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The role of kinesin family member 16B in tumorigenesis

조선대학교 대학원

의과학과

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CONTENTS

KOREAN ABSTRACT ..... Ⅴ

INTRODUCTION 1

MATERIALS AND METHODS

1. Cell culture and treatment 6

2. Small interfering RNA for KIF16B and cell transfection 7

3. RNA isolation and cDNA synthesis 8

4. Quantitative real-time PCR 9

5. Western blot analysis 10

6. MTT assay 11

7. Cell migration assay 12

8. Soft agar assay 13

9. FACS analysis 13

10. Statistical analysis·····14

RESULTS

1. The expression of KIF16B upregulated in lung cancer·····15

2. KIF16B expression inhibits by small-interference RNA (siRNA) targeting KIF16B·····18

3. Knockdown of KIF16B inhibited proliferation ability in lung cancer cells·····21

4.Knockdown of KIF16B arrested cell cycle in the G2/M phase·····24

5. Knockdown of KIF16B suppresses migration ability in lung cancer cells ·····29

DISCUSSION

Discussion·····32

ABSTRACT

Abstract·····37

REFERENCES

References·····39

CONTENTS OF FIGURES

Figure 1. KIF16B expression in lung cancer·····17

Figure 2. Knockdown using small interference RNA (siRNA) targeting KIF16B·····19

Figure 3. Effect of cell proliferation of KIF16B knockdown in lung cancer cells·····22

Figure 4. KIF16B knockdown associated with cell cycle arrest·····26

Figure 5. Effect of cell migration of KIF16B knockdown in lung cancer cells·····30

국문초록

종양형성 과정에서 kinesin family member 16B의 역할 연구

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의과학과

종양은 정상세포가 오랜기간 돌연변이가 축적되어 형질전환 된 후 계속적인 분열이 발생되고 이동이 가능한 형태로 진행된다. 많은 유전자에서 돌연변이가 발생되는데 아직도 정확한 원인 유전자들이 완전하게 규명되지 않았다. 따라서 종양 진단과 타겟치료제 개발에 현재까지 많은 한계점을 지니고 있다.

새로운 차료타겟을 찾아내기 위해 정상세포와 종양세포에서 그 발현이 현저하게

차이가 있는 유전자를 데이터베이스에서 바이오인포메틱 기법을 통하여 kinesin family member 16B (KIF16B)를 발견하였다.

이 연구에서는 정상과 종양 암 환자 조직에서 그 발현이 차이가 있는 KIF16B를 통해 이 유전자가 종양형성에 어떠한 영향을 끼치는지를 관찰하고자 하였다. 우리는 폐 암 세포에서의 KIF16B를 konckdown을 통해 mRNA와 protein의 발현이 감소하는 것을 확인하였다. 또한 MTT assay와 Soft agar colony formation assay를 통해 세포의 성장과 증식의 감소를 확인하였고, Transwell migration assay를 통해 세포의 이동성이 감소하는 것을 확인하였다. 이러한 oncogenic effect는 KIF16B가 knockdown 된 폐 암 세포에서 *in vitro* 연구를 통해 현저하게 감소되었다. 이와 같은 결과는 KIF16B가 *in vitro* assay에서 암 세포의 종양 형성 가능성과 관련 될 수 있음을 보여준다. 따라서, 이 연구결과들을 통해 향 후 KIF16B를 이용한 종양 진단 및 치료제 개발에 중요한 정보를 제공할 것으로 판단된다.

INTRODUCTION

Most cancer is the most important feature of uncontrolled growth of abnormal cells and involves the generation of genetic mutation caused by environmental and lifestyle causes, smoking, diet and obesity, infections and radiation. Lung cancer is the most common diagnosed cancer and the prognosis is very bad. On the global scale, there were 2 million new cases in 2018. Lung cancer is broadly divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [1, 2]. NSCLC is accounts for 80-85% of all lung cancer cases. Although tremendous progress has been made in the treatment of NSCLC, the 5-year survival rate is less than 15%, especially, is approximately 1% for stage Ⅳ patients. Indeed, more than 70% of lung cancer patients can develop metastasis and 30-55% can cause recurrence. However, there is no effective therapeutic target for patients with developed metastatic lung cancer. Thus, researchers are urgent to investigate the molecular mechanism of NSCLC metastasis and to find

novel therapeutic targets [3, 4].

Abnormal cell proliferation is a hallmark of cancer, and the acquisition of cancer cell mobility and invasiveness cause cancerous malignantly. This is appear by changed expression or activity of cell cycle related proteins. Constitutive activation of many signal transduction pathways stimulates cell growth [5]. Also, cell migration and invasion are important parameters for cancer cell spread and metastasis, a major cause of death in cancer patients. Cancer cells migrate through undergoing molecular and cellular changes by modifying cell-cell and cell matrix adhesion and actin cytoskeleton, a process involving the activity of various signaling networks [6, 7]. In most epithelial cancers, the loss of the cell-cell adhesion molecule, and the acquisition of mesenchymal markers are the basis for conversion to mobile and invasive cells. This process is called epithelial-to mesenchymal transition (EMT) [3, 8]. Cancer cells migrate while undergoing EMT and invasion to surrounding tissues and blood vessels. This is the early

stage of tumor metastasis [9]. Cancer metastasis is composed of many steps and during the process [3].

Kinesin superfamily members (KIFs) are motor proteins that move along microtubules and transport intracellular transport of various cargos such as membranous organelle, protein complex, and mRNA within cells. Human KIFs include a lot of proteins, organized into 14 families named from kinesin-1 to kinesin-14. KIF family genes are mainly found in eukaryotic cells, primarily in microtubules. *In vitro* experiments have demonstrated that the transport of proteins is unidirectional, moving along the negative pole of microtubule towards the positive pole. Therefore, the KIF family genes control mass protein transfer both intracellularly and extracellularly, including functions, such as transporting organelles and material vesicles, and participating in cell mitosis [10]. One of the KIFs kinesin-3 include 8 subfamily. Among them, KIF1B is reported is a plus-end-directed motor that transports synaptic vesicle precursors in the axon from

the cell body to the synapse [11, 12]. Several reports demonstrated that KIF1B is known to be associated with invasion in gastric cancer. KIF14 plays a role in many processes, such as cell division, cytokinesis, and in cell proliferation and apoptosis. Recent studies have been reported an anti-oncogenic of KIF14 effect in lung cancer [13]. KIF16B is a member of the kinesin-3 family and identified as the plus-end motor protein. KIF16B plays a role in the transport of early endosome in endocytosis [14, 15]. KIF16B regulates the recycling and degradation of receptors by controlling the localization [16-18]. However, the function of KIF16B in cancer is unknown.

In the present study, we found that KIF16B mRNA expression is up-regulated in lung cancer patients through the Cancer Genome Atlas (TCGA) data analysis. Indeed, we also confirmed that KIF16B mRNA and protein expression level in several lung cancer cell lines. These founding suggested that KIF16B is associated with lung cancer cell progression. To obtain functional role of KIF16B

in lung cancer cell, examined the KIF16B knockdown using small interference RNA (siRNA) targeting KIF16B in lung cancer cell lines. We investigated whether knockdown of KIF16B effects cell proliferation, cell cycle distribution, colony formation and migration in lung cancer cell lines. The results suggested that KIF16B may serve as a new therapeutic target for overcoming in lung cancer.

MATERIALS AND METHODS

1. Cell culture and treatment

The human fibroblast cells, IMR90 and MRC-5 in EMEM medium (WelGene), the human lung carcinoma cell lines, Calu-1 in McCoy's 5A medium (WelGene), Calu-3 and SK-MES-1 in DMEM medium (WelGene) , A549 and H1299 were cultured in RPMI1640 medium (WelGene) supplemented with 10% fetal bovine serum (FBS) and penicillin streptomycin (0.1 mg/ml). Cell growth was monitored under an inverted microscope. Upon reaching 70-90% confluency, cells were digested with 0.5% trypsin-EDTA before being passaged. All cell lines were maintained at 37℃ and in 5% CO_2 at saturated humidity. Cells in exponential growth were harvested for subsequent experiments.

2. Small interference RNA (siRNA) for KIF16B and cell transfection

siRNA was designed by targeting KIF16B (NM_001199866.2) to this study. The target sequences were 5'-GCA CCA UUC AAC GUA AAC UAA-3' for KIF16B siRNA #1 form, 5'-GCC CUU AUG UUG AGG AUU UAU-3' for KIF16B siRNA #2 form for knockdown of KIF16B in cells, A549 and H1299 cells were plated in 60mm dishes and transfected with 50nM KIF16B siRNA using Lipofectamine RNAimax (Invitrogen), and incubated 72h.

3. RNA isolation and cDNA synthesis

Following cell lysis with the TRIzol lysis reagent. After the addition of chloroform (Merk Millipore), the homogenate was separated into aqueous and organic by centrifuge at 13,000g for 15min. RNA was precipitated from the aqueous phase by the addition of isopropanol (Merk Millipore). The pellet was then washed 70% ethanol (Merk Millipore), air dried, and dissolved in 30㎕ of RNase-free water. For synthesis for cDNA prepared the following reaction mixture in a 1.5㎖ tube to the total volume of 20㎕ (template RNA 1㎍, Oligo dT 10㎛, 10 mM dNTP, 1X reverse transcriptase buffer, Reverse transcriptase), and incubate at 65℃ for 5min, place on ice for 1min, 42℃ for 1h, 70℃ for 15min.

4. Quantitative real-time PCR

Real-time PCR quantitative real-time PCR was performed using the TB Green Premix Ex Taq (Takara). cDNA of A549 and H1299 cells by PCR using the KIF16B primers 5'-CCTCATTTGAATCAGAACTTT-3' (Long form Forward primer) 5'-GCCCTTGAATTTCCTCCAA-3' (Long form Reverse primer) 5'-GCAAGATCTGGTTCAGCTT-3' (Short form Forward primer) 5'- GCTC-TACACAACAGTGAAA-3' (Short form Reverse primer). The RCR reaction consisted of 12.5㎕ of TB Green Master Mix, 0.5㎕ of forward and reverse primers, and 1㎕ of template cDNA, and 0.5㎕ of ROX reference Dye in a total volume of 25㎕. Cycling was performed using the setting conditions of the the Bio-Rad CFX Maestro software : 95℃ 30sec, (95℃ 5sec, 62℃ 10sec) X39 Cycles, 95℃ 10sec, 65℃ 5sec, 95℃ 5sec. Relative RNA expression levels were quantified using the ⊿⊿CT method.

5. Western blot analysis

Cells were washed with 1X PBS and lysed in RIPA buffer (50mM Tris-HCl (pH7.8), 150mM NaCl, 5mM EDTA, 0.5% Triton X-100, 0.5% NP-40, 0.1% sodium deoxycholate (NaDOC)) with protease inhibitors (Roche Diagnostic Corp). And amount of protein was determined using a protein assay Bio-Rad. Equal amounts of protein were electrophoresed on 6-15% SDS polyacrylamide gels (SDS-PAGE) followed by electrotransfer onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membranes were blocked for 1h with TBS-T (10mM Tris-HCl (pH7.4), 150mM NaCl and 0.1% Tween-20) containing 5% skim milk and then incubated at 4℃ with primary antibodies (1:1000) : anti-KIF16B, anti-p53, anti-cyclin B1, anti-pH3 and anti-β actin. The blots were washed four times for 15min with 0.1% Tween 20 containing TBS-T and then incubated for 3hrs with peroxidase-conjugated secondary antibodies (1:4000) at RT. The membranes were sashed four times for 15min, and devel-

oped using an enhanced chemiluminescence (ECL) detection system.

6. MTT assay

Cell proliferation was measured using the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Miphnyltetrazolium Bromide (MTT) assay. Briefly, cells ($1x10^3$) were seeded in 96-well plates. Next day, 10㎕ of 5mg/ml MTT in medium culture was added to each well. The plate was incubated for 3h at 37℃. Then, culture medium was removed, and 100㎕ 36% HCl in isopropanol was added each well for 10min and 100㎕ 3'DW was added each well for 10min. Colorimetric detection was done at a wavelength of 570nm.

7. Transwell Migration assay

In vitro cell migration assays were performed in a 24-well Transwell plate with 8㎛ Polycarbonate Membrane Tissue Culture Treated, Polystyrene separating the lower and upper culture chambers. $2X10^4$ A549 cells and $4X10^4$ H1299 cells were plated in serum free media in upper chamber. Complete media were added to the bottom wells. The plate was incubated for 24h at 37℃. Then, non-migrated cells were removed from the upper face of the filters using cotton swabs, and migrated cells in the lower face of the filters were fixed with 100% Methanol for 10min, and stained with 10㎍/ml Hochest in 1X PBS, and washed two times for 10min with 1X PBS. Number of migrated cells were counted by using InCarta software.

8. Soft agar colony formation assay

Soft agar plates were prepared 1.2% Noble Agar (BD, Difco) in 3′DW and 2X RPMI medium (with 10% FBS, 1% PS) in bottle. To prepare the base layer, 1.2% Agar with 2X RPMI in equal volumes was added into the 60mm plates. Top layer agar was prepared by 1.2% agar with 2X RPMI medium at a 1:3 ratio, and the cells suspended in this top layer of agar were plated over the base layer. Then, the plates were incubated at 37℃ under 5% CO_2 . Colony counted after two weeks.

9. Flow Cytometry Analysis

Cell cycle was measured using the Flow cytometry analysis (FACS). The cells were collected at 72h after transfection and fixed with 100% ethanol at −20℃, stained 50㎍/㎖ propidium iodide (PI) with 0.05㎍/㎖ RNase A and measured Cell Quest Pro software.

10. Statistical analysis

Each experiment was performed at least three times. The data in all expriments are represented as mean ± standard deviateion (S.D.). Statistical comparisons were carried out using an unpaired t-test. P-values<0.05 were considered statistically significant.

RESULT

1. The expression of KIF16B upregulated in lung cancer.

KIF16B is a protein of 1318 amino acid residues consist of forkhead-associated domain(FHA), pleckstrin homology or Phox homology (PX) domain at coterminus (Fig 1A) [19, 20]. But the function in cancer is not well known. To identify the associated of KIF16B expression in lung cancer progression, we analyzed the expression pattern of KIF16B between lung cancer tissue and non-cancerous lung tissue through TCGA datasets analysis. As shown in figure 1B, KIF16B is higher expressed in lung tumor compared to normal lung.

Next, we confirmed the expression of KIF16B in some lung cancer cell lines. The expression level of mRNA and protein was increased in lung cancer cell lines compared with the normal lung cell, IMR90 and MRC-5, by Real-time PCR and western blotting assay (Fig 1C, 1D). These results suggested that KIF16B may play role lung tumorigenesis that KIF16B is associated with lung cancer.

Next, KIF16B expression was relatively high in A549 and H1299, therefore, we selected these cells and carried out experiment.

Figure 1

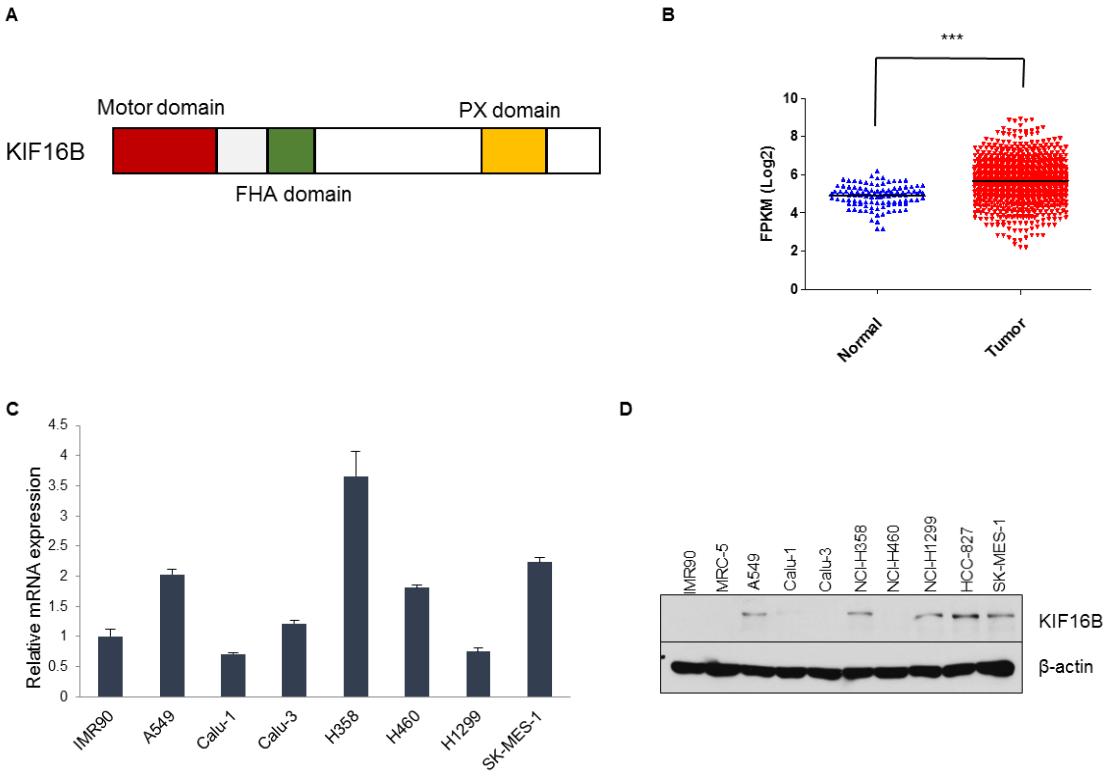
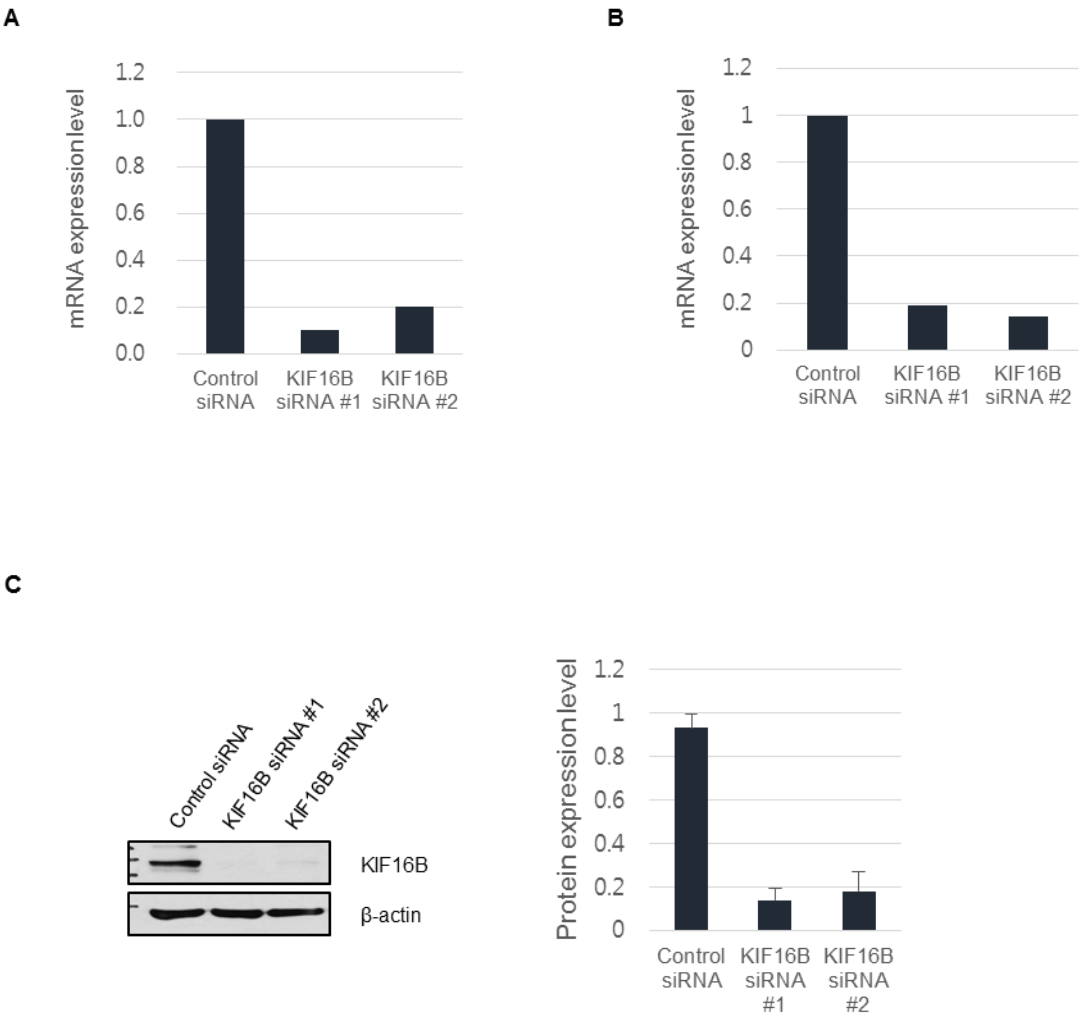


Figure 1. KIF16B expression in Lung cancer. (A) Schematic alignments of KIF16B transcript variants. (B) KIF16B mRNA expression in lung cancer patients were analyzed from TCGA datasets. . *** P<0.001. (C) KIF16B mRNA expression level in lung cancer cell line was confirmed by Real-time PCR. (D) Western blot analysis was used to identify KIF16B protein expression level in lung cancer cell line. β-actin was used as a loading control.

2. KIF16B expression inhibits by small-interference RNA (siRNA) targeting KIF16B

To investigated the functional role of KIF16B in lung cancer cell line, we performed an experiment to make KIF16B knockdown cells using specific siRNA targeted KIF16B. Two siRNA for KIF16B (KIF16B siRNA #1, #2) were transfected in lung cancer cell line A549 and H1299. After 48h, we confirmed the expression of KIF16B in cells by Real-time PCR and Western blotting assay (Figure 2). Both mRNA and protein were decreased KIF16B siRNA #1, #2 transfected A549 and H1299 cells compare to control siRNA transfected cell. We confirmed the siRNA efficiency and used in experiments.

Figure 2



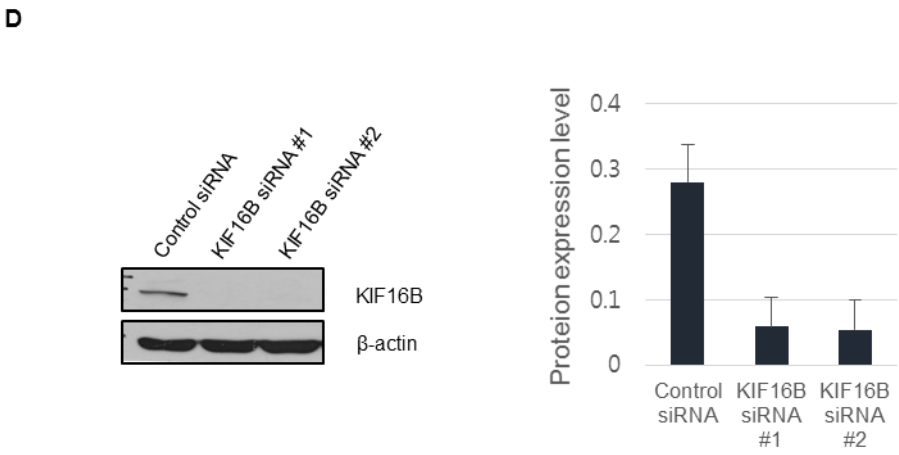


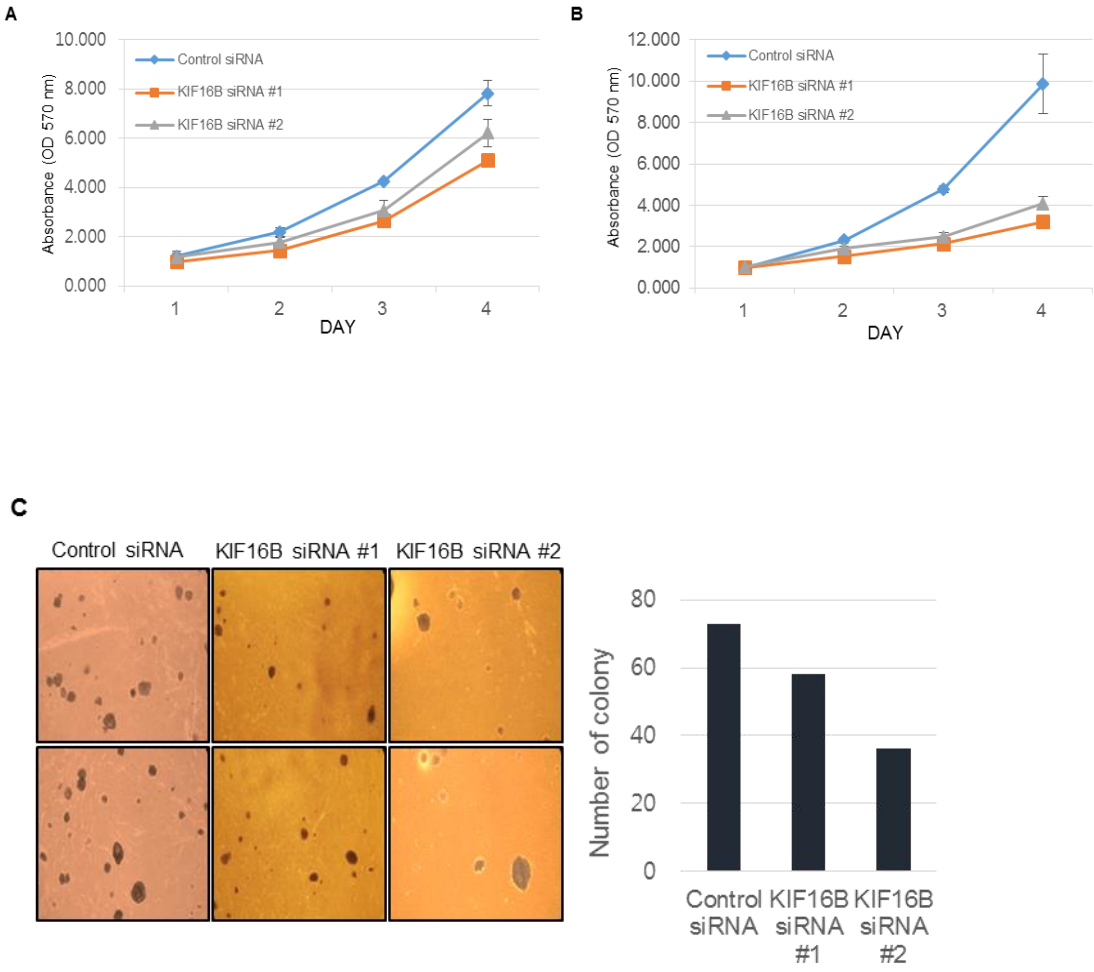
Figure 2. Knockdown using small interference RNA (siRNA) targeting KIF16B in lung cancer cell lines.

(A) Knockdown of KIF16B in A549 and H1299 cells were confirmed by Real time PCR. (B) Western blot analysis was examined to confirm the protein levels of KIF16B knockdown cells. β-actin was used as internal loading control.

3. Knockdown of KIF16B inhibited proliferation ability in lung cancer cells.

Next, we wondered whether the knockdown of KIF16B affected cancer characteristics. To this end, we were examined the proliferation of KIF16B knockdown A549 and H1299 cells using MTT assay because abnormal proliferation is a hallmarker of cancer. A549 and H1299 cell were transfected with KIF16B siRNA #1, #2. After 48h, cells were seeded on 96well plates and then performed MTT assay for 1, 2, 3, 4 days. As shown in Figure 3A and 3B, the MTT assay showed that cell proliferation was reduced KIF16B siRNA #1, #2 in A549 (Fig. 3A) and H1299 (Fig. 3B) compare to control siRNA transfected cell. To further investigate, soft agar colony formation assay was performed because cancer cells are able to proliferate without attaching in surround tissue. Consist with previous experiments, cell proliferation decreased when the knockdown of KIF16B in cells (Figure 3C, D). These results demonstrated that KIF16B is associated with cell proliferation in lung cancer.

Figure 3



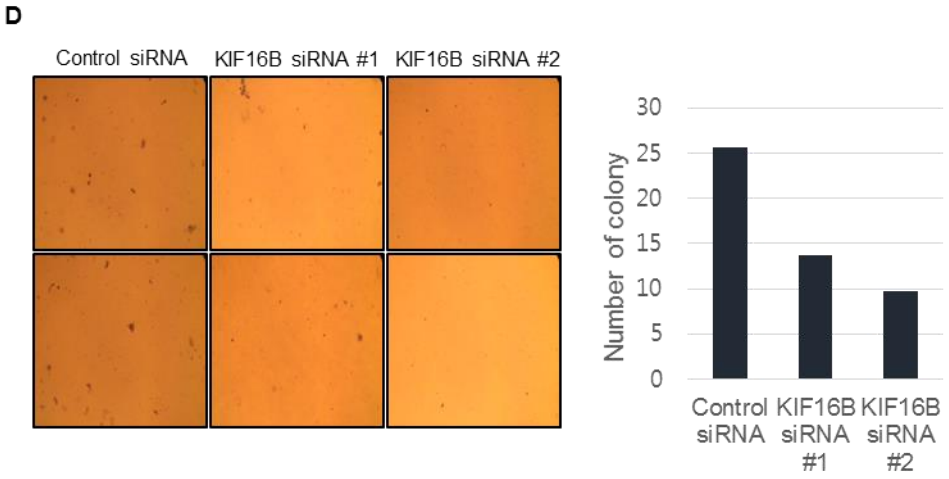


Figure 3. Effect of cell proliferation of KIF16B knockdown in lung cancer cells.

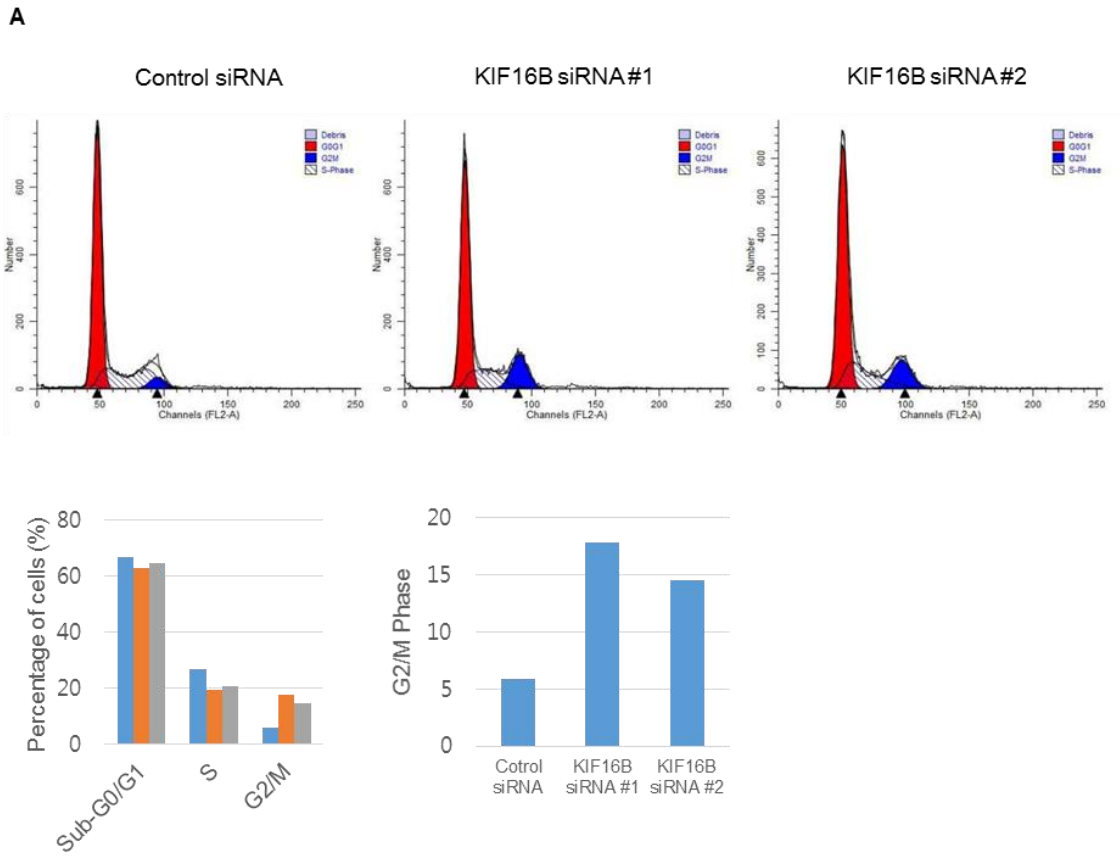
Cell proliferation determined by MTT assay and Soft agar colony assay in A549 (left panel) and H1299 (right panel) cells. (A), (B) Cell proliferation was assessed by MTT assay after 1, 2, 3 and 4 days incubation with KIF16B siRNA #1 and #2. (C), (D) KIF16B siRNA transfected cells were seeded on soft agar plates. After 2weeks, colonies were counted using microscopy.

4. Knockdown of KIF16B arrested cell cycle in the G2/M phase.

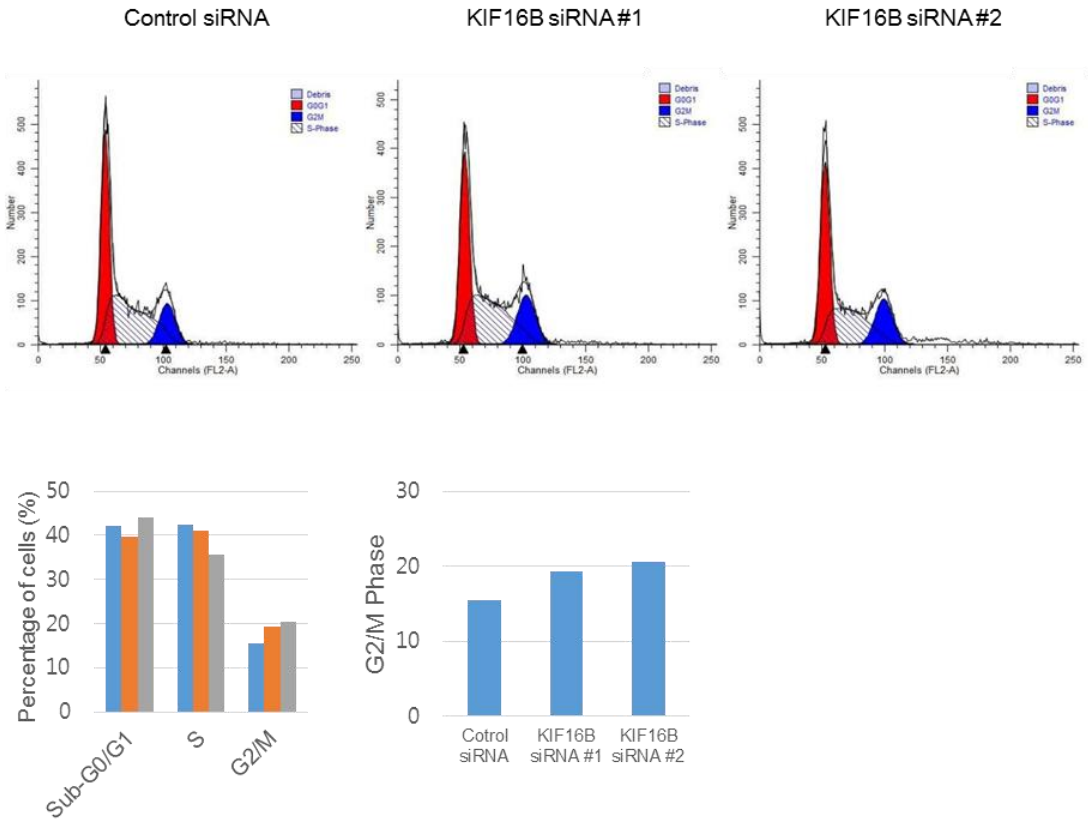
It is known that abnormalities in cell proliferation are mostly affected by the cell cycle [21, 22]. To investigate whether the cell cycle affected in decrease of cell proliferation by KIF16B knockdown, we examined the cell cycle of KIF16B knockdown cells using flow cytometry (FACS) analysis. KIF16B siRNA #1, #2 transfected with A549 and H1299, respectively, and then the cells stained using PI were subjected to FACS analysis. As shown in Figure 4A and 4B, the knock-down of KIF16B increased the number of cells in G2/M phase by 5-10%. That is, G2/M phase arrest occurred in KIF16B knockdowns cells. These results suggested that suppression of lung cancer cell growth by knockdown of KIF16B may due to interfere with cell cycle at G2/M phase. To further detailed investigate, we sought to examine expression level of proteins, p53, p-H3 and cyclin B1 by using western blotting analysis. As shown in Figure 4C and 4D, expression levels of three proteins were increased in the knockdown of KIF16B in A549 (Fig. 4C) and H1299 (Fig. 4D) cells. Therefore, above result indicated that when KIF16B

is knocked down, p53, the tumor suppressor gene, is works to and arrests the cell cycle. Also, the increase of p-H3 and cyclin B1 involved in the transition from the G2 phase to the M phase shows that the cell cycle causes a delay in the G2/M phase. These results demonstrated that KIF16B is associated with cell growth in lung cancer.

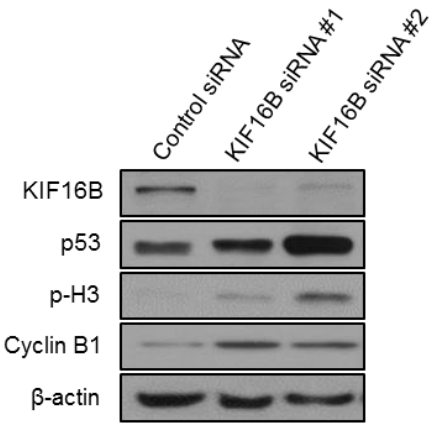
Figure 4.



B



C



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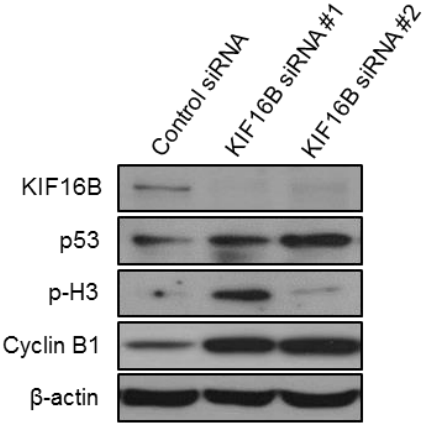

Figure 4. KIF16B knockdown associated with cell cycle arrest.

(A), (B) KIF16B siRNA transfected A549 (A) and H1299 (B) cells were analysed the cell cycle distribution through FACS analysis. The bar graphs represented the percentage of cell cycle phase. (C), (D) Western blotting analysis was examined the expression of KIF16B, p53, p-H3, and cyclin B1 in KIF16B knockdown A549 (C) and H1299 (D) cells. β-actin was used as internal loading control.

5. Knockdown of KIF16B suppresses migration ability in lung cancer.

Because cell mobility is important to tumor intensity [23, 24], we conducted cell migration ability using the transwell chamber model. In the Transwell migration assay, cell migration rate was decreased KIF16B siRNA #1, #2 in A549 (Fig. 5A) and H1299 (Fig. 5B) compared to control siRNA transfected cell. These results demonstrated that KIF16B associated with cell migration in lung cancer.

Figure 5

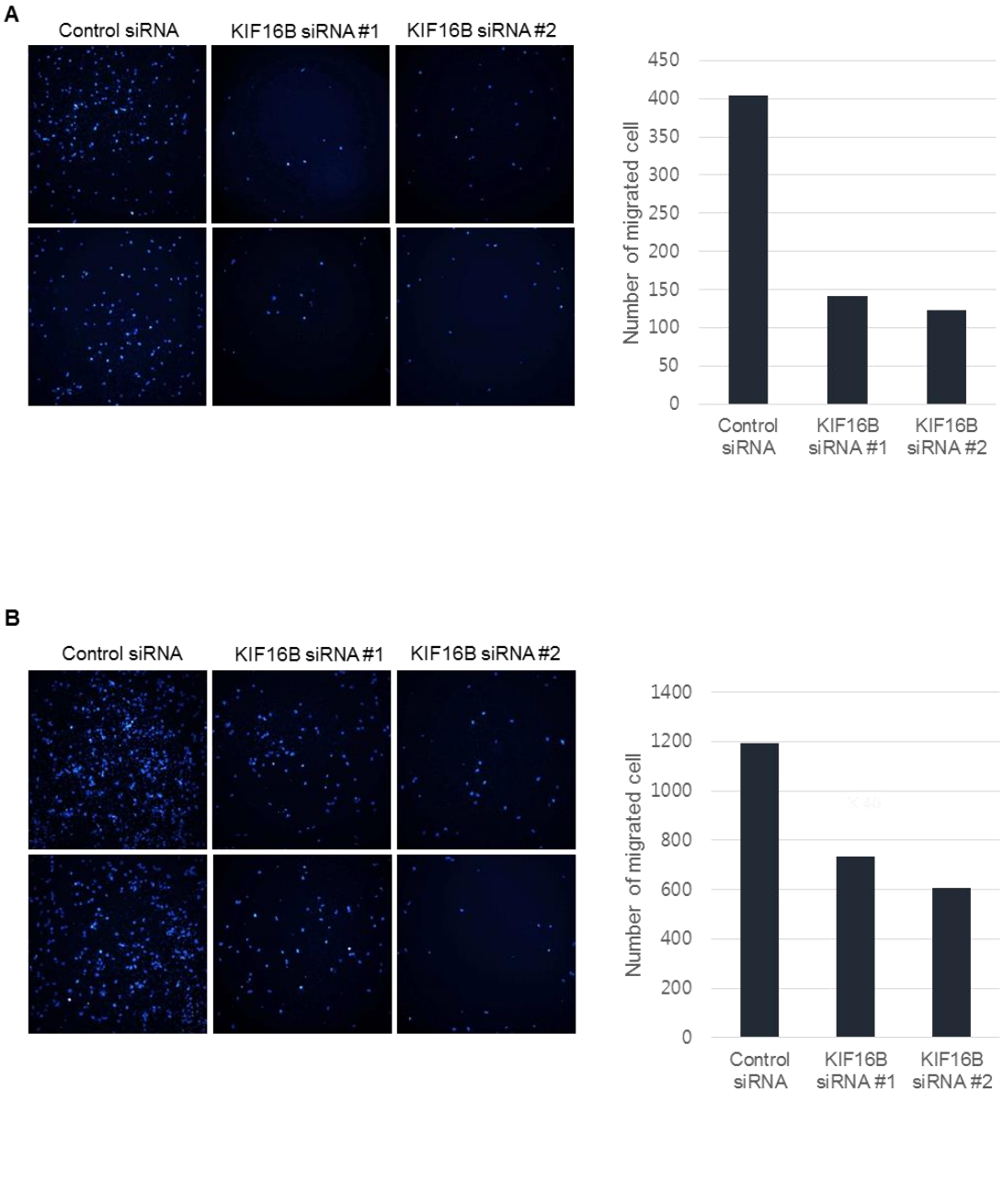

Figure 5. Effect of cell migration of KIF16B knockdown in lung cancer cells.

Cell migration assay were performed using the Transwell system in (A) A549 and (B) H1299 cells. Data represent the mean cell numbers from 4 fields.

DISCUSSION

Lung cancer is the most common diagnosed cancer and the prognosis is very bad. The identification of more therapeutic targets that contribute to the development and progression of lung cancer is clearly desirable to battle this deadly disease. The active movement of KIF family, known as microtubule-dependent molecular motor protein, supports several cellular functions, including mitosis, meiosis and the transport of cargos. Errors in these critical processes can lead to cell death, abnormalities, and cancer. Because of this, alteration of kinesin expression of function can lead to tumorigenesis. For this reason, targeting kinesin can create novel strategies for the control of human cancer [25, 26]. Among them, KIF16B is a member of the kinesin-3 family and identified as the plus-end motor protein. KIF16B plays a role in the transport of early endosome in endocytosis. KIF16B regulates the recycling and degradation of receptors by controlling the localization. However, the function of KIF16B in cancer is un-

known.

In this study, we through TCGA datasets analysis found that KIF16B mRNA is higher expressed in lung cancer tissue compared to non-cancerous lung tissue. This result suggesting that the KIF16B will cause impact in lung cancer. Next, we confirmed the expression of KIF16B in some lung cancer cell lines and then expression level of mRNA and protein was increased in lung cancer cell lines compared with the normal lung cell, IMR90 and MRC-5. These results suggested that KIF16B may play role lung tumorigenesis that is associated with lung cancer. Thus, we concentrated upon whether knockdown of KIF16B would affect the development and progression in lung cancer cell A549 and H1299 cells. First, we investigated the functional role of KIF16B in lung cancer cell line A549 and H1299 with high expression of endogeneous KIF16B. To this end, we performed an experiment to make KIF16B knockdown cells using specific siRNA targeted KIF16B. We confirmed the expression levels of mRNA and protein of KIF16B

mRNA and protein by using Real-time PCR and Western blotting assay to validate the efficiency of KIF16B knockdown. As a result, KIF16B siRNA #1, #2 transfected A549 and H1299 cells significantly decreased compared to control siRNA transfected cell. Next, we wondered whether the knockdown of KIF16B affects cell proliferation and migration ability. MTT assay and soft agar colony formation assay showed that KIF16B knockdown decrease cell proliferation. Since these abnormalities in cell proliferation were mostly known to be related to the cell cycle, we carried out that measured cell cycle by using FACS analysis. As can be seen from this data, arrest occurred in the G2/M phase when KIF16B knockdown. To further study, we investigated that expression of proteins, p53, p-H3 and cyclin B1, related to cell cycle. As a result, the expression of p53, a tumor suppressor gene, was increased and the expression of p-H3 and cyclin B1, which are involved in the progression from G2 phase to M phase, was also increased. These results demonstrated that the knockdown of KIF16B inhibits cell

growth. Cell migration is one of the processes of tumor metastasis [27]. Thus, we carried out Transwell assay and showed that the knockdown of KIF16B decrease cell migration. These above results showed that KIF16B play important role in lung cancer tumorigenesis and progression.

The active movement of KIF family, known as microtubule-dependent molecular motor protein, supports several cellular functions, including mitosis, meiosis and the transport of cargos. Errors in these critical processes can lead to cell death, abnormalities, and cancer. Because of this, alteration of kinesin expression of function can lead to tumorigenesis. For this reason, targeting kinesin can create novel strategies for the control of human cancer [25, 26, 28, 29].

Further study is required to determine whether KIF16B knockdown affects invasion ability. Moreover, the expression of proteins related to EMT must be confirmed to study more detailed mechanisms.

In conclusion, KIF16B play important role in lung cancer progression, and

these results will provide important information for the future diagnosis and treatment of tumors using KIF16B.

ABSTRACT

The role of kinesin family member 16B in tumorigenesis

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Cancer are formed in such a way that normal cells undergo transformation and accumulate long-term mutations, and then undergo continuous division and migration. Mutations occur in many genes, but the exact causal genes are not fully identified. Therefore, there are many limitations to the present diagnosis of tumor diagnosis and the development of target therapies. In order to find new car targets, kinesin family member 16B (KIF16B) was found in the database using genes with markedly different expressions in normal and tumor cells. In this study, we examined the effect of this gene on tumorigenesis through KIF16B,

whose expression differs in normal and tumor cancer tissues. Here, we show that KIF16B knockdown decrease mRNA and protein expression. In addition, cell growth and proliferation were reduced by MTT assay and soft agar colony for-mation assay, and cell mobility was decreased by Transwell migration assay. These oncogenic effects were significantly decreased in KIF16B knockdown lung cancer cells through in vitro studies. These results demonstrated that KIF16B could be associated with tumorigenic potential of cancer cells in vitro assays. Taken together, these results will provide important information for the future diagnosis and treatment of tumors using KIF16B.

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