OPEN

Experimental & Molecular Medicine (2014) 46, e97; doi:10.1038/emm.2014.23
© 2014 KSBMB. All rights reserved 2092-6413/14
www.nature.com/emm

ORIGINAL ARTICLE

Silencing of KIF14 interferes with cell cycle progression and cytokinesis by blocking the p27^{Kip1} ubiquitination pathway in hepatocellular carcinoma

Haidong Xu[1,2,6], Chungyoul Choe[1,6], Seung-Hun Shin[1], Sung-Won Park[1], Ho-Shik Kim[3], Seung-Hyun Jung[1], Seon-Hee Yim[1,4], Tae-Min Kim[5] and Yeun-Jun Chung[1]

Although it has been suggested that kinesin family member 14 (KIF14) has oncogenic potential in various cancers, including hepatocellular carcinoma (HCC), the molecular mechanism of this potential remains unknown. We aimed to elucidate the role of KIF14 in hepatocarcinogenesis by knocking down KIF14 in HCC cells that overexpressed KIF14. After KIF14 knockdown, changes in tumor cell growth, cell cycle and cytokinesis were examined. We also examined cell cycle regulatory molecules and upstream Skp1/Cul1/F-box (SCF) complex molecules. Knockdown of KIF14 resulted in suppression of cell proliferation and failure of cytokinesis, whereas KIF14 overexpression increased cell proliferation. In KIF14-silenced cells, the levels of cyclins E1, D1 and B1 were profoundly decreased compared with control cells. Of the cyclin-dependent kinase inhibitors, the p27^{Kip1} protein level specifically increased after KIF14 knockdown. The increase in p27^{Kip1} was not due to elevation of its mRNA level, but was due to inhibition of the proteasome-dependent degradation pathway. To explore the pathway upstream of this event, we measured the levels of SCF complex molecules, including Skp1, Skp2, Cul1, Roc1 and Cks1. The levels of Skp2 and its cofactor Cks1 decreased in the KIF14 knockdown cells where p27^{Kip1} accumulated. Overexpression of Skp2 in the KIF14 knockdown cells attenuated the failure of cytokinesis. On the basis of these results, we postulate that KIF14 knockdown downregulates the expression of Skp2 and Cks1, which target p27^{Kip1} for degradation by the 26S proteasome, leading to accumulation of p27^{Kip1}. The downregulation of Skp2 and Cks1 also resulted in cytokinesis failure, which may inhibit tumor growth. To the best of our knowledge, this is the first report that has identified the molecular target and oncogenic effect of KIF14 in HCC.

Experimental & Molecular Medicine (2014) **46,** e97; doi:10.1038/emm.2014.23; published online 23 May 2014

Keywords: cell cycle; cytokinesis; hepatocellular carcinoma; KIF14; p27^{kip1}; ubiquitination

INTRODUCTION

The long arm of chromosome 1 has been of particular interest in cancer biology, as gains in the region spanning 1q31-1q32 have been found in a wide variety of cancers, including hepatocellular carcinoma (HCC).[1–4] In our previous study, in which we investigated genome-wide chromosomal alterations in primary HCCs, we found that the 1q32.1 region was the most recurrently amplified region, and that the kinesin family member 14 (*KIF14*) gene, which is located in this region, was coherently overexpressed in HCCs.[5] As KIF14 was first cloned in 1994, it has been widely accepted to have a role in tumorigenesis as a chromokinesin; it binds to chromatin and microtubules during the formation of the bipolar spindle.[6,7] Therefore, the overexpression of KIF14 may lead to rapid and error-prone mitosis, which can induce aneuploidy during tumorigenesis. KIF14 has also been reported to induce anchorage-independent growth in the human ovarian cancer cell line SKOV3 and has been suggested to be a potential prognostic or therapeutic target for ovarian cancer.[8]

KIF14 is a member of the kinesin-3 superfamily.[9] Kinesin is a cytoskeletal motor protein that is involved in various

[1]Integrated Research Center for Genome Polymorphism, Department of Microbiology, Catholic University of Korea, College of Medicine, Seoul, Korea; [2]Center of Laboratory, Yanbian University Hospital, Yanji, China; [3]Department of Biochemistry, Catholic University of Korea, College of Medicine, Seoul, Korea; [4]Department of Medical Education, Catholic University of Korea, College of Medicine, Seoul, Korea and [5]MRC Cancer Evolution Research Center, Department of Medical Informatics, Catholic University of Korea, College of Medicine, Seoul, Korea

[6]These authors contributed equally to this work.

Correspondence: Professor Y-J Chung, Integrated Research Center for Genome Polymorphism, Department of Microbiology, Catholic University of Korea, College of Medicine, 222 Banpo-daero, Seoul 137-701, Korea.

E-mail: yejun@catholic.ac.kr

Received 14 July 2013; revised 26 November 2013; accepted 6 December 2013

biological phenomena, including cargo-containing vesicle transport, mitotic spindle formation, chromosome segregation, midbody formation and cytokinesis completion.[9] Depletion of KIF14 has been shown to block the completion of cytokinesis.[6,10] KIF14 has also been shown to interact with citron kinase. Because citron kinase is another form of the Rho effector kinase, which phosphorylates myosin and functions late in cytokinesis, it has been suggested that KIF14 is involved in abscission, where the cleavage furrow seals off the connection between the two daughter cells by depositing new membranes, through its interaction with other cytokinesis regulatory proteins.[10] Carleton *et al.*[6] also reported that strong RNA interference-mediated silencing of KIF14 resulted in cytokinesis failure, and that less efficacious KIF14-specific short interfering RNAs (siRNAs) induced multiple phenotypes that resulted in acute apoptosis at three distinct points in the cell cycle. This finding suggests that cell cycle progression can be disrupted by the differential modulation of KIF14 expression. All of these lines of evidence suggest that overexpression of KIF14 may have an oncogenic role because of perturbation of cell cycle control, as well as cytokinesis. However, an oncogenic mechanism of KIF14 related to cell cycle progression has not yet been well studied.

In this study, we aimed to elucidate the role of KIF14 overexpression in hepatocarcinogenesis by knocking down this gene, and we demonstrated that silencing of KIF14 repressed tumor cell growth by interfering with cytokinesis and the $p27^{Kip1}$ ubiquitination pathway in HCC. We also explored the molecular mechanism of the suppression of $p27^{Kip1}$ ubiquitination in KIF14-silenced cells.

MATERIALS AND METHODS

HCC cell lines

Six HCC cell lines, HepG2, SNU-449, SNU-475, SNU-761, SNU-878 and SNU-886, were purchased from the Korean Cell-Line Bank (Seoul, Korea) and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C under 5% CO_2. The THLE-2 cell line, a human normal liver cell line, was purchased from ATCC (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 25 mM HEPES buffer and 100 U ml^{-1} penicillin.

KIF14 knockdown

Three different KIF14-specific siRNAs (siKIF14-1, siKIF14-2 and siKIF14-3) were purchased from Invitrogen (Carlsbad, CA, USA). To verify the sequence-specific effectiveness of KIF14 RNAi, we used a negative control siRNA (Invitrogen) that has no significant homology to any known sequences in the human genome. The sequences of the three KIF14 siRNAs are available in Supplementary Table 1. HCC cells were seeded at a density of 200 000 cells per well in 6-well plates, and siRNA transfection was performed by adding a mixture of 50 nM siRNA and 1.25 μg ml^{-1} transfection reagent (Lipofectamine RNiMAX, Invitrogen) to the cells. After 72 h, HCC cells were harvested to validate the knockdown of KIF14 expression using real-time quantitative reverse transcription-PCR (qRT–PCR) and western blot analysis.

Overexpression of KIF14 and SKP2

Stable SNU-761 cells exogenously overexpressing KIF14 were generated by transfection with pCMV6-KIF14 (Origene, Rockville, MD, USA). For SKP2 overexpression, stable SNU-449 cells were transfected with pcDNA3-myc-Skp2 (Addgene, Cambridge, MA, USA). As an empty vector control, pcDNA 3.1 was transfected into the same cell lines. Following transfection, cells were selected with 1 mg ml^{-1} G418 (Duchefa, Haarlem, the Netherland) for 10 days and were then maintained in 200 μg ml^{-1} G418.

Real-time qRT–PCR

Total RNA was extracted from the HCC cells, and first-strand cDNA was synthesized using an oligo-dT primer and M-MLV reverse transcriptase (Invitrogen). Real-time qRT–PCR was performed using a Mx3000P QPCR System and MxPro Version 3.00 software (Stratagene, La Jolla, CA, USA). The real-time quantitative PCR mixture contained 10 ng cDNA, 1 × SYBR Green Tbr polymerase mixture (FINNZYMES, Vantaa, Finland), 0.5 × ROX reference dye and 20 pmol of each primer. The *GAPDH* gene was used as an internal control for each reaction. The following PCR conditions were used: denaturation at 95 °C for 5 min, followed by 40 cycles at 94 °C for 10 s, 53–55 °C for 30 s and 72 °C for 40 s. To verify specific amplification, melting curve analysis was performed (55–95 °C, 0.5 °C s^{-1}). Quantification of the relative expression was performed using the $\Delta\Delta$CT method, as described elsewhere.[11,12] Details of the primer pairs and corresponding genes are available in Supplementary Table 2.

Western blot analysis

Western blot analysis was performed as described elsewhere.[11] Briefly, the siRNA-transfected cells and negative control cells were washed with ice-cold phosphate-buffered saline (PBS) and homogenized in cell lysis buffer. After centrifugation for 10 min at 4 °C, the supernatant was harvested and the protein concentration was measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Supernatant proteins (30 μg per lane) were electrophoresed in 10% SDS-polyacrylamide gel and were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), then incubated with the following primary antibodies: anti-KIF14 (1:1000, Abcam, Cambridge, UK), anti-p16, anti-p21, anti-cyclin D1, anti-cyclin B1, anti-cyclin E1 (1:1000, Epitomics, Burlingame, CA, USA), anti-p27 (1:1000, BD Biosciences, San Jose, CA, USA), anti-Skp2 (1:1000, BD Biosciences), anti-Cks1 and anti-α-tubulin (1:1000 to 1:3000, Santa Cruz Biotechnology, Dallas, TX, USA). The membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h and visualized using the ECL detection kit (Amersham-Pharmacia Biotech, Braunschweig, Germany). Alpha-tubulin and beta-actin were used as internal controls for western blot analysis.

Colony formation and cell proliferation assay

Seventy-two hours after siRNA transfection, colony formation and cell proliferation assays were performed. For colony formation, transfected cells (1×10^4) were seeded onto 10-cm culture dishes. Nine days after seeding, the cells were washed with PBS buffer and stained with 0.5% crystal violet in 20% methanol for 20 min. Colonies larger than 1 mm in diameter were counted. The cell proliferation assay was performed using the Cell proliferation ELISA BrdU kit (Roche Diagnostics, Mannheim, Germany). Briefly, 5000 cells were seeded into each well of a 96-well plate, and the 5-bromo-2-deoxyuridine (BrdU) incorporation assay was carried out using the ELISA BrdU kit.

Nuclear staining

The cells were grown on poly-D-lysine-coated glass chamber well slides and were fixed in 3.7% formalin solution for 30 min. Then, the cells were incubated with an anti-α-tubulin (1:100, Santa Cruz) antibody in a humid chamber overnight at 4 °C. After washing with PBS, the cells were incubated with an Alexa 488-conjugated anti-mouse IgG antibody (1:100, Invitrogen) for 1 h at room temperature. The coverslips were mounted on a glass slide using VECTASHIELD mounting medium with 4′-6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA), and the cells were observed under an LSM 700 confocal microscope (Zeiss, Oberkochen, Germany). A binucleated cell was defined as a single cell containing two distinguishable 4′-6-diamidino-2-phenylindole-stained masses. The binucleated cell fraction (binucleated cell number/total cell number in a microscope field, × 200) was calculated in three to five fields for each cell.

KIF14 and $p27^{Kip1}$ double immunochemical staining

The siRNA-transfected cells were fixed and incubated with polyclonal antibodies against $p27^{Kip1}$ and KIF14 (diluted 1:25) in a humid chamber at 37 °C for 1 h. The cells were washed with PBS and then incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit or a phycoerythrin-conjugated donkey anti-mouse IgG at 37 °C for 1 h. The coverslips were mounted on a glass slide in 10% Mowiol 4–88, 1 $\mu g\,ml^{-1}$ 4′-6-diamidino-2-phenylindole and 25% glycerol in PBS, and the cells were observed using an LSM 510 Meta confocal microscope (Zeiss).

RESULTS

Elevated KIF14 expression in HCC cell lines

We first measured the baseline KIF14 expression in six HCC cell lines. In three out of the six cell lines, KIF14 protein expression was more than twofold higher than in the THLE-2 normal liver cells (Figure 1a). Among the three HCC cell lines in which KIF14 was overexpressed, the SNU-449 cell line was selected for the KIF14 knockdown studies to explore its biological role in hepatocarcinogenesis, as SNU-449 showed the highest expression of KIF14 (approximately fivefold higher than the normal liver cells).

Specific suppression of KIF14 expression in HCC by siRNA

To knock down KIF14, we transfected three siRNA constructs (siKIF14-1, siKIF14-2 and siKIF14-3) into the SNU-449 cells. As a control, a negative oligonucleotide construct (siNEG) was transfected into the same cell line. All three siRNAs specifically and efficiently repressed KIF14 mRNA expression compared with the siNEG according to real-time qRT–PCR analysis (Figure 1b). Of the three siRNAs, siKIF14-2 suppressed KIF14 expression most efficiently; the relative KIF14 mRNA expression ratio (siKIF14-2/siNEG) was 0.01 ($P < 0.0001$). Therefore, all of the downstream functional analyses were performed using siKIF14-2 (hereafter referred to as siKIF14). We further measured KIF14 protein expression in a time series after siKIF14 transfection into SNU-449 cells. KIF14 protein expression was repressed by approximately 65% 24 h after siKIF14 transfection, and expression was almost completely suppressed by 48 h after siKIF14 transfection. In contrast, siNEG did not repress KIF14 expression even after 72 h (Figure 1c).

Effect of siKIF14 knockdown on tumor cell growth

To test the potential role of KIF14 in tumor cell growth, we first observed the colony formation of the siKIF14-treated cells.

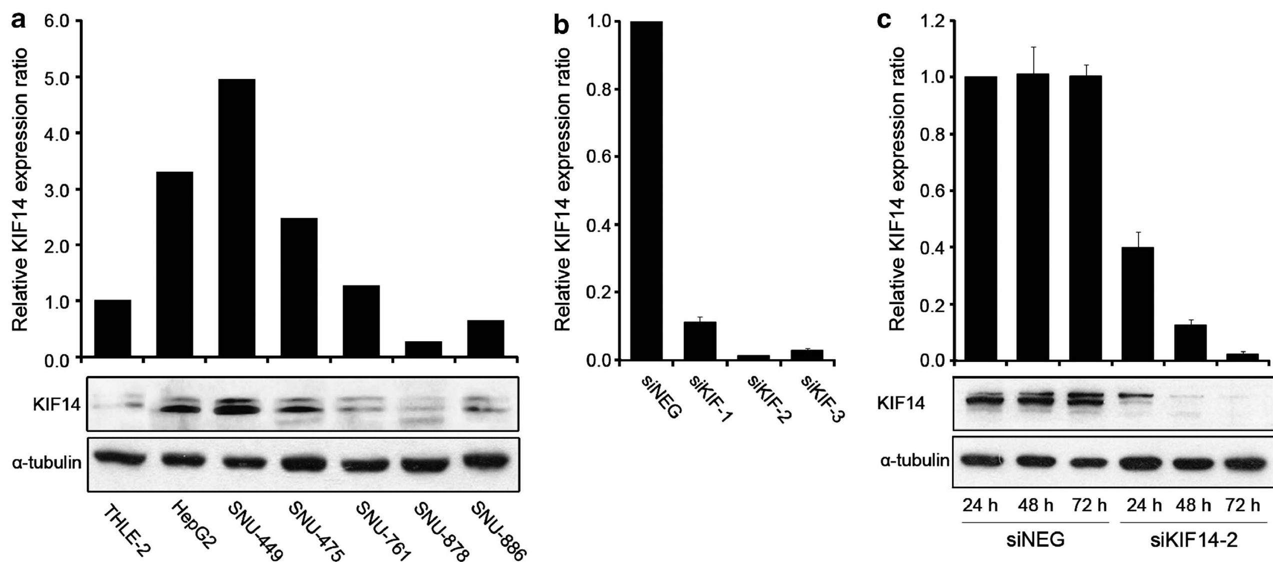

Figure 1 Kinesin family member 14 (KIF14) expression in various hepatocellular carcinoma (HCC) cell lines and short interfering RNA (siRNA)-mediated downregulation of KIF14 expression. (**a**) KIF14 protein expression in six HCC cell lines and a normal liver cell line (THLE-2). Alpha-tubulin was used as an internal control for western blot analysis. The *y* axis represents the relative KIF14 protein expression ratio (siKIF14/siNEG). (**b**) Downregulation of KIF14 by transfection of three KIF14 siRNAs into SNU-449 cells. After 72 h, the cells were harvested to validate the knockdown of KIF14 expression using quantitative reverse transcription-PCR analysis. The *y* axis represents the relative KIF14 expression ratio (siKIF14/siNEG). (**c**) KIF14 protein expression after siKIF14 transfection in a time series. Alpha-tubulin was used as an internal control for western blot analysis. The error bars indicate the s.d. of the mean of three individual experiments.

The number of colonies formed by the siKIF14-treated cells was significantly lower than that formed by the siNEG-treated cells (25 versus 150, $P<0.0001$; Figure 2a). This result supports the hypothesis that the overexpression of KIF14 can influence tumorigenesis. Therefore, we compared the cell proliferation of siKIF14- and siNEG-treated SNU-449 cells by measuring BrdU incorporation. Consistent with the colony formation results, BrdU incorporation in the siKIF14-transfected cells was significantly lower than in the siNEG-transfected cells ($P<0.0001$; Figure 2b). In addition, to explore the effects of KIF14 on tumor cell proliferation, we transfected a KIF14 overexpression vector into SNU-761 cells (an HCC cell line with a level of KIF14 expression similar to that of normal liver cells). A KIF14 empty vector was also transfected as a control. After observing the overexpression of KIF14 in the SNU-761 cells, we performed a cell proliferation assay. The KIF14-overexpressing SNU-761 cells showed significantly higher levels of BrdU incorporation than those transfected with the control vector ($P=0.006$, Figure 2c).

Cytokinesis failure in siKIF14-treated cells

KIF14 is a mitotic kinesin that promotes the completion of cytokinesis. Therefore, it is likely that the cellular basis for the repression of SNU-449 cell growth by siKIF14 transfection involves the inhibition of cytokinesis. To test this possibility, we measured the binucleated cell fraction of the siKIF14-

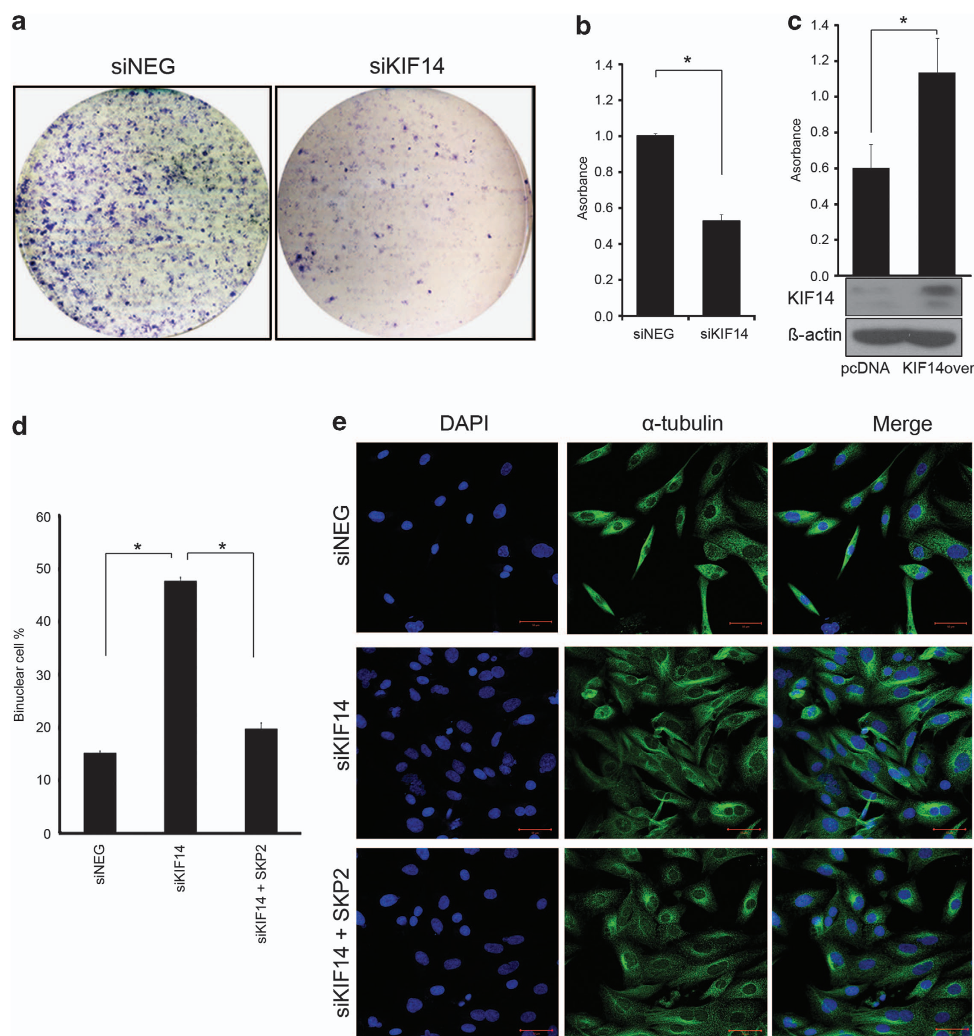

Figure 2 Effects of kinesin family member 14-specific short interfering RNA (siKIF14) knockdown and overexpression on tumor cell growth and cell cycle progression. (**a**) Colony formation assay after siKIF14 knockdown in SNU-449 cells. (**b**) Proliferation assay after siKIF14 knockdown in SNU-449 cells. All measurements were repeated three times, and the mean optical density values with s.d. were plotted for each case. *$P<0.01$. (**c**) Proliferation assay after transfection of SNU-761 cells with the KIF14 overexpression vector. KIF14 overexpression was confirmed by western blot analysis (bottom plot). Beta-actin was used as an internal control. KIF14over, pCMV6-KIF14-transfected cells; pcDNA, KIF14 empty vector (pcDNA)-transfected cells. (**d** and **e**) Effects of KIF14 depletion and siKIF14 + pcDNA3-myc-Skp2 co-transfection on cytokinesis. (**d**) The bar chart represents the average frequencies of the binucleated cells in the siKIF14-, siKIF14 + pcDNA3-myc-Skp2- and siNEG-transfected cells. *$P<0.05$. (**e**) Examples of the binucleated cells in the three different condition: siNEG-, siKIF14- and siKIF14 + pcDNA3-myc-Skp2-transfected cells. DAPI, 4′-6-diamidino-2-phenylindole.

treated SNU-449 cells. In line with our expectations, the fraction of binucleated cells in the KIF14 knockdown cells was significantly higher than that in the siNEG control cells (47.7% versus 15.1%, $P = 1.65 \times 10^{-5}$; Figures 2d and e), suggesting that cells deprived of KIF14 were more prone to cytokinesis failure.

KIF14 knockdown increases the expression of $p27^{Kip1}$ and decreases the expression of cyclins

To identify the molecular mechanism by which KIF14 knockdown repressed tumor cell growth and caused cytokinesis failure, we measured the expression of cyclins. In the siKIF14-treated SNU-449 cells, the levels of cyclins E1, D1 and B1 were profoundly decreased compared with the siNEG-treated cells (Figure 3a). We also measured the levels of cyclin-dependent kinase inhibitors, such as p16, $p21^{Cip1}$ and $p27^{Kip1}$. The level of $p27^{Kip1}$ was specifically increased after siKIF14 transfection, whereas there was no change in the expression of the other two cyclin-dependent kinase inhibitors (Figure 3a). The negative correlation between KIF14 and $p27^{Kip1}$ was confirmed by double immunostaining; siKIF14-treated cells showed decreased levels of KIF14 and increased levels of $p27^{Kip1}$, whereas the opposite was observed in the siNEG-treated cells (Figure 3b).

Mechanism of elevated $p27^{Kip1}$ expression in KIF14 knockdown cells

To understand the molecular mechanism of the decreased cyclin expression and $p27^{Kip1}$ overexpression, we examined the mRNA expression levels of the target molecules using reverse transcription-PCR. The mRNA expression levels of cyclins E1, D1 and B1 were consistently downregulated, in agreement with their protein levels; however, contrary to our expectations, the mRNA level of $p27^{Kip1}$ was not elevated (Figure 4a). This result suggests that the increased expression of $p27^{Kip1}$ protein in the siKIF14-treated cells was acquired at the post-transcriptional level. To verify the possibility that the elevation in $p27^{Kip1}$ in the absence of changes in $p27^{Kip1}$ transcription might result from inhibition of $p27^{Kip1}$ degradation in the siKIF14 knockdown cells, we measured $p27^{Kip1}$ in a time series in the SNU-449 cells after treatment with the 26S proteasome inhibitor MG132. After MG132 treatment, $p27^{Kip1}$ accumulated, and the accumulation increased in a time-dependent manner (Figure 4b). Because the posttranscriptional alteration of the $p27^{Kip1}$ level is known to be regulated, in part, by a translational control mechanism,[18] we also examined whether MG132 could block the decrease in $p27^{Kip1}$ in the presence of the translation inhibitor cycloheximide (CHX). When the SNU-449 cells were treated with CHX only, the $p27^{Kip1}$ level gradually decreased in a time-dependent manner, but treatment with both MG132 and CHX blocked the decrease in $p27^{Kip1}$ (Figure 4b). If the increase in $p27^{Kip1}$ in the KIF14 knockdown cells was mainly due to the inhibition of the $p27^{Kip1}$ degradation pathway, treatment with both siKIF14 and CHX should show a similar effect as that observed in the MG132 and CHX co-treated cells. To explore this possibility, SNU-449 cells were treated with both siKIF14 and CHX (CHX was added 72 h after the siKIF14 treatment). As we anticipated, cells treated with both siKIF14 and CHX showed an accumulation of $p27^{Kip1}$ (Figure 4b).

Effect of KIF14 knockdown on the Skp1/Cul1/F-box (SCF) complex

To investigate the molecular mechanism by which KIF14 knockdown causes $p27^{Kip1}$ accumulation, we measured the expression of five components of the SCF complex (Skp1, Skp2, Cul1, Roc1 and Cks1). After KIF14 knockdown, the mRNA levels of Skp2 and its cofactor Cks1 decreased significantly, but the mRNA levels of Cul1, Roc1 and Skp1 did not change (Figure 5a). Consistent with the changes in

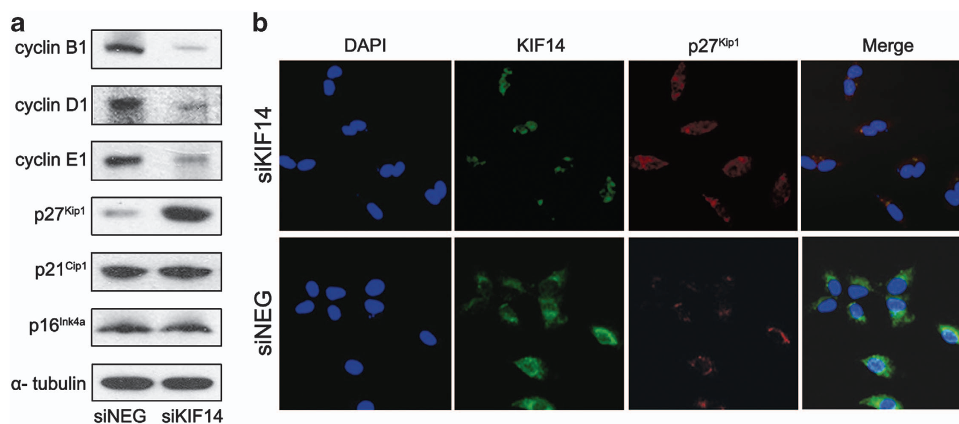

Figure 3 Effects of kinesin family member 14 (KIF14) knockdown on expression of cyclins and cyclin-dependent kinase inhibitors. (**a**) Western blot analysis shows the downregulation of cyclins B1, D1 and E1 and the upregulation of $p27^{Kip1}$ in the KIF14-specific short interfering RNA (siKIF14)-treated cells. There was no difference in the levels of $p16^{Ink4a}$ and $p21^{Cip1}$ between the siKIF14- and siNEG-transfected cells. (**b**) Double immunochemical staining shows a negative correlation between KIF14 (green) and $p27^{Kip1}$ (red). DAPI, 4′-6-diamidino-2-phenylindole.

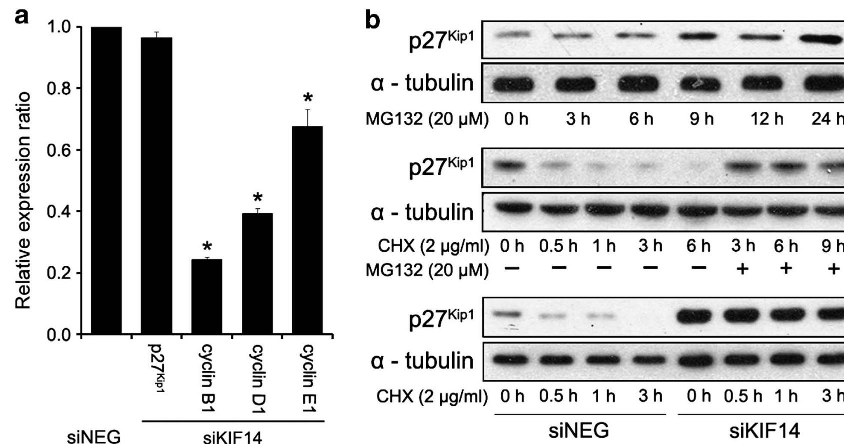

Figure 4 Mechanism of the elevated level of $p27^{Kip1}$ in the kinesin family member 14 (KIF14) knockdown cells. (**a**) RNA expression levels of $p27^{Kip1}$ and cyclins. The *y* axis represents the relative expression ratio of the target genes (KIF14-specific short interfering RNA (siKIF14)- or siNEG-treated SNU-449/original SNU-449). The ratio values were subsequently normalized by setting the mean ratio of the value from the siNEG-treated cells equal to 1. (**b**) KIF14 knockdown mimics a proteasome inhibitor in SNU-449 cells. Upper plot: $p27^{Kip1}$ expression level in MG132-treated cells. Intracellular $p27^{Kip1}$ accumulated in a time-dependent manner after treatment with the proteasome inhibitor MG132. Middle plot: $p27^{Kip1}$ expression level in cycloheximide (CHX)-treated and MG132 + CHX co-treated cells. In the CHX treatment only cells, the $p27^{Kip1}$ level gradually decreased in a time-dependent manner. However, MG132 + CHX co-treatment blocked the decrease of $p27^{Kip1}$. Bottom plot: effect of siKIF14 treatment on the decreased expression of $p27^{Kip1}$ caused by treatment with CHX. Seventy-two hours after transfection with siKIF14 or siNEG, the cells were co-treated with CHX for 0, 0.5, 1 or 3 h.

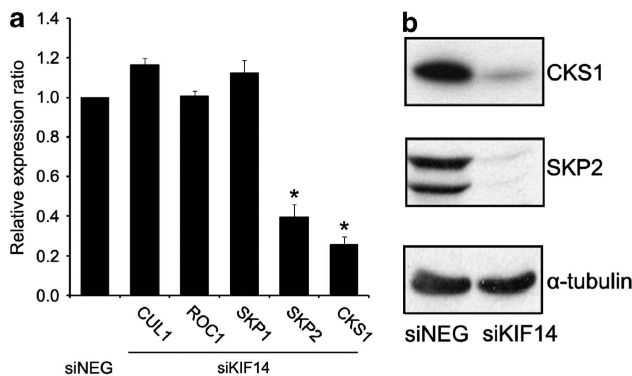

Figure 5 Effects of kinesin family member 14 (KIF14) knockdown on Skp1/Cul1/F-box (SCF) complex components in SNU-449 cells. (**a**) KIF14 knockdown downregulated the mRNA expression of Skp2 and Cks1 but not the mRNA expression of Cul1, Roc1 and Skp1. $*P<0.05$. (**b**) Skp2 and Cks1 protein expression after KIF14 knockdown.

mRNA expression, the protein levels of Skp2 and Cks1 were also profoundly decreased in KIF14 knockdown cells (Figure 5b). We further tested whether overexpression of Skp2 attenuated the failure of cytokinesis that resulted from KIF14 knockdown. We transfected pcDNA3-myc-Skp2 into SNU-449 cells and confirmed the overexpression of Skp2 (Supplementary Figure 1). Then, siKIF14 was co-transfected into the Skp2-overexpressing cells. The fraction of binucleated cells in the co-transfected cells was significantly lower than that in cells transfected with siKIF14 only (19.7% versus 47.7%, $P=0.0003$), but it was not significantly different from that in the siNEG cells (Figures 2d and e).

DISCUSSION

In this study, we propose that the suppression of KIF14 expression can inhibit tumor growth in at least two ways: by increasing the level of $p27^{Kip1}$ and causing failure of cytokinesis. Although the overexpression of KIF14 in various tumors and tumor cell lines has suggested the oncogenic potential of KIF14, to the best of our knowledge, this is the first report to demonstrate the molecular target and oncogenic mechanism of KIF14 overexpression in HCC.

Consistent with our previous study, in which we showed that KIF14 copy number and expression were increased in primary HCCs,[5] KIF14 overexpression (>2 times) was observed in half of the six HCC cell lines examined in this study. Our previous and current results indicate that KIF14 is involved in hepatocarcinogenesis, and KIF14 overexpression can be a genetic marker of HCC. Elevated BrdU incorporation after KIF14 overexpression in SNU-761 cells and repressed incorporation after KIF14 knockdown in SNU-449 cells consistently support the oncogenic potential of KIF14. Considering that BrdU is incorporated into DNA during S phase, the inhibition of BrdU incorporation suggests that the repressed growth of SNU-449 cells after KIF14 knockdown may be due to inhibition of cell cycle progression through S phase. In addition, KIF14 is a mitotic kinesin that promotes the completion of cytokinesis.[9] Therefore, it is likely that the cellular basis for the repression of SNU-449 cell growth by siKIF14 transfection can be attributed to the inhibition of cytokinesis. In line with our expectations, a substantial number of KIF14 knockdown cells were bi-nucleated. Carleton *et al.* reported previously that strong silencing of KIF14 resulted in cytokinesis failure and that less efficacious

silencing of KIF14 resulted in acute apoptosis at some points in the cell cycle.[6] On the basis of these findings, we can speculate that overexpression of KIF14 contributes to uncontrolled cell cycle progression and division in hepatocytes, which may cause initiation or progression of hepatocarcinogenesis. It is well known that the pRb pathway is functionally inactivated in most cases of HCC by genetic, epigenetic and/or viral mechanisms.[13,14] We tried to examine the effect of KIF14 knockdown on the level of phosphorylated Rb, but we could not observe the phosphorylated Rb signal (data not shown).

The activity of CDKs is regulated by the abundance of their cyclin partners and by association with the CDK inhibitors of the Cip/Kip ($p21^{Cip1}$ and $p27^{Kip1}$) or Ink families.[15,16] In our study, among the cyclins and cyclin-dependent kinase inhibitors, the levels of cyclins E1, D1 and B1 were profoundly decreased by KIF14 knock down, whereas the level of $p27^{Kip1}$ was specifically increased. However, the mRNA level of $p27^{Kip1}$ was not elevated. This result suggests that the increased level of $p27^{Kip1}$ in the siKIF14-treated cells was acquired at the post-transcriptional level. It is well known that proteasome-dependent degradation of cell cycle regulators is critical for the tight control of cell division.[17] Post-transcriptional alteration of the $p27^{Kip1}$ level is also known to be regulated, in part, by the translational control mechanism.[18] Based on our findings, we hypothesized that the elevated level of $p27^{Kip1}$, which occurred without a change in $p27^{Kip1}$ transcription, might be a result of the inhibition of $p27^{Kip1}$ degradation in the siKIF14 knockdown cells. Consistent with our speculation, when we examined the $p27^{Kip1}$ level after MG132, CHX and/or siKIF14 treatment, all of the results suggested that the elevation of $p27^{Kip1}$ in the KIF14 knockdown cell was not due to upregulation at the transcription or translation level but was instead due to inhibition of the proteasome-dependent $p27^{Kip1}$ degradation pathway.

There is a large body of evidence that the degradation of $p27^{Kip1}$ occurs in the nucleus via the ubiquitin protein ligase complex SCF^{Skp2} (Skp1-Cul1-Roc1-F box protein), a major class of E3 ubiquitin ligases, which triggers protein degradation by covalently attaching ubiquitin onto lysine residues of the target proteins.[19,20] In this study, the expression levels of Skp2 and its cofactor Cks1 specifically decreased after KIF14 knockdown, but the levels of Cul1, Roc1 and Skp1 did not change. Overexpression of Skp2 attenuated the failure of cytokinesis after KIF14 knockdown. These data suggest that expression of Skp2 is, at least in part, regulated by upstream KIF14 expression, and that both molecules contribute to hepatocarcinogenesis. Skp2 has been reported to recognize multiple targets, including $p27^{Kip1}$, $p21^{Cip1}$ and some cyclins; of these proteins, only $p27^{Kip1}$ requires the cofactor protein Cks1 for its recognition and ubiquitination by SCF^{Skp2}.[20–22] Although our observation that the level of $p27^{Kip1}$ was specifically changed in the siKIF14-transfected cells is consistent with previous reports, we did not further examine the molecular mechanisms of the phenomenon in this study. Further studies of the molecules that interact with KIF14 will be required to identify the detailed mechanisms. On the other hand, the decreased ubiquitination that results from reduced Skp2 and Cks1 levels may have an additional effect on tumor growth. Considering that ubiquitination has been reported to have a key role in the completion of cytokinesis and that Skp2 has been reported to degrade the components of the actomyosin ring, which is important for cytokinesis,[23–26] the reduced level of Skp2 might contribute to the observed failure of cytokinesis following siKIF14 treatment.

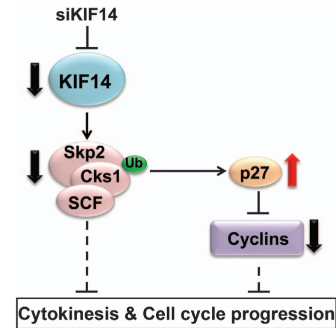

Figure 6 Schematic summary of the potential molecular mechanism of growth repression caused by kinesin family member 14 (KIF14) knockdown. KIF14 knockdown downregulates the expression of Skp2 and Cks1, which inhibits the proteasome-dependent $p27^{Kip1}$ ubiquitination pathway, leading to its accumulation. The increase in $p27^{kip1}$ inhibits the expression of cyclins, including E1, D1 and B1, which leads to the suppression of cell cycle progression, resulting in the suppression of hepatocellular carcinoma tumorigenicity. Downregulated Skp2 and Cks1 may also influence cytokinesis failure.

Taking our results and previous findings together, we postulate the following molecular mechanism of growth suppression by siKIF14 (Figure 6). KIF14 knockdown down-regulates the expression of Skp2 and its cofactor Cks1, which target $p27^{Kip1}$ for degradation by the 26S proteasome, leading to the accumulation of $p27^{Kip1}$. In addition, the downregulation of Skp2 and Cks1 also causes cytokinesis failure, which may also inhibit tumor growth. Further studies based on our findings will help us to understand the mechanism of hepatocarcinogenesis and the role of KIF14 in cytokinesis and cell cycle progression, which may highlight its potential role as a target for therapeutic intervention.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This study was supported by a grant from the MRC for Cancer Evolution Research Center (2012R1A5A2047939), Ministry of Education, Science and Technology, Republic of Korea. Haidong Xu was also supported by the Natural Science Foundation of China (81260305).

1 Corson TW, Huang A, Tsao MS, Gallie BL. KIF14 is a candidate oncogene in the 1q minimal region of genomic gain in multiple cancers. *Oncogene* 2005; **24:** 4741–4753.
2 Komatsu S, Imoto I, Tsuda H, Kozaki KI, Muramatsu T, Shimada Y *et al.* Overexpression of SMYD2 relates to tumor cell proliferation and malignant outcome of esophageal squamous cell carcinoma. *Carcinogenesis* 2009; **30:** 1139–1146.
3 Roversi G, Pfundt R, Moroni RF, Magnani I, van Reijmersdal S, Pollo B *et al.* Identification of novel genomic markers related to progression to glioblastoma through genomic profiling of 25 primary glioma cell lines. *Oncogene* 2006; **25:** 1571–1583.
4 Schlaeger C, Longerich T, Schiller C, Bewerunge P, Mehrabi A, Toedt G *et al.* Etiology-dependent molecular mechanisms in human hepatocarcinogenesis. *Hepatology* 2008; **47:** 511–520.
5 Kim TM, Yim SH, Shin SH, Xu HD, Jung YC, Park CK *et al.* Clinical implication of recurrent copy number alterations in hepatocellular carcinoma and putative oncogenes in recurrent gains on 1q. *Int J Cancer* 2008; **123:** 2808–2815.
6 Carleton M, Mao M, Biery M. Warrener P, Kim S, Buser C *et al.* RNA interference-mediated silencing of mitotic kinesin KIF14 disrupts cell cycle progression and induces cytokinesis failure. *Mol Cell Biol* 2006; **26:** 3853–3863.
7 Nomura N, Miyajima N, Sazuka T, Tanaka A, Kawarabayasi Y, Sato S *et al.* Prediction of the coding sequences of unidentified human genes. I. The coding sequences of 40 new genes (KIAA0001-KIAA0040) deduced by analysis of randomly sampled cDNA clones from human immature myeloid cell line KG-1. *DNA Res* 1994; **1:** 27–35.
8 Theriault BL, Pajovic S, Bernardini MQ, Shaw PA, Gallie BL. Kinesin family member 14: An independent prognostic marker and potential therapeutic target for ovarian cancer. *Int J Cancer* 2012; **130:** 1844–1954.
9 Miki H, Okada Y, Hirokawa N. Analysis of the kinesin superfamily: insights into structure and function. *Trends Cell Biol* 2005; **15:** 467–476.
10 Gruneberg U, Neef R, Li X, Chan EH, Chalamalasetty RB, Nigg EA *et al.* KIF14 and citron kinase act together to promote efficient cytokinesis. *J Cell Biol* 2006; **172:** 363–372.
11 Choi HS, Yim SH, Xu HD, Jung SH, Shin SH, Hu HJ *et al.* Tropomyosin3 overexpression and a potential link to epithelial-mesenchymal transition in human hepatocellular carcinoma. *BMC Cancer* 2010; **10:** 122.
12 Jung SH, Lee A, Yim SH, Hu HJ, Choe C, Chung YJ. Simultaneous copy number gains of NUPR1 and ERBB2 predicting poor prognosis in early-stage breast cancer. *BMC Cancer* 2012; **12:** 382.
13 Burkhart DL, Sage J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat Rev Cancer* 2008; **8:** 671–682.
14 Viatour P, Ehmer U, Saddic LA, Dorrell C, Andersen JB, Lin C *et al.* Notch signaling inhibits hepatocellular carcinoma following inactivation of the RB pathway. *J Exp Med* 2011; **208:** 1963–1976.
15 Hirai H, Roussel MF, Kato JY, Ashmun RA, Sherr CJ. Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. *Mol Cell Biol* 1995; **15:** 2672–2681.
16 Kato JY, Matsuoka M, Polyak K, Massague J, Sherr CJ. Cyclic AMP-induced G1 phase arrest mediated by an inhibitor (p27Kip1) of cyclin-dependent kinase 4 activation. *Cell* 1994; **79:** 487–496.
17 Reed SI. Ratchets and clocks: the cell cycle, ubiquitylation and protein turnover. *Nat Rev Mol Cell Biol* 2003; **4:** 855–864.
18 Hengst L, Reed SI. Translational control of p27Kip1 accumulation during the cell cycle. *Science* 1996; **271:** 1861–1864.
19 Bashir T, Dorrello NV, Amador V, Guardavaccaro D, Pagano M. Control of the SCF(Skp2-Cks1) ubiquitin ligase by the APC/C(Cdh1) ubiquitin ligase. *Nature* 2004; **428:** 190–193.
20 Schulman BA, Carrano AC, Jeffrey PD, Bowen Z, Kinnucan ER, Finnin MS *et al.* Insights into SCF ubiquitin ligases from the structure of the Skp1-Skp2 complex. *Nature* 2000; **408:** 381–386.
21 Ganoth D, Bornstein G, Ko TK, Larsen B, Tyers M, Pagano M *et al.* The cell-cycle regulatory protein Cks1 is required for SCF(Skp2)-mediated ubiquitinylation of p27. *Nat Cell Biol* 2001; **3:** 321–324.
22 Spruck C, Strohmaier H, Watson M, Smith AP, Ryan A, Krek TW *et al.* A CDK-independent function of mammalian Cks1: targeting of SCF(Skp2) to the CDK inhibitor p27Kip1. *Mol Cell* 2001; **7:** 639–650.
23 Mukai A, Mizuno E, Kobayashi K, Matsumoto M, Nakayama KI, Kitamura N *et al.* Dynamic regulation of ubiquitylation and deubiquitylation at the central spindle during cytokinesis. *J Cell Sci* 2008; **121:** 1325–1333.
24 Blondel M, Bach S, Bamps S, Dobbelaere J, Wiget P, Longaretti C *et al.* Degradation of Hof1 by SCF(Grr1) is important for actomyosin contraction during cytokinesis in yeast. *EMBO J* 2005; **24:** 1440–1452.
25 Jiang F, Caraway NP, Li R, Katz RL. RNA silencing of S-phase kinase-interacting protein 2 inhibits proliferation and centrosome amplification in lung cancer cells. *Oncogene* 2005; **24:** 3409–3418.
26 Kaiser P, Moncollin V, Clarke DJ, Watson MH, Bertolaet BL, Reed SI *et al.* Cyclin-dependent kinase and Cks/Suc1 interact with the proteasome in yeast to control proteolysis of M-phase targets. *Genes Dev* 1999; **13:** 1190–1202.

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/3.0/

Supplementary Information accompanies the paper on Experimental & Molecular Medicine website (http://www.nature.com/emm)