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Inhibition of Lung Carcinoma A549 Cell Growth by Knockdown of Hexokinase 2 In Situ and In Vivo

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Hexokinase 2 (HK2) has been identified as an oncogene in some malignant diseases such as breast cancer and ovarian cancer. However, the role of HK2 in lung cancer remains unclear. In this study, we explored the functional role of HK2 in lung cancer cell proliferation and tumorigenesis and determine its expression profile in lung cancer. HK2 expression was increased in primary lung cancer tissues of patients. Knocking down HK2 expression by small interfering RNA (siRNA) inhibited cell proliferation in lung cancer cells and nude mice. Thus, HK2 is required for sustained proliferation and survival of tumor cells in vitro and in vivo, and its aberrant expression may contribute to the pathogenesis of lung cancer. Thus, our study provided evidence that HK2 functions as a novel oncogene in lung cancer and may be a potential therapeutic target for lung cancer.

Key words: Hexokinase 2 (HK2); Lung cancer; Tumorigenesis

INTRODUCTION

Lung cancer has been one of the most ranked cancers in China, as in the rest of the world, with a high rate of death and low 5-year survival rate (1). Understanding the molecular mechanisms promoting tumor proliferation and tumorigenesis will enable the development of new therapeutic strategies (1). Stable biomarkers for risk assessment or predication of clinical outcome and further investigations are necessary and urgent.

Cancer cells have increased rates of glucose metabolism compared to normal cells. A variety of mechanisms have been proposed for the accelerated glucose seen in growing tumors and in transformed and malignant cells (2,3). Furthermore, increased concentrations of hexokinase with decreased rates of glucose-6-phosphatase have been reported to accelerate glucose phosphorylation, which results in increased glucose consumption (4). Among several subtypes, hexokinase 2 (HK2) is regarded as one of the most important subtypes for glucose metabolism in cancer cells (5). Studies in rats suggest that it is involved in the increased rate of glycolysis seen in rapidly growing cancer cells (6). However, the role of HK2 in lung cancer remains unclear.

To search for the underlying relationship between HK2 and lung cancer, in vitro and in vivo experiments were carried out. HK2 was found to be closely related to tumor proliferation and tumorigenesis in lung cancer. Knocking down HK2 with siRNA can inhibit tumor proliferation and tumorigenesis.

MATERIALS AND METHODS

Cells and Antibodies

Human lung cancer cell lines H460, L78, H1975, PG-LH7, H157, and A549 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cell lines mentioned above were cultured in DMEM (Invitrogen) supplemented with 10 % (w/v) FBS and incubated at 37°C with 5% CO_2. Antibody of HK2 and actin were used in immunohistochemistry and Western blot.

Clinical Samples

From May 2008 to January 2010, 38 primary lung cancer and corresponding noncancerous lung tissue samples were collected from the Shanghai Pudong New Area People's Hospital. All specimens were collected after

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obtaining written informed consent according to a protocol approved by the Institutional Review Board of the Shanghai Pudong New Area People's Hospital. The specimens were frozen in liquid nitrogen and stored at −80°C for quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis. All of the 38 patients received follow-up. The median follow-up was 60 months (range: 8 to 62 months).

siRNA for HK2

A549 cells were transfected with either two siRNAs against HK2 (Sigma-Aldrich) or one nontargeting siRNA (Sigma-Aldrich) and cultured in 48-well plates according to the manufacturer's instructions. Puromycin selection was performed at a concentration of 0.5 μg/ml for 10 days.

Colony Formation Assay

The transfected A549 cells cultured in plates with DMEM (containing 10% FBS) and DMEM was changed every 2 days. After 15 days of incubation, the A549 cells were fixed with methanol and stained with 0.1% crystal violet. Visible colonies were manually counted. Triplicate plates were measured for each group.

Western Blotting Analysis

Western blotting was employed to detect the protein level of HK2 as previously described. β-Actin (Abcam) was used as the protein-loading control. The protein complex was detected with enhanced chemiluminescence reagents. Digital images were visualized using the electrochemilumine scene detection system (Invitrogen, USA).

Immunohistochemistry

The tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight and subsequently embedded in paraffin wax. Sections were cut at a thickness of 4 μm, air dried, and rehydrated with PBS before being incubated in 3% H_2O_2/PBS to block endogenous peroxidase. Sections were blocked in 5% normal goat serum in PBS followed by incubating with HK2 antibody overnight. Slides were colorated by DAB, counterstained in hematoxylin, dehydrated in ethanol, cleared in xylene, and mounted in gum.

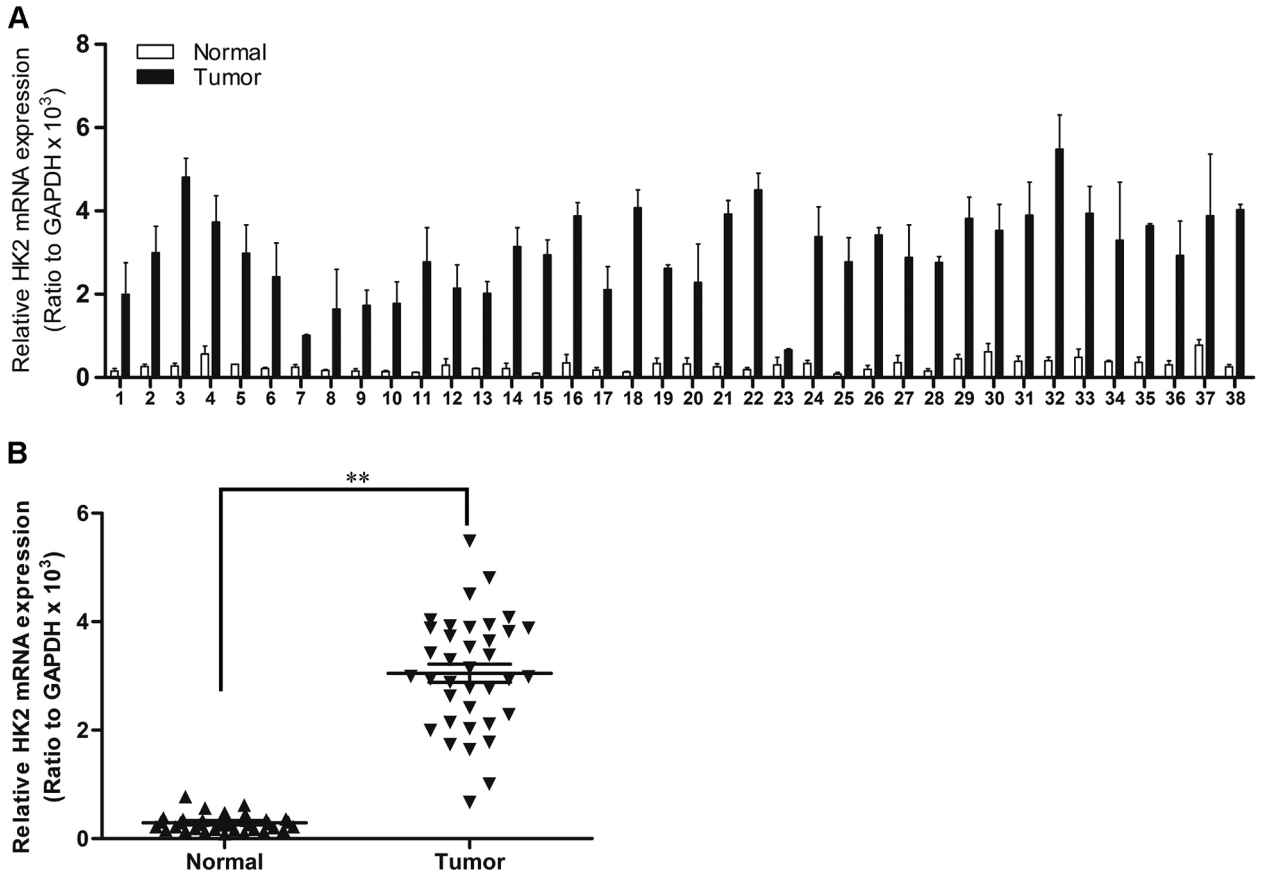

Figure 1. HK2 mRNA is overexpressed in human lung cancer. (A) HK2 mRNA levels in lung tumor tissues and matched normal tissue lesions, as assessed using qRT-PCR analyses. (B) HK2 mRNA expression levels are significantly elevated in osteosarcoma tissues compared to normal tissues. Data were presented as mean ± SD. $^{**}p < 0.01$ versus surrounding normal tissue group.

Tumor Formation In Vivo

Nude mice, 5 weeks old, 18.0–22.0 g in weight, were manipulated and housed according to protocols approved by the Local Medical Experimental Animal Care Commission. Mice were injected subcutaneously with 2×10^6 transfected A549 cells. Every 2 days post-inoculation, the length and width of the implanted tumor were measured with a vernier caliper. After 5 weeks, mice were sacrificed, and tumors were removed and weighed.

Statistical Analysis

All data were presented as mean ± standard error of mean (SEM). Statistical significance was determined using *t*-test or analysis of variance (ANOVA) using the SPSS20.0 program. A value of $p < 0.05$ was considered as critical statistical signification.

RESULTS

HK2 Overexpressing in Lung Cancer Patient Tissues

To evaluate the role of HK2 in lung cancer, we performed qRT-PCR to detect the expression of HK2 in 38 clinical samples of lung cancer tissues and noncancerous tissues. Compared with noncancerous tissues, lung cancer tissues showed increased expression levels of HK2 (Fig. 1A and B). Protein expression level of HK2 was also evaluated in these samples. The results showed increased levels of HK2 in lung cancer tissues (Fig. 2A and B). Furthermore, we performed an immunohistochemistry assay to detect expression of HK2 in these clinical samples of lung cancer tissues and noncancerous tissues. The results showed that there were more HK2-positive cells in lung cancer tissues (Fig. 3A and B). These results suggest that there is a possible role for HK2 in the development or progression of lung cancer.

HK2 Expression and Overall Survival Rate

To explore the prognostic value of HK2 expression for lung cancer, we assessed the association between the levels of HK2 expression and overall survival. We found that HK2 expression was obviously associated with lung cancer patients' overall survival. Patients with low HK2 expression had better overall survival than those with high expression of HK2 (Fig. 4).

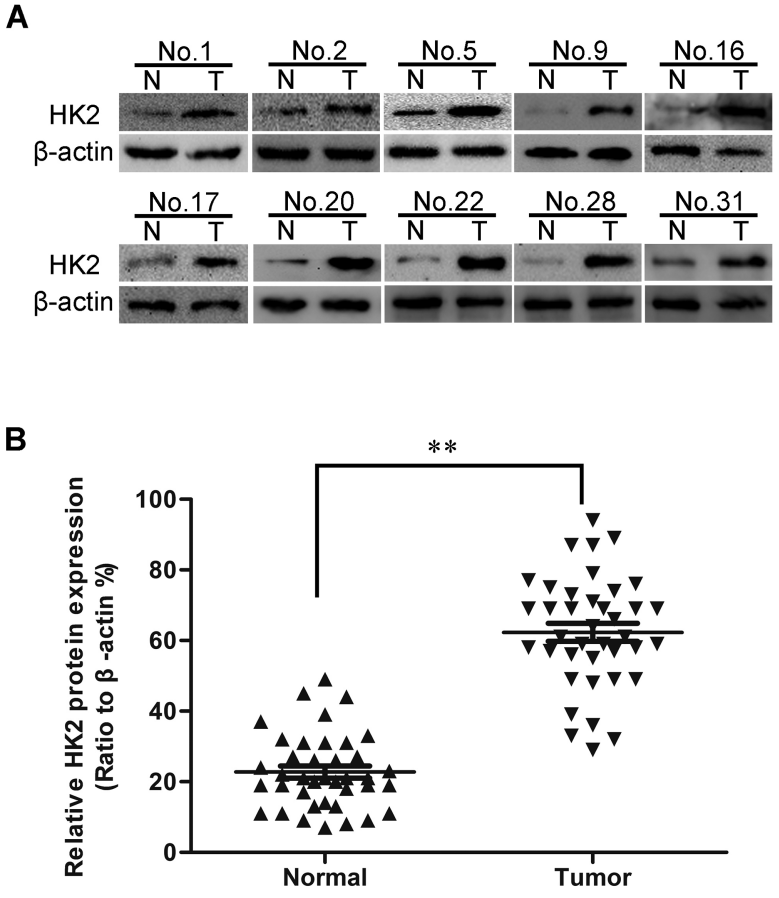

Figure 2. HK2 protein is overexpressed in human lung cancer. HK2 and β-actin (the loading control) expression in nine human lung cancer tissues ("T") or in surrounded normal tissues ("N") was tested by Western blots (A), relative HK2 expression (vs. β-actin, 38 clinical tissues for each group) was quantified (B). Data were presented as mean ± SD. $^{**}p < 0.01$ versus surrounding normal tissue group.

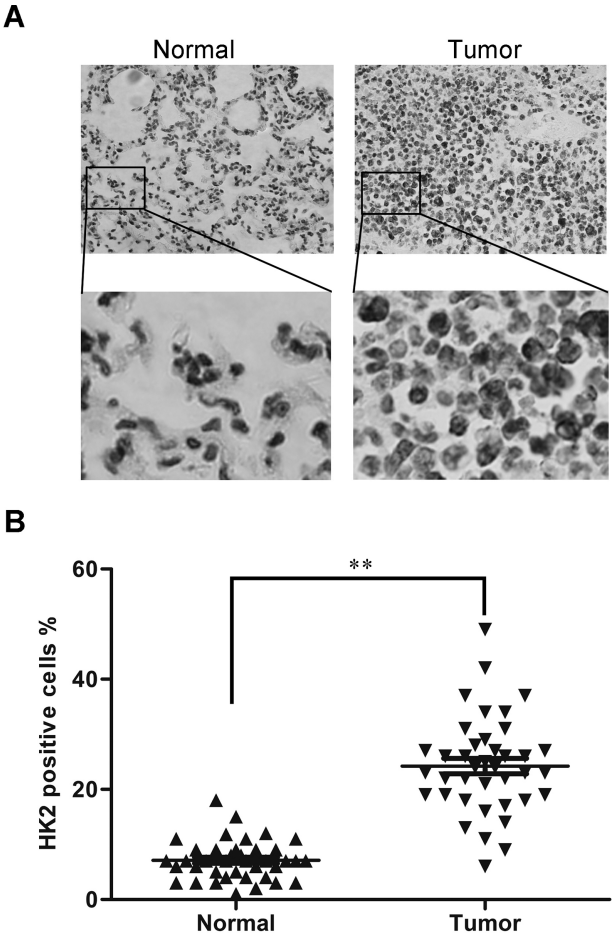

Figure 3. The expression of HK2 was measured by immunohistochemistry assay. Representative IHC images showed HK2 protein expression in human lung cancer tissues or in normal tissue (A). HK2-positive cell was statistically analyzed in human lung cancer tissues or in normal tissue (B). Data were presented as mean ± SD. $^{**}p<0.01$ versus surrounding normal tissue group (B).

HK2 Expression in Different Lung Cancer Cell Lines

Next, we measured the HK2 expression levels in six lung cancer cell lines (H460, L78, H1975, PG-LH7, H157, and A549). The results showed that the A549 cell line expressed the highest level of HK2 (Fig. 5A). We used siRNA to generate a HK2 knockdown in the A549 lung cancer cell line. The transfection efficiency was confirmed using Western blotting and qRT-PCR analyses. As shown in Figure 2B and C, A549 cells that had been transfected with the HK2 siRNA plasmid displayed significantly decreased HK2 expression at both the mRNA and protein levels compared with control cells.

Silence of HK2 Can Inhibit Lung Cancer Cell Proliferation In Vitro

We first explored the effects of HK2 expression on cell growth using the MTT assay.

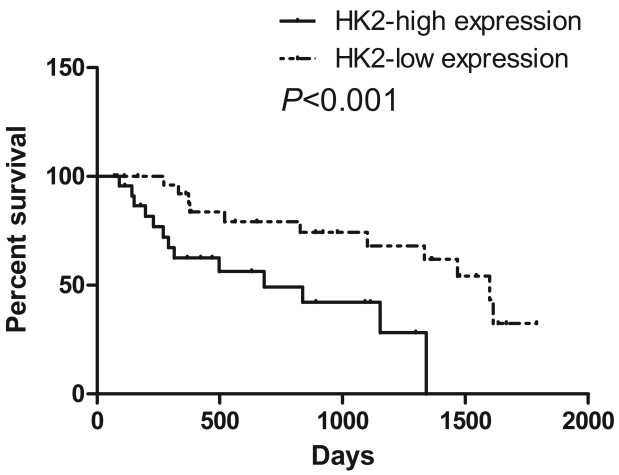

Figure 4. HK2 expression and overall survival rate. Patients with low HK2 expression had better overall survival than those with high expression of HK2.

As shown in Figure 6A, HK2 knockdown significantly inhibited the growth of A549 cells. Next, we performed a clonogenic assay to confirm the effects of HK2 on proliferation. We found that HK2 knockdown dramatically increased the colony formation efficiency of A549 cells (Fig. 6B). These results suggested that HK2 could significantly promote the proliferation of lung cancer cells.

Silence of HK2 Can Inhibit Tumor Proliferation In Vivo

To explore the effects of HK2 on tumorigenesis in vivo, different cells were injected subcutaneously into the flanks of nude mice. In representative tumor pictures as shown in Figure 7A, the diameters of the tumors were measured every 3 days. We found that the HK2 knockdown cells formed tumors later and that the tumor volumes were much smaller in those that were formed from the knockdown cells than in those that were formed from the control cells (Fig. 7B). We also analyzed the weight of the tumors, and the weight of HK2 knockdown cell-formed tumors was less than that of tumors formed by control cells (Fig. 7C). These results suggested that HK2 may promote lung cancer cell xenograft formation and growth in vivo.

DISCUSSION

Lung cancer is one of the most common cancers in the world (7). Despite the decline in lung cancer mortality, a number of lung cancer patients develop metastatic tumors even after surgical removal of the primary tumors (7). In the present study, we identified HK2 as a candidate target gene for the promotion of lung cancer growth.

Metabolism alterations in cancer cells distinguish them from normal cells. High levels of glucose metabolism are common in all tumors (8). Many of these studies provide

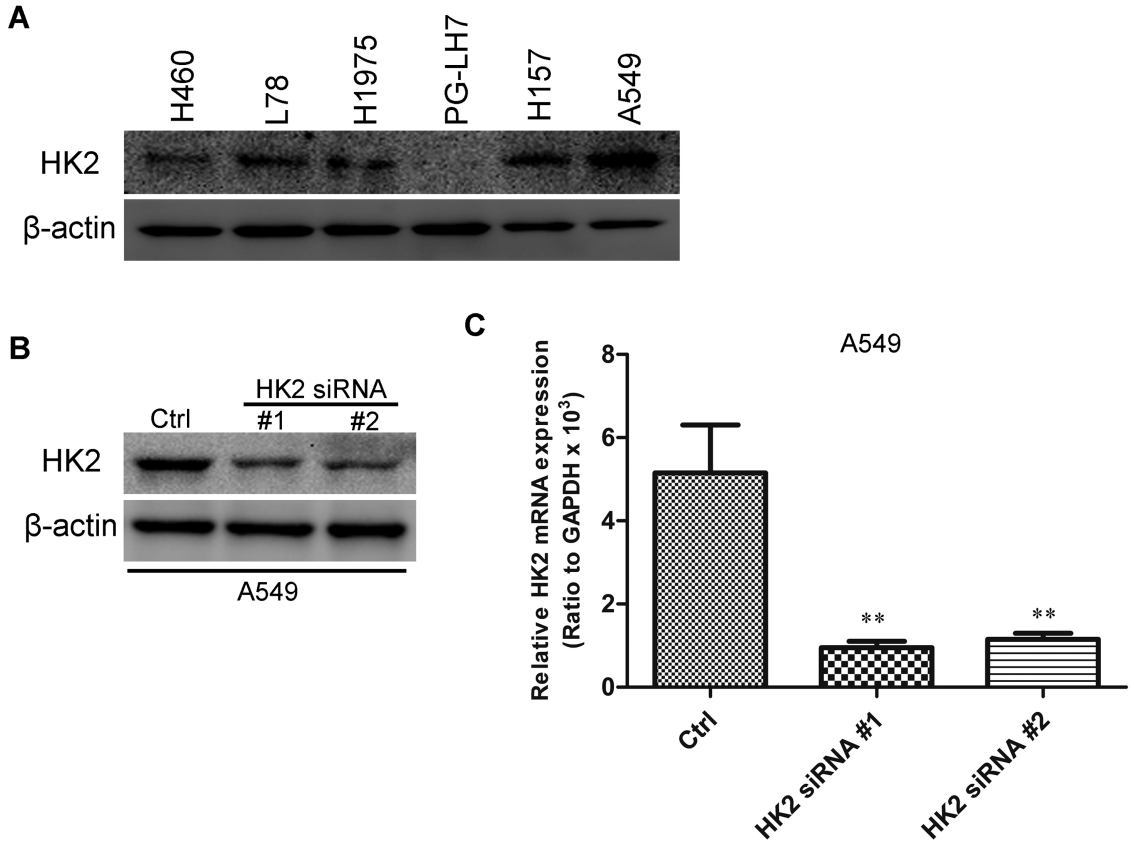

Figure 5. Transfection efficiency of HK2 in lung cancer cell lines. HK2 protein levels in six lung cancer cells, as assessed using Western blotting analyses. β-Actin was used as a loading control (A). HK2 expression in untransfected control A549 cells ("NO siRNA") or A549 cells transfected with indicated siHK2 were tested by Western blots (B) and qRT-PCR (C). The data represent the means ± SD of three independent experiments $^{**}p < 0.01$.

rationales for targeting certain glycolytic enzymes for cancer therapy (9–11). However, glycolysis is the fundamental biological activity in normal metabolism; concerns arise regarding potential adverse homeostatic consequences of targeting specific glycolytic enzymes in a systemic manner (12). Selective cancer-associated expression of specific isoforms of certain glycolytic enzymes could render these enzymes as potential selective targets for cancer therapy if their expression is critical for the neoplastic phenotype (12). Evidence indicates that HK2 overexpression is related to tumorigenesis in several types of cancers, and although it is abundantly expressed in embryonic tissues, it constitutes the predominant hexokinase isoform in only a limited number of adult tissues (12–14). In contrast, the HK1 isoform is ubiquitously expressed and constitutes the major hexokinase in most normal adult tissues (15).

Previous studies have demonstrated the ability of RNAi-mediated HK2 silencing to inhibit tumor growth in a xenograft model of glioblastoma (2). However, it is unknown whether these results can be extrapolated to other forms of cancer, and the specific contributions of (and requirements for) HK2 and the other major hexokinase isoform, HK1, in both tumor initiation and maintenance are incompletely understood (2). In addition, it is unknown whether systemic targeting of HK2 is a viable approach to treating cancer.

Oncogenes can enhance tumor growth and have invasive and metastatic potential (16,17). Overexpression of oncogenes may lead to a malignant cancer phenotype (18). Previous studies have reported that the expression levels of oncogenes were increased in tumors compared with normal tissues (18). To explore the relationship between HK2 and lung cancer, the expression level of HK2 was detected in clinical samples, and a high level of HK2 was shown in tumor tissues. A high level of HK2 is related to poor prognosis and lower overall survival. HK2 could promote cell proliferation in vitro and tumorigenesis in nude mice.

As we can see in this study, HK2 is highly expressed in lung cancer tissues but not in normal lung tissues. Knockdown of HK2 inhibited cancer cell proliferation and tumorigenesis in nude mice. Therefore, HK2 could be a promising target in lung cancer therapy.

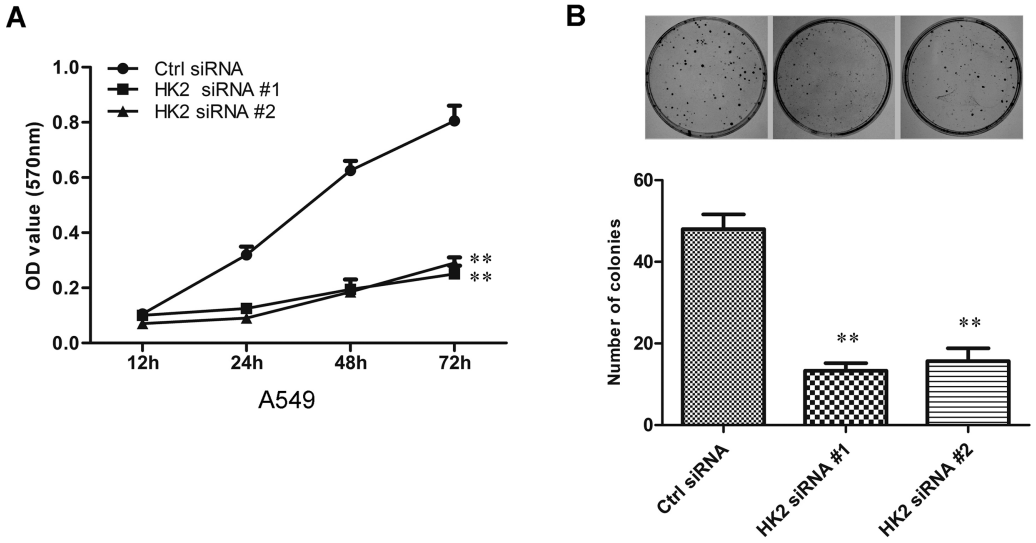

Figure 6. siRNA knockdown of HK2 inhibits lung cancer cell proliferation in vitro. The same amount of A549 cells, with or without indicated siRNA, were maintained in growth medium, cells were further cultured for different hours, cell number was measured by MTT (A). A549 cells were also subjected to colony formation assay (B, C) to test cell proliferation. Representative colony formation images are shown (C). Experiments in this and all figures were repeated three times, with similar results obtained. Data were presented as mean ± SD. $^{**}p < 0.01$ versus control group.

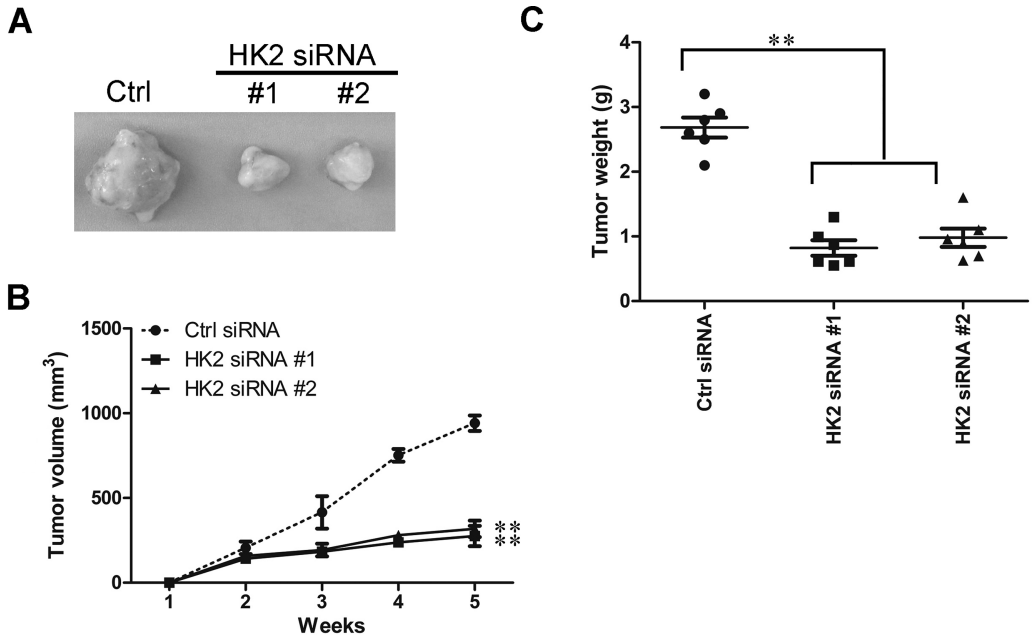

Figure 7. Knockdown of HK2 inhibits lung cancer cell tumorigenesis in vitro. Stable HK2-silenced A549 and its control cells were inoculated subcutaneously into the axilla of five male BALB/c-nu/nu mice, and the representative tumors are presented (A). Growth curves of tumors after the injection of HK2-silenced A549 and control cells into SCID mice (B). Tumor weight was measured (C). $^{**}p < 0.01$ versus Control group.

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