Abstract. We investigated the role of microRNA-26b signaling in mediating the tumor growth of human liver cancer. MicroRNA-26b expression was observably downregulated in human liver cancer tissue. Forced upregulation of microRNA-26b had anticancer effects, inhibited cell proliferation, induced apoptosis and promoted Bax and caspase-3/-9 protein expression in a liver cancer cell line as determined by MTT assay, flow cytometry and western blot analysis. MicroRNA-26b significantly suppressed the PI3K/Akt and NF-κB/MMP-9/VEGF pathways. PI3K inhibitor significantly facilitated the effects of microRNA-26b regarding the suppression of the PI3K/Akt pathway, inhibition of cell proliferation, induction of apoptosis and increases in Bax and caspase-3/-9 protein expression in the liver cancer cell line. NF-κB inhibitor also significantly enhanced the effects of microRNA-26b regarding suppression of the NF-κB/MMP-9/VEGF pathway, inhibition of cell proliferation, induction of apoptosis, and promotion of Bax and caspase-3/-9 protein expression in the liver cancer cell line. The present study demonstrated that microRNA-26b inhibits the tumor growth of human liver cancer through the PI3K/Akt and NF-κB/MMP-9/VEGF pathways.

Introduction

Liver cancer is the major type of primary liver cancer as well as one of the most common malignant tumors worldwide (1). China is a country with the highest incidence of liver cancer in the world and its overall morbidity and mortality show increasing trends (2). Although therapeutic approaches targeted at liver cancer have been continuously advanced in recent years, the overall therapeutic effects on liver cancer have shown no obvious improvement, and recurrence and metastasis remain the major factors affecting the prognosis of patients with liver cancer (3).

Tumor metastasis is a multi-step, multi-stage and multi-pathway complex process, which includes the detachment of tumor cells from the primary lesion, invasion into blood vessels or lymph vessels, migration and adhesion to appropriate sites, induction of tumor angiogenesis, anti-host antitumor immunity, and the eventual formation of distal metastasis (4). Multiple genes are involved during this process. A vast majority of current studies have focused on the effects and functions of related genes in tumor invasion and metastasis, such as oncogenes, tumor-suppressor genes and pro-metastatic genes (5). Research has demonstrated that, apart from these protein-coding genes which can regulate tumor invasion and metastasis, various non-coding genes, particularly microRNA molecules, are also involved in regulating tumor invasion and metastasis. MicroRNAs have been found to be abnormally expressed in multiple malignant tumors, including colorectal, lung, breast, liver, brain and prostate cancer, and they play an important role in tumor proliferation, differentiation, invasion, metastasis and treatment response (6).

The PI3K/Akt signaling pathway plays a vital role in the genesis, development and survival of tumors (7). With multiple biological activities, activated Akt can catalyze the phosphorylation of a series of proteins, promote tumor cell growth and proliferation, inhibit apoptosis, facilitate invasion and metastasis, regulate endothelial growth and angiogenesis, and increase sensitivity to radiation. PTEN molecule is a significant inhibitory regulator of PI3K (8).

As is indicated in research, PTEN gene deletions or mutations can be observed in multiple human malignant solid tumors (including prostate cancer, glioblastoma multiforme, melanoma, thyroid cancer and bladder cancer) as well as hereditary tumor susceptible syndrome (7). The PTEN gene exerts certain effects on regulating phosphatase activities of multiple intracellular protein molecules, rendering gene deletion and mutation, which plays an important role in tumor genesis and development (9).
As a type of nuclear transcription factor that is extensively distributed in multiple cells, NF-κB plays an important role in aspects of cell carcinogenesis and apoptosis regulation (10). It has been indicated in research that the NF-κB signal transduction pathway is involved in the transcription expression of oncoproteins and tumor-suppressor genes in the liver, which can participate in the genesis and development of liver cancer by inhibiting cell apoptosis. As a type of polyphonic transcription factor with multi-function, NF-κB can regulate cell apoptosis- and proliferation-associated genes, thus playing an important role in cell carcinogenesis (11). The NF-κB protein family is comprised of 5 distinct subunits, among which, the p50/p65 heterodimer is the most common one. Abnormal regulation is induced when NF-κB is inappropriately activated and located at the cell nucleus where it cannot return to the cytoplasm (12). Furthermore, we examined the role of microRNA-26b signaling in mediating the tumor growth of human liver cancer.

Materials and methods

Liver cancer tissue. Written informed consent was obtained from all patients according to the guidelines approved by the Institutional Research Board of The First Affiliated Hospital of Dalian Medical University and information regarding the clinical samples are shown at Table I. Liver cancer tissue and the adjacent normal tissue were collected from patients at The First Affiliated Hospital of Dalian Medical University from May 2016 to August 2016. All specimens were immediately frozen in liquid nitrogen and stored at -70°C until use.

qRT-PCR detection. Total RNA isolation from tissues was conducted using the TRIzol kit (Invitrogen, Carlsbad, CA, USA), followed by RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA). RNA (100 ng) was reverse-transcribed into cDNA using an iScript CDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). MicroRNA-26b expression was quantified using miR-qRT PCR using the Hairpin-it™ miRNA qPCR Quantitation kit (GenePharma Co., Ltd., Shanghai, China) and measured as using the 2ΔΔCt method.

HepG2 cell culture and controlled microRNA-26 expression. HepG2 cells were obtained from the Cell Culture Center of Chinese Peking Union Medical College (Beijing, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) (All from thermo Fisher Scientific, Inc., Shanghai, China) at 37°C in 5% CO₂. Anti-microRNA-26 and control inhibitor mimics were purchased from RiboBio Co., Ltd. (Guangzhou, China). MicroRNA-26 and control mimics were transfected into HepG2 cells using Invirogen™ Lipofectamine 2000 reagent (Thermo Fisher Scientific). After cells were transfected for 6 h, the cells was treated with 10 μM of 3-methyladenine (PI3K inhibitor) or 5 μM of PDTC (NF-κB inhibitor) for 48 h.

Proliferation assay. Cells transfected with the mimics (1x10⁵/ml) were added to each well of a 96-well plate for 24, 48 and 72 h. MTT (20 μl) (5 mg/ml; Sigma-Aldrich China, Inc., Shanghai, China) was added and incubation was carried our at 37°C for 4 h and 150 μl DMSO was added and agitated for 20 min in the dark for dissolution. The optical density (OD) was then measured at an absorbance of 570 nm.

Apoptosis assay. Cells transfected with the mimics (1x10⁵/ml) were added to each well of a 6-well plate for 48 h. Cells were washed twice with PBS, and then centrifuged at 1,000 x g for 5 min. Annexin V/FITC (10 μl) and propidium iodide (PI) (5 μl) (Becton-Dickinson, San Jose, CA USA) were added and maintained at room temperature for 15 min in the dark. Apoptosis were then analyzed by flow cytometry (BD FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

Western blotting. Cells transfected with the mimics (1x10⁵/ml) were added to each well of a 6-well plate for 48 h, and then incubated with RIPA lysis buffer (Beyotime, Inc., Jiangsu, China) for 30 min. Protein contents were quantified using the Bicinchoninic acid (BCA) protein kit (Beyotime, Inc., Shanghai, China) for 30 min. Protein (50 μg) was separated by 8-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with anti-PI3K (4249), anti-p-Akt (4060), anti-NF-κB (8242), anti-MMP-9 (13667), anti-pVEGF (2463) and anti-GAPDH (5174) (Cell Signaling Technology, Inc., Danvers, MA, USA) antibodies overnight at 4°C. The membrane was then incubated with the anti-rabbit secondary antibody at 37°C for 1 h and protein bands were visualized using an enhanced chemiluminescence system.

Statistical analysis. Data are shown as mean ± SEM. The correlations were analyzed using the analysis of variance (ANOVA). A probability value of <0.05 was chosen for statistical significance.

Results

MicroRNA-26b expression in human liver cancer tissue. To analyze the clinical samples of human liver cancer tissue...
and the adjacent normal tissue, we performed qRT-PCR to detect the expression of microRNA-26b. As shown in Fig. 1, microRNA-26b expression was observably downregulated in the human liver cancer tissues, compared with that noted in the adjacent normal tissues.

Correlation between microRNA-26b and cell growth of liver cancer. In order to assess whether microRNA-26b possess anticancer effects on liver cancer, microRNA-26b expression was upregulated in HepG2 cells using microRNA-26b mimics. Fig. 2 indicates that microRNA-26b was upregulated in the HepG2 cells using microRNA-26b mimics, and upregulation of microRNA-26b significantly decreased cell proliferation and induced apoptosis of the HepG2 cells.

Correlation between microRNA-26b and Bax and caspase-3/-9 protein expression in liver cancer. Then, we analyzed the correlation between microRNA-26b and Bax and caspase-3/-9 protein expression in liver cancer. As shown in Fig. 3, statistical analyses demonstrated that microRNA-26b significantly induced Bax and caspase-3/-9 protein expression in the HepG2 cells.

Correlation between microRNA-26b and PI3K/Akt and NF-κB/MMP-9/VEGF pathways. To investigate the correlation between microRNA-26b and PI3K/Akt and NF-xB/MMP-9/VEGF pathways. PI3K, p-Akt, NF-xB, MMP-9 and VEGF protein levels were significantly suppressed in the HepG2 cells by the upregulation of microRNA-26b, compared with the negative control group (Figs. 4 and 5). These results suggest that microRNA-26b may have a functional role in the development of human liver cancer.

PI3K inhibitor regulates the anticancer effects of microRNA-26b on PI3K/Akt pathways of liver cancer. We examined whether the function of PI3K participates in the anticancer effects of microRNA-26b on liver cancer. PI3K inhibitor significantly further suppressed PI3K and p-Akt protein expression in the HepG2 cells by microRNA-26b, when compared with the negative control group (Fig. 6).

PI3K inhibitor regulates the anticancer effects of microRNA-26b on liver cancer cell growth. We further examined the

Figure 2. Effect of the microRNA-26b mimic on the growth of HepG2 liver cancer cells. (A) microRNA-26b expression in HepG2 cells transfected with the microRNA-26b mimics. Effects of microRNA-26b mimic on (B) cell growth by MTT assay and (C and D) apoptosis by flow cytometry and (E) DAPI staining assay; **P<0.01 vs. negative control group.
function of PI3K in the anticancer effects of microRNA-26b on liver cancer cell growth and apoptosis. Analysis of MTT assay and flow cytometry showed that, as compared to the negative control, the PI3K inhibitor significantly accelerated...
the anticancer effects of microRNA-26b on the inhibition of liver cancer cell growth and induction of apoptosis (Fig. 7).
cancer. We analyzed the effects of PI3K on Bax and caspase-3/-9 protein levels in the HepG2 cells by microRNA-26b using western blotting. The PI3K inhibitor further significantly induced Bax and caspase-3/-9 protein expression in the liver cancer cells by microRNA-26b, compared with the negative control group (Fig. 8). These data demonstrated that PI3K is correlated with the anticancer effects of microRNA-26b on liver cancer.

**NF-κB inhibitor regulates the anticancer effects of microRNA-26b on liver cancer cell growth.** The results of analyses for liver cancer cell growth and apoptosis using MTT assay and (B and C) apoptosis by flow cytometry and (D) DAPI staining assay; ###P<0.01 vs. control group, ###P<0.01 vs. microRNA-26b group.

**NF-κB inhibitor regulates the anticancer effects of microRNA-26b on NF-κB/MMP-9/VEGF pathways in liver cancer.** We examined whether NF-κB influences the anticancer effects of microRNA-26b in liver cancer. The NF-κB inhibitor significantly further suppressed NF-κB, MMP-9 and VEGF protein expression in liver cancer cells by microRNA-26b, compared with the negative control group (Fig. 9).

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In addition, we analyzed Bax and caspase-3/-9 protein expression in liver cancer by microRNA-26b. As shown in Fig. 11, NF-κB inhibitor significantly further induced Bax and caspase-3/-9 protein expression in the hepG2 cells by microRNA-26b, compared with the negative control group.

**Discussion**

Liver cancer is a major disease that severely threatens human health. Liver cancer shows an annually increasing trend due to the influence of factors such as viral hepatitis and environment (13). Although liver cancer is treated by conventional surgery combined with drugs (chemotherapy) in the clinic, recurrence and metastasis, together with the resistance of cancer cells to drugs has resulted in bleak prospects for treating liver cancer. Consequently, unveiling the molecular mechanisms of the drug resistance of liver cancer will bring new hopes for treating liver cancer (14). In the present study, we showed that microRNA-26b expression was observably downregulation in human liver cancer tissue.

microRNAs exert effects that are analogous to tumor suppressors or oncogenes by binding with oncogenes or tumor suppressor genes, and are closely related to tumor genesis and development (15). Participating in tumor invasion and metastasis, microRNAs play a regulatory role in multiple steps during tumor metastasis (16). Abnormal microRNA expression has been reported to exist in human cancers, and the differential expression profile has been reported in multiple cancers successively, including breast, colorectal and liver cancer (17). microRNAs are located in tumor-associated fragile sites in the genome, prone to develop deletion, amplification or translocation. microRNAs exert effects that are analogous to tumor suppressors or oncogenes through locating in onco- or tumor suppressor gene (18). These data demonstrated that microRNA-26b has anticancer effects, by inhibiting cell proliferation and inducing apoptosis of liver cancer cells.

Bel-2 protein can form a heterodimer with the Bax protein (the pro-apoptotic factor), which inhibits tumor apoptosis, prolongs the cell lifespan, and thus participates in tumor
Matrix metalloproteinases (MMPs) are a class of zinc-dependent proteases which can degrade the extracellular matrix (ECM) as well as a majority of proteins on the basement membrane, and they play important roles in the invasion and metastasis of malignant tumors (11). MMP-9 belongs to the gelatinase family, which mainly degrades gelatin, along with type IV, V, VII and X basement membrane collagen (25). Its high expression can result in accelerated degradation of ECM and the basement membrane of blood vessels, stimulates tumor cells to move out of the carcinoma nest along with the injured basement membrane and infiltrate to surrounding tissues, which is benefit to tumor cells going in and out of blood vessels, thus promoting distal metastasis (26). High MMP-9 expression is related to the enhanced invasion and metastatic abilities of multiple tumors, including gastric, colon and liver cancer (22). Therefore, our results suggest that the inhibition of NF-κB significantly enhanced the effects of microRNA-26b on suppression of the NF-κB/MMP-9/VEGF pathway, inhibition of cell proliferation, induction of apoptosis, and promotion of Bax and caspase-3/-9 protein expression in a liver cancer cell line. Li et al revealed that microRNA-26b suppresses small cell lung cancer metastasis through the NF-κB/MMP-9/VEGF pathway (27).

In conclusion, our findings indicate that microRNA-26b has anticancer effects, inhibits cell proliferation, induces apoptosis and promotes Bax and caspase-3/-9 protein expression in liver cancer cells mainly through the PI3K/Akt and NF-κB/MMP-9/VEGF pathways (Fig. 12). Those findings suggest the microRNA-26b may be a novel biomarker for the early diagnosis or may aid in developing new clinical treatments for liver cancer.

References


