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NKAP plays an oncogenic function partly through AKT signaling pathway in hepatocellular carcinoma

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NF-κB activating protein (NKAP) is a highly conserved protein involved in transcriptional repression, immune cell development, maturation, T cell acquisition of functional competency and maintenance of hematopoiesis. Here, we first explore the function of NKAP in hepatocellular carcinoma (HCC). We found that NKAP was highly expressed in HCC tissues and associated with a poor patient survival. CCK8 assay showed that NKAP knockdown significantly decreased cell viability of HuH7 and Hep3B HCC cell lines. Cell invasion, tested by transwell assays, was significantly inhibited by NKAP knockdown in HuH7 and Hep3B cells ($p<0.05$). Percentage of cell apoptosis was significantly increased by NKAP knockdown in HuH7 cells (6.5% to 12.5%) and in Hep3B cells (8.3% to 27.3%). Furthermore, western blot results indicated that NKAP silencing upregulated the expression of pro-apoptotic proteins Bax and Caspase3-P17 while downregulated anti-apoptotic protein Bcl2. Finally, AKT signaling pathway was evaluated to reveal the underlying mechanism of NKAP in HCC cells. It was suggested that NKAP knockdown decreased the phosphorylation level of AKT and the expression of its downstream members p70S6K and Cyclin D1. Furthermore, we demonstrated that NKAP knockdown also played an oncogenic role in human gastric cancer AGS and MKN45 cells. In conclusion, our study for the first time reveals that NKAP promotes the proliferation and invasion in HCC cell lines at least partly through AKT signaling pathway.

Key words: NKAP, HCC, apoptosis, AKT

Hepatocellular carcinoma (HCC) is one of the most life-threatening tumors worldwide, leading to one third of cancer cases and 695, 900 cancer deaths worldwide every year with China accounting for nearly 50% [1, 2]. Previous studies have revealed many factors contributing to HCC pathogenesis, such as hepatitis B/C virus infection, alcohol abuse, liver cirrhosis, gene mutation, and so on [3]. Despite great advances in HCC treatment strategies such as sorafenib, liver transplantation and percutaneous ablation, the survival rate of HCC patients remains very poor, 3–5% within 5 years [3–7]. With the development of bioinformatics, gene expression profile of HCC has been fully characterized and many of the genetic abnormalities contributing to HCC, such as potential oncogenes and tumor suppressor genes, are well known [8–10]. These efforts have greatly facilitated the early diagnosis and accurate treatment of HCC.

NF-κB activating protein (NKAP) was initially identified as a positive regulator of NF-κB activation [11]. Recent studies of NKAP mostly focus on immune cells, including T cells, Invariant Natural Killer T (iNKT) cells and regulatory T (Treg) cells [12–16]. It is reported that NKAP plays an important role in T cells and iNKT development by repression of Notch pathway [13, 14]. T cells maturation and acquisition of functional competency also requires NKAP but not through Notch pathway [15]. NKAP regulates iNKT cell proliferation and differentiation into ROR-γt expressing NKT17 cells [12]. Tregs also undergo a maturation process, which requires NKAP to acquire complement resistance after thymic egress [16]. Other study also shows that NKAP is required for the maintenance and survival of adult hematopoietic stem cells and a potentially important regulator of adult neurogenesis by Notch and/or Notch and/or NF-κB pathways [17, 18]. In addition, NKAP is also reported to participate in some fundamental biological processes. For instance, NKAP acts as a nuclear speckle protein involved in RNA splicing and processing [19] and anchors CENP-E to kinetochores to ensue chromosome alignment in mitosis and its dysregulation can lead to chromosomal instability, which might result in tumorigenesis [20]. However, there is no direct evidence explaining the roles of NKAP in tumor progression.

In this study, we show that NKAP was upregulated in HCC tissues compared with paracarcinoma tissues, and high expression of NKAP predicts a poor prognosis in patients with HCC. The function analysis revealed that NKAP restrained the proliferative and invasive abilities of human HuH7 and Hep3B cells. The underlying action mechanism of NKAP was also explored, which indicated that NKAP knockdown led to the activation of apoptosis pathway and inactivation of AKT pathway. In conclusion, our study extends the understanding of the role of NKAP as a tumor promoter.

Materials and methods

Cell culture and transfection. HCC cell lines, HuH7 and Hep3B and human gastric cancer cell lines AGS and MKN45 were purchased from ATCC. The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2. After the cells reached 80% confluence, NKAP was knocked down in HuH7 and Hep3B cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, Lipofectamine 2000 (10 µl) was added to 250 µl DMEM medium without serum and incubated for 5 min at room temperature. NKAP siRNA (siNKAP, 5 ml) or a scrambled siRNA (siNC, 5 ml) was added to 250 ml RPMI-1640 medium without serum. After the 5 min incubation, the diluted siRNA was mixed with the diluted Lipofectamine 2000 and incubated for 30 min at room temperature. The final concentration of siRNA was 50 nM. Cell medium was replaced with the above siRNA-contained medium. Cells were then cultured at 37 °C in a CO_2 incubator for 24 h.

Analysis of mRNA expression. Total RNA was isolated from cells using a high pure RNA isolation kit (Bioteke Corporation, Beijing, China).One mg of RNA was then reverse transcribed to cDNA using a Transcriptor first strand cDNA synthesis kit (Roche, Shanghai, China). Real-time PCR amplification was used via ABI Prism 7300 sequence detector (Applied Biosystems) and SYBR Green reagent. The PCR reaction was performed as follows: an initial stage of 95 °C for 30 sec, then a two-step program of 95 °C for 5 sec, 60 °C for 31 sec over 40 cycles and were performed in triplicate. β-actin was used as an internal control.

Immunohistochemical assay. Immunohistochemical analysis was performed by using PV9000 two-step immunohistochemical method (ZSGB-BIO Cor., Beijing; catalog number: PV-9000) according to manufacturers' instructions. The tissues were obtained from Changhai Hospital of Second Military Medical University of Chinese PLA. This work has been approved by the ethics committees at Changhai Hospital of Second Military Medical University of Chinese PLA. All patients provided written informed consent. The sections of HCC tumor or paracancerous tissues were prepared with 8 µm thickness. Sodium citrate buffer (pH 6.0) and 3% H_2O_2 were used for antigen retrieval and endogenous peroxidase clearance, respectively. Then the sections were blocked with goat serum for 1 h at room temperature and incubated with primary antibody, anti-NKAP (Cat#ab229096, Abcam). DAB developer was used for slides development. After doubled staining with hematoxylin, dehydrated with gradient ethanol and mounted with neutral resins, tissue sections were observed under microscope and Image Pro Plus6.0 (Media Cybernetics) was used to analyze images. 3–5 random fields from each slice (×400) were selected to evaluate NKAP expression. NKAP expression was assessed as 1) positive (+) when the percentage of immuno-reactive cells was more than 10%, and 2) negative (−) when immuno-reactive cells accounted for less than 10%. Two investigator blind to each other recorded and calculated cell number.

CCK8 assay. Cell Counting Kit-8 (CCK-8) was used to test cell proliferation. Cells were transfected with siNKAP or siNC following plating into 96-well plate at a density of 5000 per well. Ten µl CCK8 reagent was added to each well and incubated for 2 h every 24 h. OD values at 450 nm were measured to plot proliferation curve.

Transwell invasion assay. To determine the invasive ability of cancer cells, the upper chamber of a transwell was pre-coated with Matrigel. Five hundred µl serum-free medium containing cells and 500 µl complete (10% FBS) were respectively added to the upper and lower compartment of the transwell. After 24 h, the non-invaded cells were removed and invaded cells were stained with crystal violet. Then invaded cells were visualized using an inserted microscope and counted.

Cell apoptosis assay. HuH7 and Hep3B cells were transfected with siNKAP for 48 h and collected by trypsinization. After washed with PBS twice, cells were washed with PBS buffer twice, centrifuged and resuspended in 1X binding buffer containing dyes of Annexin V-FITC and propidium iodide (PI). After incubation for 15 min in dark, cell distribution was analyzed by using flow cytometry.

Western blot. HuH7 and Hep3B cells transfected with siNKAP or siNC were collected, lysed and centrifuged at 4 °C. Total proteins were separated by SDS-PAGE. Then the protein bands were transferred to PVDF membranes, blocked in non-fat milk for 2 h, incubated with primary antibodies overnight at 4 °C and secondary antibodies for 1 h. After washing with TBST for 3 times, protein bands were detected using an ECL development system. The primary antibodies included anti-NKAP (Cat#ab229096, Abcam), anti-Bcl-2 (Cat#ab32124, Abcam), anti-Bax (Cat#ab32503, Abcam), anti-Caspase3-P17-specific (Cat#25546-1-AP, Proteintech), anti-p-Akt (Cat#ab81283, Abcam), anti-Akt (Cat#ab32505, Abcam), anti-p70S6K (Cat#ab32529, Abcam), anti-CyclinD1 (Cat#ab40754, Abcam) and anti-GAPDH (Cat#ab8245, Abcam). The secondary antibodies were obtained from Proteintech. The Image J software was utilized to quantify the density of each band.

Statistical analysis. All data were the representative of at least 3 independent experiments. Differences were evaluated by the Student's t-test. A p-value <0.05 was considered as statistically significant.

Results

NKAP is upregulated in HCC and represents a poor prognosis. Along with advances in RNA sequencing technology, a large number of tumor-related RNA expression information is mined by such projects as TCGA and GTEx, providing us with great insight into gene function and convenience of finding diagnostic biomarkers or therapeutic targets for diseases [21–23]. GEPIA (http://gepia.cancer-pku.cn) is an interactive RNA expression analysis website that integrates RNA expression information from TCGA and GTEx projects and provides customers with a convenient and fast search pattern [24]. Here, we searched on GEPIA the expression of NKAP in 369 HCC samples and 160 normal controls. The result is shown in Figure S1A, which indicates a significant upregulation of NKAP in HCC. GEPIA also provides patient survival analysis, based on gene expression levels, to assess the prognosis value of a given gene. For NKAP in HCC, we found that the survival rate of patients with high NKAP expression is significantly lower than those with a low NKAP expression (p=0.024), both groups containing 182 samples (Figure S1B). In addition, we investigated the expression of NKAP in HCC tumor tissues by IHC of paired

Table 1. NKAP expression and the relationship between clinical pathological features of HCC patients.

Clinic pathologic feature	Cases (n)	NKAP level		p-value
		High expression (n)	Low expression (n)	
Age				
≤50	38	23	15	0.521
>50	40	27	13	
Sex				
Male	60	45	15	<0.001*
Female	18	5	13	
Tumor size				
<5 cm	45	35	10	0.006*
≥5 cm	33	15	18	
HBV infection				
Absent	20	15	5	0.176
Present	58	35	23	
Metastasis				
Absent	52	40	12	<0.001*
Present	26	10	16	
Pathological grade*				
I–II	44	22	22	0.003*
III–IV	34	28	6	

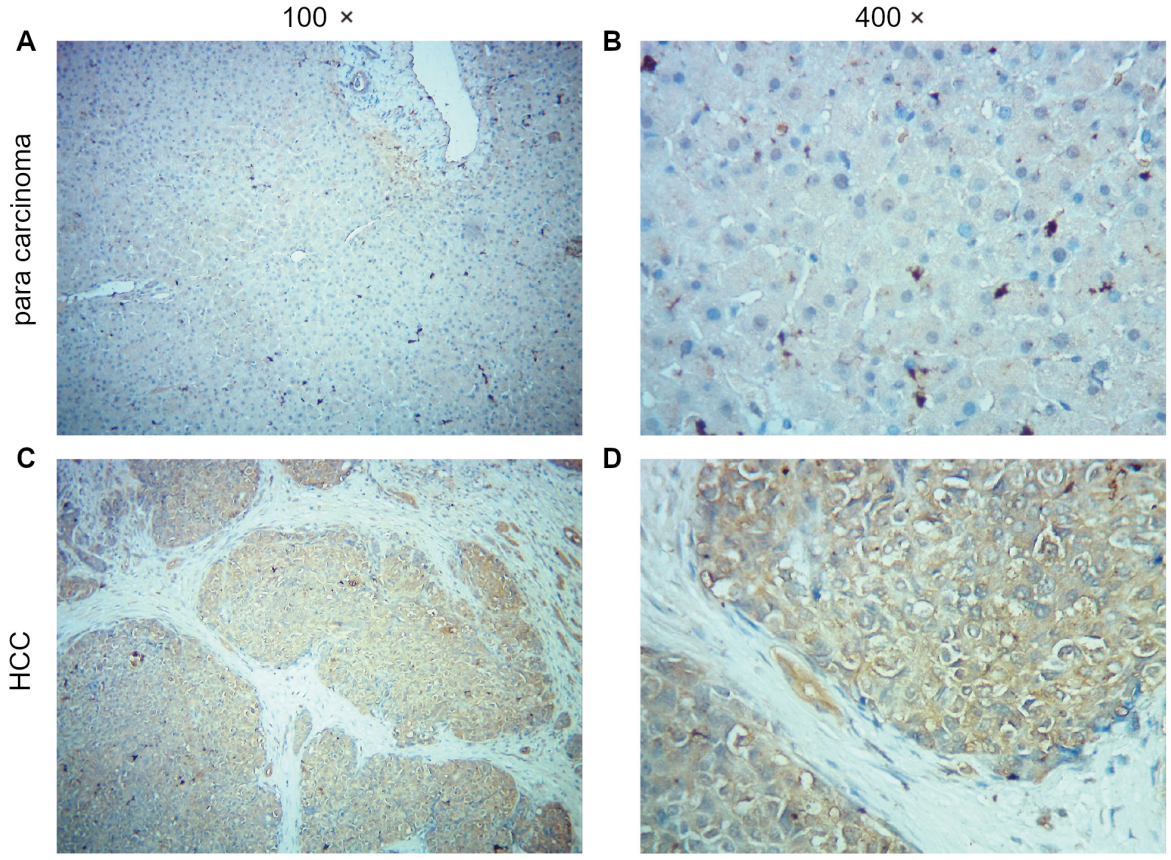

Figure 1. Endogenous levels of NKAP in HCC and paracarcinoma tissues is tested by immunohistochemical assay. A) NKAP expression in paracarcinoma tissue in patients with HCC (×100); B) NKAP expression in paracarcinoma tissue in patients with HCC (×400); C) NKAP expression in cancer tissue in patients with HCC (×100); D) NKAP expression in cancer tissue in patients with HCC (×400). HCC, hepatocellular carcinoma.

carcinoma and paracarcinoma tissues using an anti-NKAP antibody. It is suggested that compared to paracarcinoma tissues, the expression of NKAP is prominently accelerated in HCC tumor tissues (Figure 1).

The correlation between NKAP expression and clinical pathological features of HCC patients was also investigated (Table 1). Overall, high-expression rate of NKAP in HCC tumor patients is 64.10% (50/78). NKAP expression has no connection with age but male patients tend to highly express NKAP ($p<0.001$). In addition, NKAP expression is significantly associated with tumor size, pathological grade and metastasis but has no connection with HBV infection. So it suggests that NKAP is significantly upregulated in HCC and represents a poor prognosis.

NKAP knockdown inhibits HCC cell proliferation and invasion. By using RNA interference technology, we knocked down NKAP in HCC cell lines, HuH7 and Hep3B. The interference efficiency was assessed in RNA level by qRT-PCR assay and protein level by western blot assay. As shown in Figures 2A and 2B, both RNA and protein expression of NKAP were significantly decreased in HuH7 and Hep3B cells.

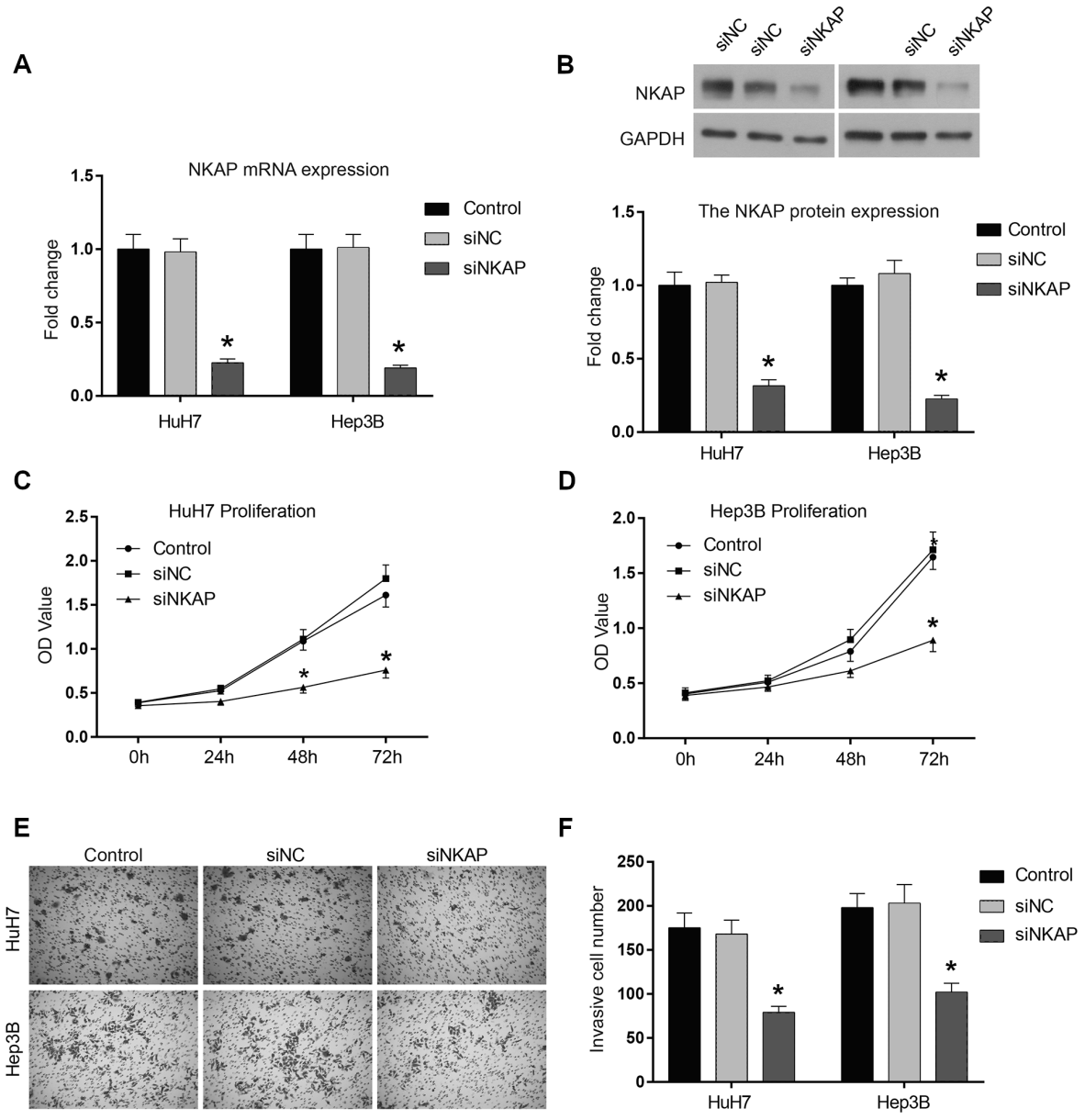

Figure 2. NKAP knockdown significantly inhibits HCC cell proliferation and invasion. A) qPCR demonstrating siNKAP inhibits mRNA expression of NKAP in both HuH7 and Hep3B cells; B) Western blot verifies the inhibition of NKAP expression in protein level. HCC, hepatocellular carcinoma; C and D) Cell proliferation of HuH7 and Hep3B is detected by CCK8 assay; E and F) Cell invasion HuH7 and Hep3B is detected by transwell assay. HCC, hepatocellular carcinoma. * represents $p<0.05$.

Next, we evaluated the impact of NKAP knockdown on cell proliferation of two cell lines. As shown in Figures 2C and 2D, cell viability of siNKAP group is decreased to about 50% at 72 h in both HuH7 and Hep3B cells. Cell invasion was assessed by using transwell assay. As shown in Figure 2E, compared to dense cell distribution in siNC group, cell density in siNKAP group is significantly lower in both HuH7 and Hep3B cells. Quantification result of cell invasion is shown in Figure 3F, which indicates that invasive cell number of siNKAP group is decreased to 9.54% and 15.01% in HuH7 and Hep3B cells, respectively. In order to determine whether the oncogenic function of NKAP was specific to HCC cells, we also performed proliferation and invasion assays in NKAP silenced gastric cancer cells. As shown in Figure 3, the results indicated that NKAP knockdown also significantly inhibited the proliferation and invasion in human AGS and MKN45 gastric cancer cells.

NKAP knockdown induced HCC cell apoptosis by regulating apoptosis related protein expression. In order to determine whether cell apoptosis contributed to the inhibitory effect of NKAP knockdown on HCC cell proliferation, a flow cytometry apoptosis analysis was performed. The

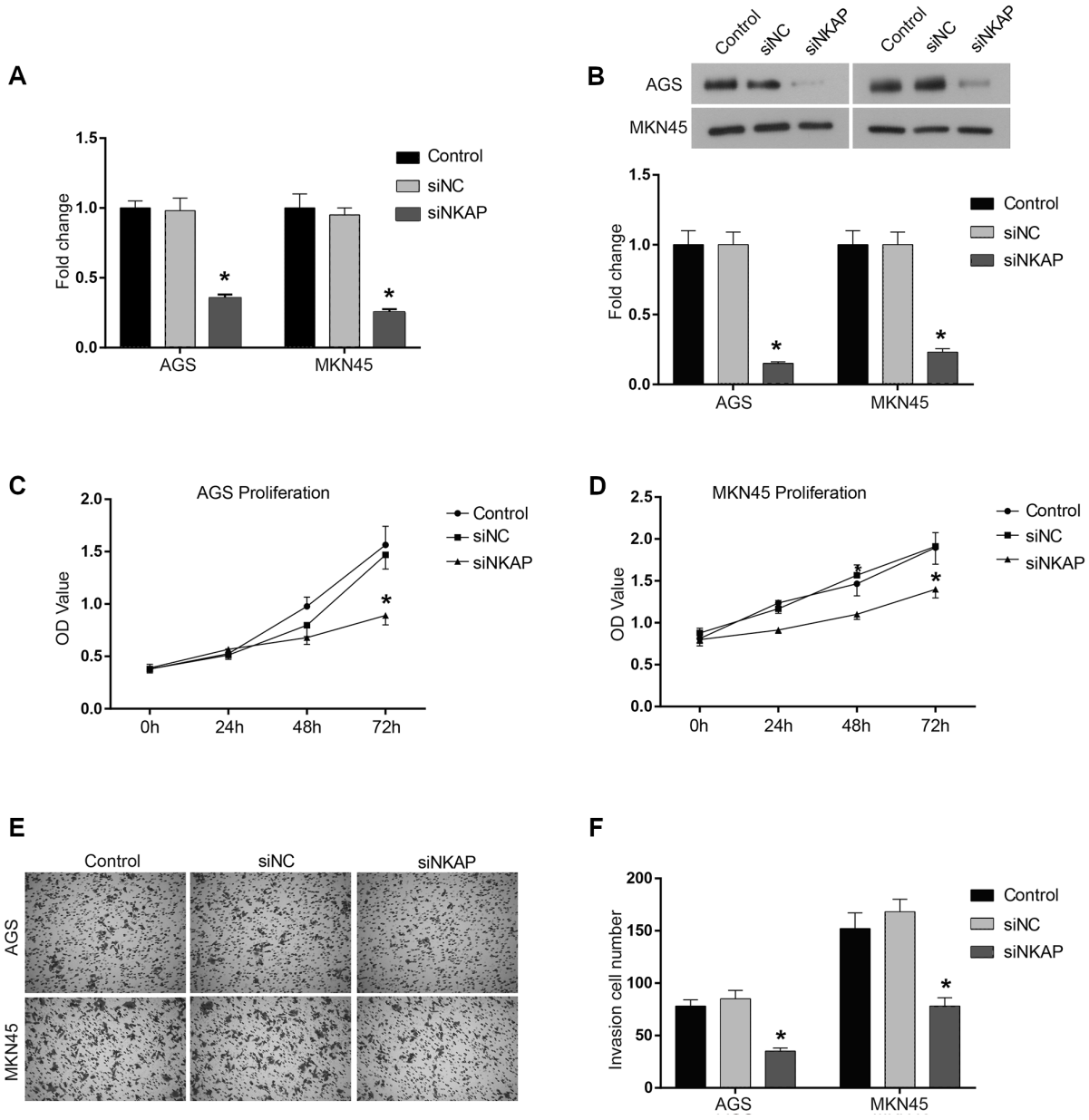

Figure 3. NKAP knockdown significantly inhibits the proliferation and invasion of gastric cancer cells. NKAP was knocked down by using siRNA transfection and NKAP expression was detected on mRNA (A) and protein (B) level. C and D) Cell proliferation of AGS and MKN45 is detected by CCK8 assay; E and F) Cell invasion AGS and MKN45 is detected by transwell assay. * represents $p<0.05$.

results in Figures 4A and 4B show that compared to siNC group, cell apoptosis percentage is significantly increased in siNKAP group in HuH7 cells (6.5% to 12.5%) and in Hep3B cells (8.3% to 27.3%). We further investigated the expression of apoptosis related protein by western blot. As shown in Figures 4C–E, in both HuH7 and Hep3B cells, anti-apoptotic protein Bcl2 is significantly downregulated in NKAP knockdown group while pro-apoptotic protein Bax and Caspase-P17 are significantly upregulated.

NKAP knockdown inhibits the activation of AKT signaling pathway. To elucidate the regulating mechanism of NKAP in HCC, we investigated the effect of NKAP knockdown on AKT signaling pathway in HuH7 and Hep3B cells. When the signaling pathway is activated, AKT is phosphorylated. p-AKT further upregulated the downstream effector proteins, like p70S6K and CyclinD1, which promote protein synthesis or cell cycle progression [25]. As shown in Figure 5, it is suggested that p-AKT and the downstream effectors CyclinD1 and p70S6K are all downregulated by knockdown of NKAP in both cell lines, while AKT expression does not change. The results demonstrate that NKAP knockdown inhibits HCC by suppressing AKT signaling pathway.

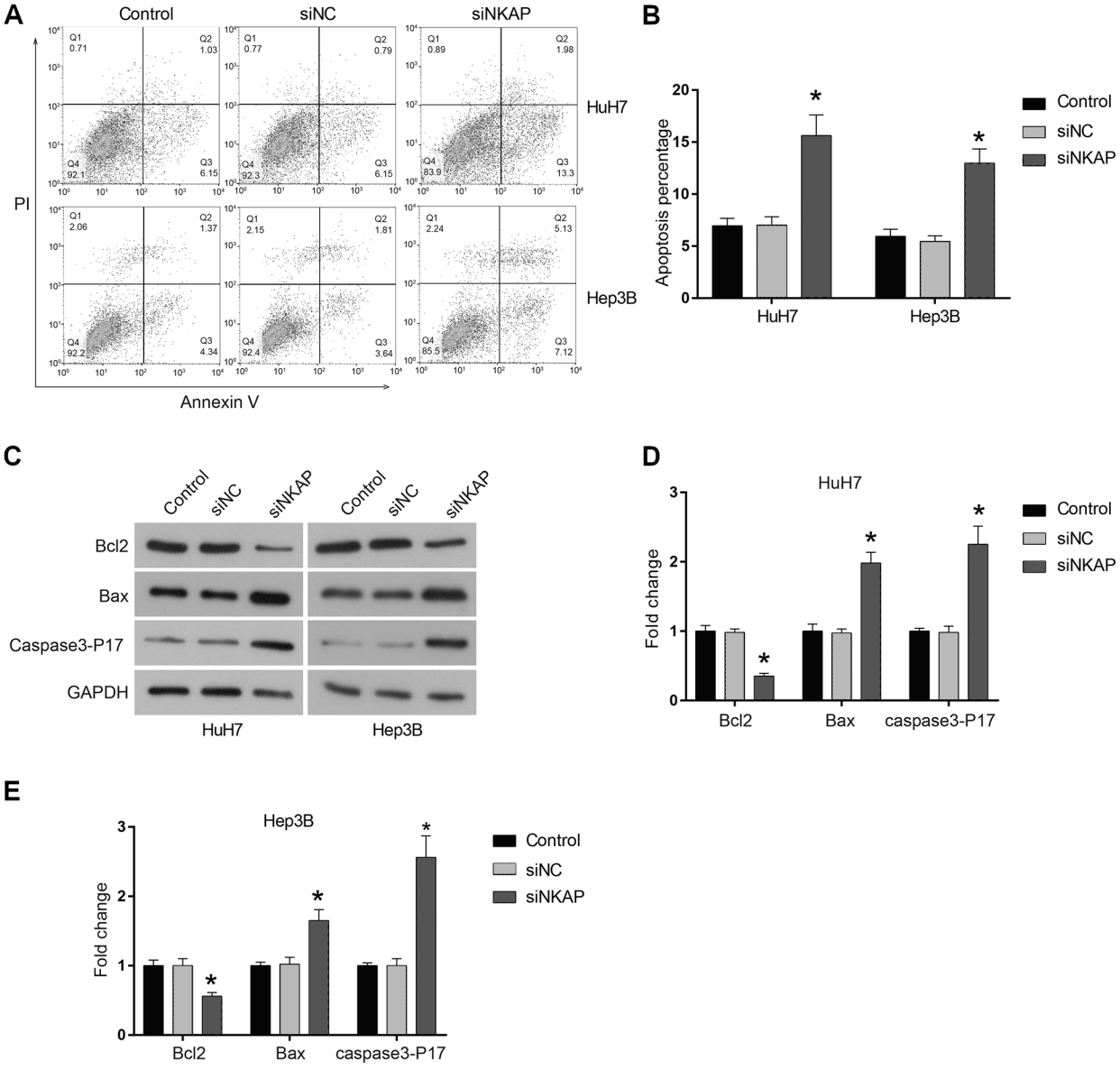

Figure 4. NKAP knockdown induced cell apoptosis by changing apoptosis related protein expression. A and B) Cell apoptosis of HuH7 and Hep3B is detected by flow cytometry; C–E) The expression of apoptosis related proteins Bcl2, Bax and Caspase3-P17 is detected by western blot. HCC, hepatocellular carcinoma. * represents p<0.05.

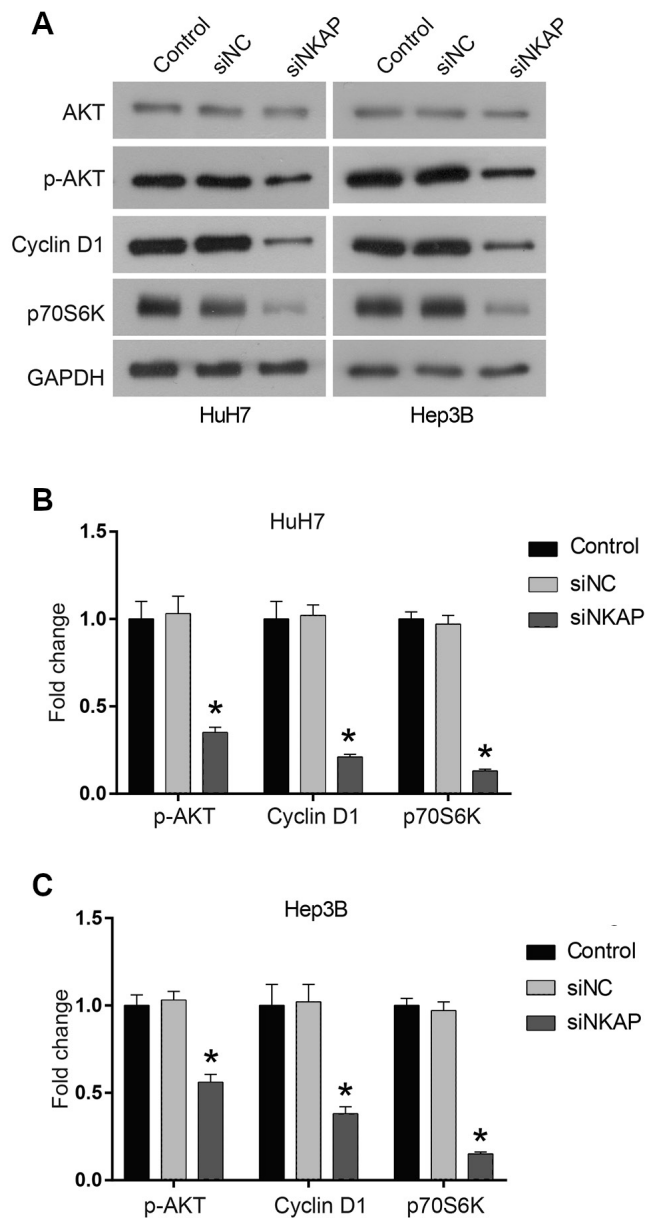

Figure 5. NKAP knockdown inhibited HCC through AKT signaling pathway. Western blot image (A) and quantification analyses (B) and (C) indicates that NKAP knockdown reduced the phosphorylation of AKT and the expression of the downstream effector CyclinD1 and p70S6K. HCC, hepatocellular carcinoma. * represents p<0.05.

Discussion

It has been reported that NKAP plays an important role in fundamental cell functions such as mitosis, RNA splicing and processing [19, 20]. So it is speculated that the dysfunction of NKAP can cause important changes in cellular and organic functions, including tumorigenesis. Indeed, NKAP gene deficiency has been observed in soft tissue sarcomas as well as several other types of human cancer [20]. However, our understanding of NKAP functions in tumor remains scarce. Here for the first time, we identify the function of NKAP in HCC cell lines HuH7 and Hep3B.

By searching on GEPIA and IHC analysis, we find that the expression of NKAP is upregulated in HCC tissues compared with the paracancerous tissues, and is associated with gender and stage of patients. In terms of prognosis, HCC patients with high NKAP expression tend to have a lower survival rate. These data suggest that NKAP may play a positive role in HCC progression. CCK8 and flow cytometry assay show that NKAP knockdown significantly inhibits cell proliferation and promotes cell apoptosis in HuH7 and Hep3B cells. As shown in previous study, NKAP is critical for chromosome alignment in mitosis and its dysfunction will impair chromosome stability [20]. Here we demonstrate NKAP knockdown inhibits cell proliferation and induces cell apoptosis, which may be associated with chromosome instability induced by NKAP impairment. Western blot analysis shows that cell apoptosis related protein expression is also changed by NKAP knockdown, including increased Bax and Caspase3-P17 and decreased Bcl2. As is well-known, Caspase3-P17 and Bax are key apoptosis initiation proteins in mitochondrial apoptosis pathway [26, 27]. And Bcl2 functions as an anti-apoptotic protein by antagonizing Bax [28, 29]. The changes of apoptosis related proteins are consistent with the pro-apoptotic effect of NKAP knockdown. In addition, we also examine the effect of NKAP knockdown on cell invasion of two HCC cell lines. Cell invasion is one of the most important factors in tumor metastasis, which is the leading cause of death in HCC patients [30]. Our data show that NKAP knockdown significantly inhibits cell invasion in HuH7 and Hep3B cells, which suggests that NKAP functions as a promoter in HCC metastasis. Moreover, the oncogenic function of NKAP was also proved in human AGS and MKN45 gastric cancer cells.

As described in previous studies, NKAP knockdown will lead to an increase in cellular pre-mRNA proportion and chromosome misalignment which further cause cell cycle arrest [19, 20]. However, the accurate mechanism by which NKAP functions in tumor remains unclear. As is commonly known, AKT signaling pathway has been reported to regulate numerous biological processes such as cell proliferation, survival, protein synthesis, and so on [31, 32]. Here, we found that NKAP knockdown significantly inhibited the activation of AKT signaling pathway, including decreased p-AKT level and downstream proteins, Cyclin D1 and p70S6K.

In summary, our study for the first time reveals that NKAP plays an oncogenic function through AKT signaling pathway in HCC *in vitro*. Our results may provide a potential target for the treatment of HCC.

Supplementary information is available in the online version of the paper.

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