*. 160 normal liver tissues). We found that the expression of *NOL6* in HCC tissues was higher than that in normal liver tissues. Especially, in patients with higher tumor stage, grade or lymph node positive, *NOL6* expression was also higher [Figure 1A]. Above results were also verified in the TIMER database (Tumor Immune Estimation Resource, https://cistrome.shinyapps.io/timer/). In an RNA sequencing data that included multiple tumor types, the expression level of *NOL6* was relatively high in HCC and many other malignant tumors [Figure 1A]. Then, to investigate the role of *NOL6* in HCC, we tested the expression of *NOL6* in liver cancer cell lines by real-time quantitative PCR. We found that *NOL6* was highly expressed in a variety of liver cancer cell lines in comparison with normal liver cell [Figure 2A]. To facilitate the study of the subsequent regulation of cell gene expression levels, we chose the BEL-7404 and SMMC-7721 cell lines as the subjects of subsequent experiments.

The survival rate of HCC with high NOL6 expression is lower

To determine if *NOL6* gene expression is related to prognostic characteristics, the gene expression profiles in the Kaplan-Meier plotter database (http://kmplot.com/analysis/index.php?p=service&cancer=liver), which contains 364 HCC patients, were analyzed using the univariate Cox proportional hazards regression model. High expression of *NOL6* (at the mRNA level) was found to be significantly negatively related to overall survival (hazard ratio: 1.71, 95% confidence interval: 1.43–2.06, $P < 0.001$) [Figure 1B]. Similar results were found in 341 HCC samples in the Human Protein Atlas. Based on these *NOL6* protein expression data, it can be concluded that high *NOL6* expression is associated with poor prognosis ($P_{\text{log-rank}} < 0.001$), especially in patients with stage I–II ($P_{\text{log-rank}} < 0.001$) [Figure 1C].

Knockdown of NOL6 inhibits HCC cell growth

shRNAs containing *NOL6*-targeting or non-silencing sequences were cloned into GV115 plasmid vectors. Then, *NOL6* shRNA lentivirus or non-silencing lentivirus (negative control) expressing GFP was generated and applied to the human HCC cell lines, BEL-7404 and SMMC-7721. As shown in Figure 2B, 3 days after infection, Western blot analysis showed that *NOL6* protein expression was downregulated in BEL-7404 and SMMC-7721 cells infected with *NOL6*-shRNA lentivirus compared with those infected with non-silencing lentivirus. The qRT-PCR assay suggested that the *NOL6* mRNA level was reduced by approximately 60% in both BEL-7404 and SMMC-7721 cells treated with *NOL6* shRNA lentivirus compared with those in the negative control group.

To assess the role of *NOL6* in the proliferation of HCC cell lines, they were infected with shRNA lentivirus and counted continuously for 5 days by Cellomics Arrayscan (Thermo). Green fluorescence was observed over the 5-day period, and a 5-day cell proliferation curve and a cell/fold curve were plotted. As shown in Figure 2C, the number of BEL-7404 cells increased approximately five-fold in the control group and increased by approximately two-fold in the *NOL6*-shRNA group from day 0 to day 5, indicating that *NOL6* knockdown inhibits the proliferation of BEL-7404 cells. Similar results were found in SMMC-7721 cells, as the control group cell count increased much more than that in the *NOL6*-shRNA group.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were also carried out. The optical density at 490 nm (OD490), which represents the proliferation activity of cells, was lower in the *NOL6* downregulated group than in the control group in both cell lines [Figure 2D].

Knockdown of NOL6 inhibits the colony formation of HCC cells

We then studied the colony formation ability of HCC cells treated with *NOL6* shRNA lentivirus. Cells treated with *NOL6* shRNA or control lentivirus were allowed to grow for 12 days to form colonies. As shown in Figure 3A, compared with control cells, *NOL6* knockdown resulted in a significant reduction in the number of colonies in both HCC cell lines ($P < 0.01$). These results suggested that the *NOL6* gene is associated with the colony-forming ability of HCC cells.

Knockdown of NOL6 increases HCC cell apoptosis

We detected the effect of *NOL6* shRNA on HCC cell apoptosis with annexin V-APC staining by FACS 72 h after infection. As a result, the percentage of apoptotic cells in the *NOL6* knockdown group increased several times

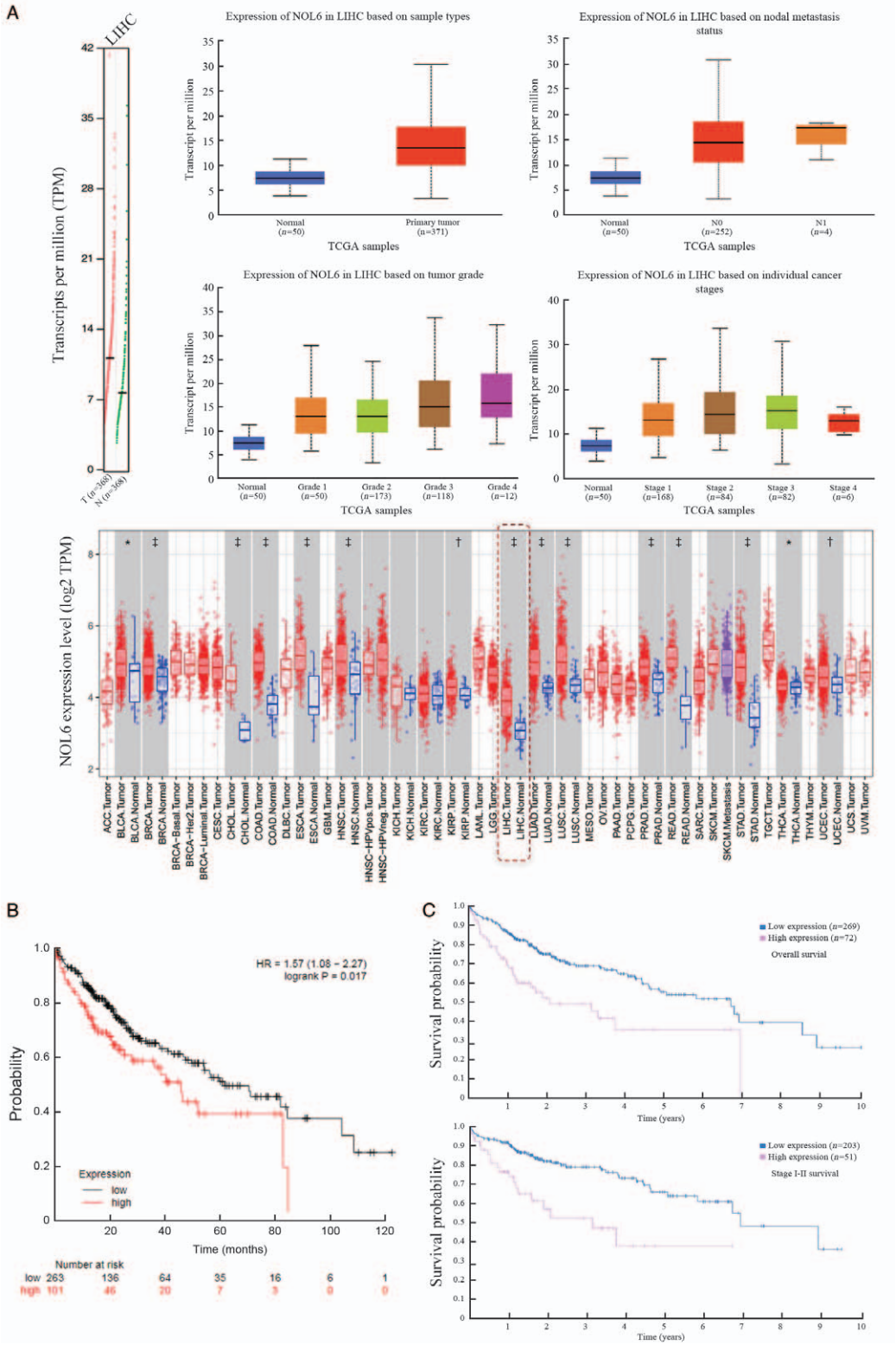

Figure 1: *NOL6* was highly expressed in HCC tissues, and patients with high expression of *NOL6* in HCC have a lower survival rate. (A) The dot chart on the upper left was the expression of *NOL6* in 368 pairs of liver hepatocellular carcinoma (LIHC) and normal control tissues in the GEPIA database. *NOL6* had a higher average number of transcripts in LIHC; Data from TCGA (Boxplots) that involved 528 samples also showed that *NOL6* expression was higher in LIHC tissues; and the expression of *NOL6* was higher in patients with high tumor stages, grades, or with positive lymph nodes. The lower part showed the *NOL6* expression in a variety of tumor tissues, which still supports the conclusion that *NOL6* is highly expressed in liver cancer. (Data of liver cancer *vs.* liver normal tissue can be seen in the dashed red box; $^{*}/^{\dagger}/^{\ddagger}$ represents $P < 0.05/P < 0.01/P < 0.001$). (B) The survival curve that involved 364 patients was generated in the Kaplan-Meier plotter website ($P_{\text{log-rank}} = 0.017$). Cases of high *NOL6* expression in HCC had a lower survival rate. (C) The survival curves that involved 341 patients were generated in the Human Protein Atlas website. Cases of high *NOL6* expression in HCC also had a lower survival rate. For patients whose tumors were stage I–II, the above-mentioned difference in survival was even more obvious. HCC: Hepatocellular carcinoma; *NOL6*: Nucleolar protein 6; TCGA: The Cancer Genome Atlas.

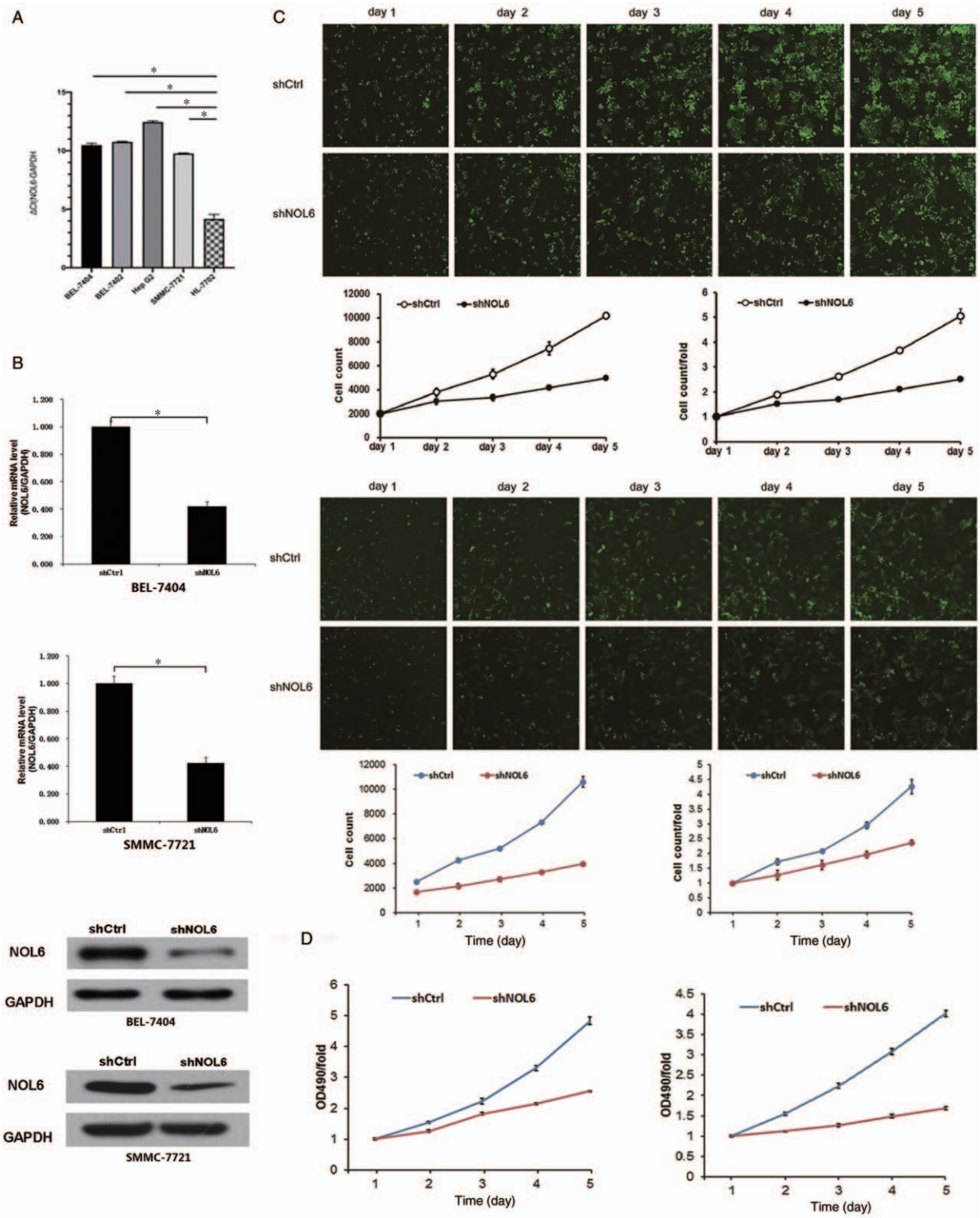

Figure 2: Knockdown of *NOL6* inhibits HCC cell proliferation in HCC cells. (A) In comparison with normal cells (HL-7702), increased *NOL6* mRNA expressions were demonstrated in multiple HCC cell lines; (B) After treated with *NOL6* shRNA lentivirus, the relative expression of *NOL6* was significantly reduced in HCC cells. *Represents $P < 0.01$. Western Blot results also showed that the *NOL6* protein expression was significantly reduced by *NOL6* shRNA lentivirus. (C) Fluorescent cell counting experiment showed that downregulated *NOL6* inhibited the proliferation rate in HCC cells; the upper graph shows the growth difference of HCC cells in 5 consecutive days, and the lower graph corresponds to the difference in cell count and fluorescence intensity. (D) MTT result also proved that lower level of *NOL6* inhibited the proliferation of HCC cells. The experiments in Figure 2 were repeated three times. HCC: Hepatocellular carcinoma; *NOL6*: Nucleolar protein 6; shRNA: Short hairpin RNA.

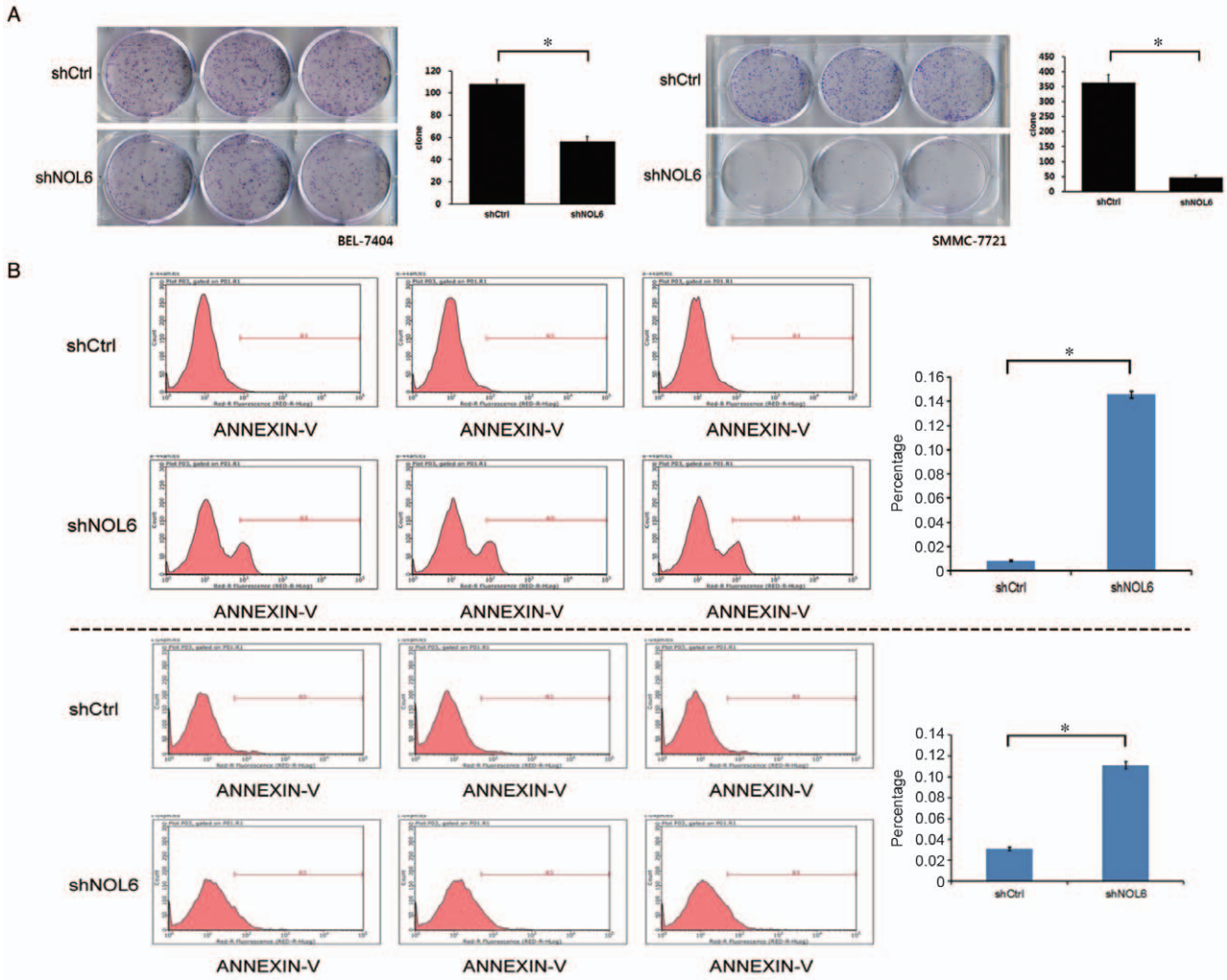

Figure 3: *NOL6* expression was positively correlated with HCC cell's clone formation and negatively correlated with cell apoptosis. (A) Knockdown of *NOL6* inhibits colony formation in BEL-7404 cells (Left) and SMMC-7721 cells (right). (B) The flow cytometry results showed that downregulated *NOL6* increased HCC cells apoptosis. Statistics showed that apoptotic cells increased several times in *NOL6* knockdown cells. The experiment in the figure was repeated more than three times, where * represents $P > 0.01$. The left side is a presentation of selected typical results, and the right side is the bar graph of statistical data. Results of BEL-7404 cells are shown above the dotted line, and those of SMMC-7721 cells are under the dotted line. HCC: Hepatocellular carcinoma; *NOL6*: Nucleolar protein 6.

[Figure 3B]. This result suggests that *NOL6* knockdown promotes HCC cell apoptosis.

MAPK8, CEBPA, and FOSL1 were identified as potential NOL6 downstream acting effectors

After *NOL6* shRNA lentivirus or non-silencing lentivirus (negative control) were generated and applied to the human HCC cell line BEL-7404, we divided the cells into two groups (3 *vs*. 3) and used human gene expression chips to detect differentially expressed genes between the groups. IPA (http://www.ingenuity.com) was then performed.

After data filtering and hierarchical clustering (with |Fold Change|≥1.2 and FDR <0.05 as standard), the disease function analysis revealed genes related to cell proliferation and apoptosis (data not shown). Among them, genes that inhibit tumor cell proliferation or promote tumor cell apoptosis were selected, and a gene relationship network was drawn. As shown in Figure 4A, genes such as *MAPK8*, *CEBPA*, and *FOSL1* were at the center of the network.

Next, we performed a Western blot assay based on those potential downstream genes [Figure 4B]. *MAPK8*, *CEBPA*, and *FOSL1* expression changed in HCC cells with differential expression of *NOL6*, which means that *NOL6* affects the proliferation and apoptosis of HCC cells through the effects of these genes.

We also performed a pathway enrichment analysis of differentially expressed genes between groups. As Figure 4C shows, the top four downregulated functional pathways included cancer, organismal injury and abnormalities, gastrointestinal disease, and hepatic system disease.

Discussion

NOL6 is a nucleolar protein that was highly conserved throughout evolution. Previous studies of *NOL6* have

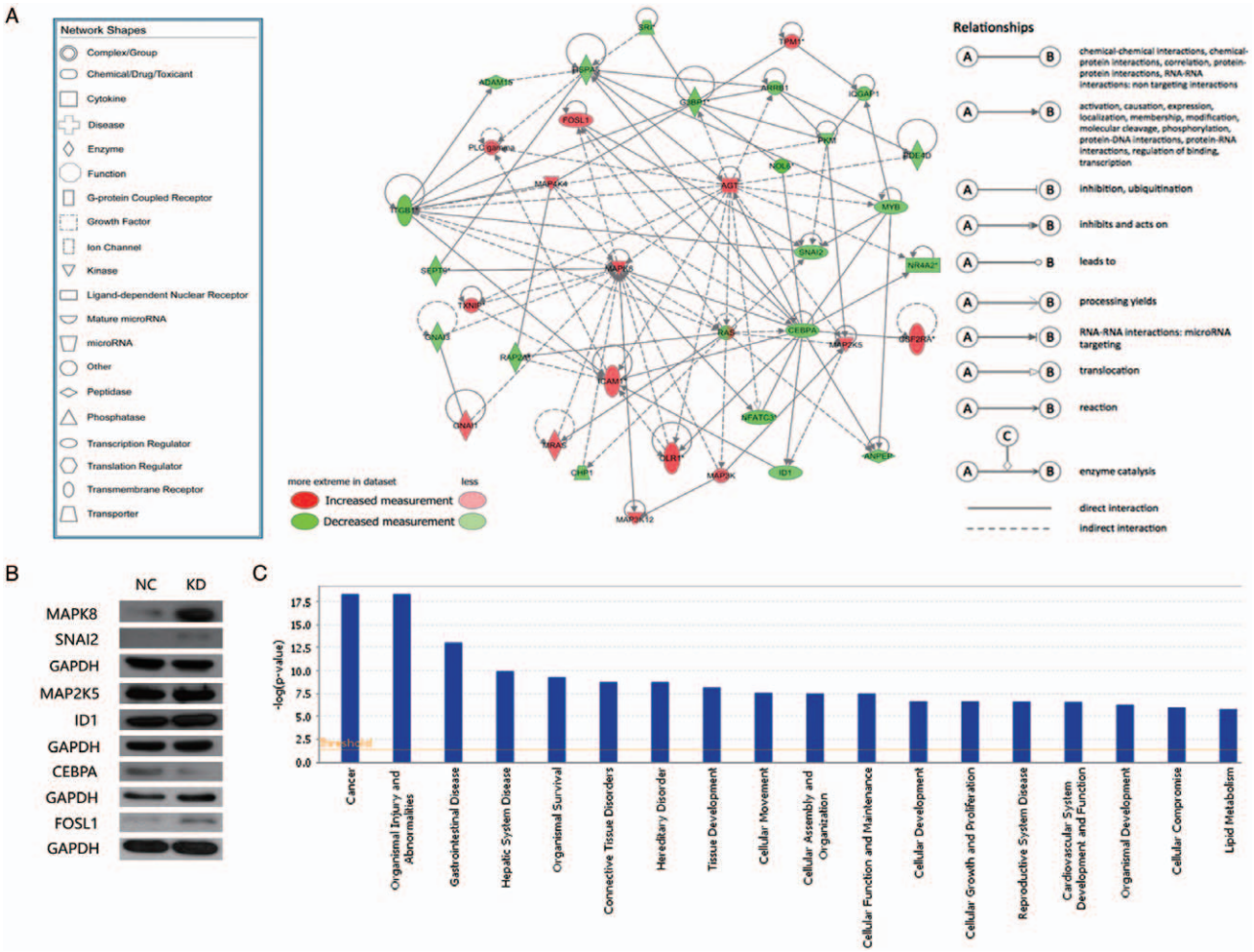

Figure 4: IPA gene analysis and IPA-based upstream regulation analysis results. (A) A gene interacting network with *NOL6* was presented by bioinformatics analysis. The red genes represent the same trend as *NOL6* expression, and the green ones represent the opposite expression trend. The solid line indicates direct correlation, and the dotted line indicates indirect relationship. (B) Western blot analysis showed that in comparing with the normal control (NC) group, the expressions of *MAPK8*, *CEBPA*, and *FOSL1* changed accordingly when *NOL6* was knocked down (KD). (C) A downstream pathway enrichment analysis table showed the significant enrichment pathways of those differential genes between groups. The abscissa is the path name, and the ordinate is the level of significance of the enrichment (the negative logarithm of the base 10). IPA: Ingenuity pathway analysis; NOL6: Nucleolar protein 6.

linked its nucleolar localization to ribosomal biogenesis. Confocal microscopy was adopted to observe the localization of *NOL6* in sub-nucleoli. Li *et al*[13] showed that loss of *NOL6* expression within these cells resulted in G_1 phase arrest and cell death induction. These findings demonstrate that *NOL6* is involved in rRNA processing and cell cycle progression.

In this study, we proved that *NOL6* was highly expressed in HCC cells. Through survival analysis, we found that *NOL6* is associated with poor prognosis in HCC patients. Then, we proved that the knockdown of *NOL6* significantly inhibited cell proliferation and colony formation and promoted apoptosis in HCC cells. These results suggest that *NOL6* was associated with tumor progression in HCC. It may become a target for anti-HCC treatment.

Through GeneChip and IPA analysis, *MAPK8*, *CEBPA*, and *FOSL1* were strongly considered as genes downstream of *NOL6*. By consulting the literature, we learned that these three genes are all closely related to cell apoptosis. FRA1, which encoded by *FOSL1* gene, can directly bind to *CEBPA*'s promoter.[14,15]*NOL6* can upregulate FRA1 and JNK1 (protein of *MAPK8*), both of which can downregulate *CEBPA* and finally affect apoptosis.[16]-*CEBPA* can prevent the growth and division of cells which are too fast or uncontrolled. Therefore, it should be a tumor suppressor. *CEBPA* is closely related to CCAAT enhancer-binding protein α, which is a transcription factor that can bind to specific regions of DNA and regulate its function. CCAAT enhancer-binding protein alpha is involved in the maturation (differentiation) of blood cells, which is also related to the occurrence and development of HCC.[17,18] These data, together with the pathway enrichment analysis results of our IPA analysis, indicate that *NOL6* plays a role in regulating HCC growth and apoptosis.

Targeted molecular therapy is a personalized medical therapy designed to treat cancer by blocking unique molecular abnormalities that drive cancer growth.[19] Gene-targeted therapies based on RNA interference have shown great potential because they can specifically and

effectively reduce the expression of the target gene.[20] Drugs used in targeted therapy are designed to interfere with specific biochemical pathways that are critical to the development, growth, and spread of cancers.[21-25] Like conventional chemotherapy agents, the lentivirus that mediating *NOL6* knockdown also targets the proliferating of body cells, but it has no genetic toxicity. Therefore, it may serve as a useful target in anti-HCC therapy.

In summary, we found that the *NOL6* gene is involved in cell proliferation and apoptosis in HCC cell lines. And the expression of *NOL6* is correspondingly linked to the prognosis of HCC patients. *NOL6* may regulate cell proliferation and apoptosis through the *MAPK8*, *CEBPA*, and *FOSL1* genes. *NOL6* plays a role in the occurrence and development of liver cancer. Of course, there is still much work to be done regarding the specific and detailed function of *NOL6*.

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Conflicts of interest

None.

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