E3 ubiquitin ligase Hakai regulates cell growth and invasion, and increases the chemosensitivity to cisplatin in non-small-cell lung cancer cells

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Received November 10, 2017; Accepted April 26, 2018
DOI: 10.3892/ijmm.2018.3683

Abstract. Hakai was originally identified as an E3 ubiquitin ligase of the E-cadherin complex implicated in cell adhesion and invasion. Recently, emerging evidence has strongly suggested that Hakai serves a pivotal role in the tumorigenesis of certain tumors. However, the role of Hakai in non-small-cell lung cancer (NSCLC) and its underlying molecular mechanism have not been clarified. In the present study, it was observed that Hakai was highly expressed in NSCLC cell lines compared with human normal bronchial epithelial cells, and transfection with Hakai small interfering RNA significantly inhibited the growth of A549 and NCI-H460 NSCLC cells. In addition, the inhibition of Hakai suppressed NSCLC cell migration and invasion through upregulation of E-cadherin and downregulation of N-cadherin. Notably, it was also revealed that knockdown of Hakai led to a decrease in the expression of phosphorylated AKT (Ser473), and a significant enhancement of chemosensitivity to cisplatin was observed following Hakai suppression. In conclusion, the present study demonstrated for the first time that knockdown of Hakai inhibited the proliferation, migration and invasion of NSCLC cells, and sensitized NSCLC cells to cisplatin. Thus, Hakai may serve as a potential therapeutic target for the treatment of NSCLC.

Introduction

Lung cancer is the most frequent cause of cancer-associated mortalities worldwide, and non-small-cell lung cancer (NSCLC) is the most commonly occurring type of lung cancer (1,2). NSCLC patients are often diagnosed at a late stage of the disease, and exhibit a poor prognosis and resistance to chemotherapy (3). Despite advances associated with the use of targeted therapy, chemotherapy and immunotherapy, the treatment of advanced NSCLC remains challenging (2,4,5). Therefore, it is urgent to elucidate the molecular mechanisms that underlie the malignant behavior of NSCLC and identify novel approaches to inhibit aggressiveness and increase chemosensitivity of NSCLC to anticancer agents.

E3 ubiquitin ligases are engaged in the regulation of the turnover of numerous target proteins and serve an important role in controlling various biological processes (6-9). Dysregulated expression of E3 ligases, such as Mdm2 RING finger E3 ligase (10) and Skp2 SCF E3 ligase (11,12), has been convincingly shown to contribute to cancer development. Thus, targeting E3 ubiquitin ligases for cancer therapy has gained increasing attention. Hakai, a RING finger E3 ubiquitin ligase, was originally identified as an E3 ligase for the E-cadherin complex in 2002 (13). Since its identification, an increasing number of studies have focused on the role of Hakai in cell-cell contacts and cell invasion (14). Recently, emerging evidence suggested that Hakai promotes tumorigenesis. It was reported that Hakai was highly expressed in human colon and gastric adenocarcinomas, and regulated cell proliferation by enhancing the RNA-binding function of polypyrimidine tract binding protein-associated splicing factor (PSF) in an E-cadherin-independent manner (15,16). Furthermore, several drugs, such as vinflunine or silibinin, have been reported to inhibit cell invasion by upregulation of E-cadherin and downregulation of Hakai (17,18). These results demonstrated the increasingly important role of Hakai in cancer development and progression, and that Hakai may be a potential molecular target for cancer treatment. However, little is known regarding the role of Hakai and its direct mechanisms in human lung adenocarcinoma.

In the current study, the expression and potential function of Hakai in NSCLC cell lines were investigated. The results demonstrated that Hakai was significantly upregulated in human NSCLC cell lines, while knockdown of Hakai inhibited the proliferation, migration and invasion of NSCLC cells. Furthermore, it was demonstrated that Hakai knockdown decreased the levels of phosphorylated protein kinase B (ppAKT) and sensitized NSCLC cells to cisplatin (19,20). These findings provide new insights into the role of Hakai in NSCLC and suggest potential therapeutic strategies for the treatment of this disease.
(pAKT) and enhanced the cytotoxicity of cisplatin on NSCLC cells. The results suggested that the development of small molecules or RNA interference (RNAi)-based therapies targeting Hakai is promising for future treatment of NSCLC.

Materials and methods

Reagents. Anti-Hakai antibody (21179-1-AP) was purchased from ProteinTech Group, Inc. (Chicago, IL, USA). Anti-pAKT (Ser473) antibody (sc-7985-R), anti-AKT antibody (sc-8312), and the goat anti-rabbit (sc-2004) and anti-mouse (sc-2005) horseradish peroxidase-conjugated IgG secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-N-cadherin (AF0243) and anti-E-cadherin (AF0138) antibodies were obtained from Beyotime Institute of Biotechnology (Shanghai, China). Anti-actin (A5316), cisplatin (P4394) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Vetec V900888) were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Lipofectamine® 2000 (Invitrogen 11668-019) was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Cell lines and cell culture. The NSCLC cell lines A549 and NCI-H460 were obtained from the American Type Culture Collection (Manassas, VA, USA). The human normal bronchial epithelial cell line 16HBE was purchased from the Cell Resource Center of the Chinese Academy of Medical Sciences (Beijing, China) and cultured according to standard protocols. The human normal bronchial epithelial cells Beas-2B were provided by Professor Guangbiao Zhou at the Institute of Zoology, Chinese Academy of Sciences. Cells were cultured in Dulbecco modified Eagle medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Beyotime Institute of Biotechnology) under 5% CO₂ and 37°C in an incubator.

Small interfering RNA (siRNA) assay. NSCLC cells were plated in 6-well plates with growth medium without antibiotics at densities ranging between 1x10⁵ and 2x10⁵ cells/well. Following incubation for 24 h, cells were transfected with synthesized Hakai siRNA or negative control (NC) siRNA (GenePharma Co., Ltd., Shanghai, China) at a concentration of 100 nM using Lipofectamine® 2000 for siRNA delivery, according to the manufacturer's protocol. The specific transfection procedure was as follows: First, siRNA oligomer was diluted in 250 µl Opti-MEM I Reduced Serum Medium (Gibco; Thermo Fisher Scientific, Inc.) without serum. Next, 4 µl Lipofectamine® 2000 was diluted in 250 µl Opti-MEM I Reduced Serum Medium, mixed gently and incubated for 5 min at room temperature. After 5 min of incubation, the diluted oligomer and Lipofectamine® 2000 solutions were combined, mixed gently and incubated for 20 min at room temperature. Oligomer-Lipofectamine® 2000 complexes were added to each well containing the cells, along with 500 µl growth medium without antibiotics, and cells were incubated at 37°C in a CO₂ incubator. After 6 h of transfection, the medium was replaced with fresh growth medium, and the cells were harvest for further analysis after 48-72 h of cultivation. The sequences for Hakai RNAi were as follows: Hakai RNAi-1, 5'-CUCAUGCGCGUACGAGAA-3'; and Hakai RNAi-2, 5'-ACACCACGAAUAGUAGCUA-3'. The efficiency of siRNA transfection in downregulating the targeted gene was detected by a western blot assay, with an >50% reduction observed in the targeted protein expression.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Isolation of total RNA from cell lines was conducted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and the phenol-chloroform extraction method, according to the manufacturer's protocols. The 28S and 18S bands were detected by agarose gel electrophoresis for measuring the quality of the extracted RNA. cDNA was subsequently generated from total RNA using a PrimeScript 1st Strand cDNA Synthesis kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The RT conditions were as follows: 42°C for 1 h and 95°C for 5 min, followed by storage at -20°C. Next, the resultant cDNAs were used as templates for PCR amplification in 25 µl reaction mixture, including 10X ExTaq PCR buffer, deoxynucleoside triphosphate mixture (2.5 mM each), 0.2 µM of each primer and 1 U ExTaq polymerase (RR001A; TaKaRa Biotechnology Co., Ltd.). The primers for Hakai were synthesized by Jinsirui Biotechnology Co., Ltd. (Nanjing, China), and the sequences were as follows: Hakai: 5'-GGACACCTTTTTGGGACTT-3' (forward) and 5'-CACCTCTGAACATGTCAC-3' (reverse); Actin: 5'-ATCGTCCACCGAAA TGCTTCTA-3' (forward) and 5'-AGCCATGCCAATCTC ATCTTGTT-3' (reverse). The PCR amplification conditions for Hakai and Actin were 94°C for 10 sec, 55°C for 15 sec and 72°C for 30 sec, for a total of 35 and 22 cycles, respectively. Actin was used as an internal control. PCR products were separated by electrophoresis on 1.5% (w/v) agarose gels and visualized under UV light via Goldview staining (Beijing Solarbio Science & Technology Co. Ltd., Beijing, China).

Trypan blue exclusion assay. Cell viability was evaluated using the trypan blue dye exclusion assay (cat. no. C0011; Beyotime Institute of Biotechnology). Cells after 48 and 72 h of transfection were respectively detached with a trypsin-EDTA solution (HyClone; GE Healthcare Life Sciences). The mixture of detached cells was centrifuged at 1,000 x g for 1 min at room temperature and resuspended with fresh DMEM supplement with 10% FBS and penicillin/streptomycin. Subsequently, 100 µl of cell suspension was combined with 100 µl trypan blue solution (2X) and mixed gently. After 3 min staining at room temperature, viable cells that were not stained were counted manually using an inverted light microscope (Leica Microsystems GmbH, Wetzlar, Germany) using a x10 objective in 6-8 random fields of view in each group.

Colony formation assay. A549 or NCI-H460 cells were transfected with NC or Hakai-specific siRNA using the aforementioned method. After 48 h, the transfected cells were digested with trypsin and seeded onto 35 mm plates in triplicate (1,000 cells per plate). The medium were refreshed every 5 days. After 10 days of incubation, the cells were fixed with methanol for 10 min and stained with 0.005% crystal violet solution for ~20 min. Stained clones containing >50 cells were counted under a microscope.
Wound healing assay. A549 (2x10⁵/well) and NCI-H460 (4x10⁵/well) cells were seeded into 6-well plates and transfected the next day with Hakai RNAi or NC RNAi, while there was also a control group without siRNA transfection. After 48 h, when the cells approximated full confluence, the bottom chamber was filled with DMEM with 20% FBS. After 24 h of incubation at 37˚C in a Lakes, NJ, USA), and the bottom chamber was filled with DMEM with 20% FBS. After 24 h of incubation at 37˚C in a 5% CO₂ atmosphere, the optical density was measured at an absorbance wavelength of 490 nm using a microplate reader (Multiskan FC; Thermo Fisher Scientific, Inc.). The cell survival rate was calculated as: Cell viability (%) = (average A490 of the experimental group - average A490 of the blank group)/(average A490 of the blank group) x 100.

In vitro cell invasion assay. A 24-well transwell unit (pore size, 8 µm; Costar; Corning Life Science, Woburn, MA, USA) was used to evaluate the ability of cell invasion. A total of 2x10⁵ transfected cells were seeded into the upper chamber coated with Matrigel (cat. no. 354248; BD Biosciences, Franklin Lakes, NJ, USA), and the bottom chamber was filled with DMEM with 20% FBS. After 24 h of incubation at 37˚C in a 5% CO₂ atmosphere, invasive cells on the bottom sides of the membrane were fixed with ethanol for 10 min and stained with 0.2% crystal violet for 20 min. Invasive cells were manually counted under a microscope (Leica Microsystems GmbH, Wetzlar, Germany) at a x20 objective in 4-5 random fields-of-view in each group.

MTT assay. At 48 h post-transfection, A549 and NCI-H460 cells from the two different groups (Hakai RNAi and NC RNAi) were seeded into 96-well plates at a density of 5,000-10,000 cells/well, with three replicate wells used per group. When the cells in each well reached 70-80% confluence, different concentrations of cisplatin (0, 5, 10 and 15 µM) were added followed by 48 h incubation. Next, 10 µl MTT (5 mg/ml) was added to each well for an additional 2-4 h. The medium was discarded and 150 µl DMSO was added to dissolve the formazan crystals for measurement. Subsequently, the optical density was measured at an absorbance wavelength of 490 nm using a microplate reader (Multiskan FC; Thermo Fisher Scientific, Inc.). The cell survival rate was calculated as: Cell viability (%) = (average A490 of the experimental group - average A490 of the blank group)/(average A490 of the control group - average A490 of the blank group) x 100.

Western blot analysis. Proteins from different cell lines in the different transfection groups were extracted using radioimmunoprecipitation assay buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM Na₃VO₄, 1 mM NaF and 1 mM PMSF (19). The protein levels were determined using Bradford Protein Assay kit (Beyotime Institute of Biotechnology). Proteins (25-50 µg) were then subjected to SDS-polyacrylamide gel electrophoresis (8 and 10%), and transferred to a nitrocellulose membrane (Pall Corporation, East Hills, NY, USA). Subsequent to blocking with 5% non-fat milk for 1 h at room temperature, the membranes were incubated overnight at 4˚C with the indicated primary antibodies, then washed with Tris-buffered salin containing 1% Tween-20 for 20 min, followed by incubation with the corresponding secondary antibodies for 2 h at room temperature. Detection was performed using an enhanced chemiluminescence substrate (CW0049M; Cwbiotech, Beijing, China). Bands intensities were quantified using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data were collected and analyzed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA), and are expressed as the mean ± standard deviation. Student’s t-test was used to compare data between two groups. One-way or two-way analysis of variance, followed by Sidak post hoc test, was used to analyze data of multiple groups. P<0.05 was considered to be an indicator of a statistically significant difference.

Results

Elevated expression of Hakai in NSCLC cell lines. Hakai expression was assessed by RT-PCR and western blot analysis in the NSCLC cell lines A549 and NCI-H460, as well as in human normal bronchial epithelial cells 16HBE and Beas-2B. The results demonstrated that Hakai mRNA was highly expressed in A549 and NCI-H460 cell lines compared with its expression in 16HBE and Beas-2B cells (Fig. 1A). Consistent with mRNA expression, western blot analysis revealed significantly higher protein expression levels of Hakai in A549 and NCI-H460 cell lines, with weaker expression observed in 16HBE and Beas-2B cells (P<0.0001; Fig. 1B and C). These results indicated that Hakai is overexpressed in NSCLC cells.

Downregulation of Hakai inhibits proliferation in A549 and NCI-H460 cells. In order to investigate the potential function of Hakai in NSCLC cell lines, A549 and NCI-H460 cells were transfected with siRNA against Hakai, and the results revealed that the two siRNAs were able to decrease the expression of Hakai (Fig. 2A). Thereafter, Hakai RNAi-1 was used for further experiments, owing to its higher effect. Hakai silencing was found to markedly inhibit the proliferation of A549 and NCI-H460 cells, and an enhanced inhibitory effect was observed when the incubation time increased, as demonstrated by trypan blue dye exclusion assay (P<0.05; Fig. 2B and C). Similarly, a flat colony formation assay indicated that Hakai knockdown in A549 and NCI-H460 cells led to a significant decrease in the colony numbers (P<0.01 and P<0.0001, respectively; Fig. 2D). These data demonstrated that Hakai serves a critical role in NSCLC cell proliferation.

Hakai depletion inhibits migration and invasion in NSCLC cells. Hakai is implicated in the regulation of cell substratum adhesions and in epithelial cell invasion (14). Thus, the present study next investigated whether Hakai was involved in the cell migration progress in NSCLC cells. When comparing with the cells of the NC and non-transfected control groups, the results of wound healing assay demonstrated that Hakai depletion led to a significant decrease of the migration ability of A549 and NCI-H460 cells transfected with Hakai RNAi (P<0.001 and P<0.01, respectively; Fig. 3A). A transwell invasion assay revealed a low level of invasion in Hakai RNAi cells compared with the NC and non-transfected control cells (P<0.01 and P<0.0001; Fig. 3B).

Hakai was firstly described as the E3 ubiquitin ligase for the E-cadherin and mediated its degradation (13). Thus,
Figure 1. Overexpression of Hakai in NSCLC cell lines. (A) Relative Hakai mRNA expression levels measured by reverse transcription-polymerase chain reaction. (B) Western blotting of Hakai protein in NSCLC cell lines (A549 and NCI-H460) and human normal bronchial epithelial cells (16HBE and Beas-2B). (C) Densitometric Hakai protein data were normalized to actin protein levels. The values are expressed as a ratio of the Beas-2B cells. ***P<0.0001, vs. Beas-2B cells; ****P<0.0001 vs. 16HBE cells (one-way analysis of variance). NSCLC, non-small-cell lung cancer.

Figure 2. Knockdown of Hakai inhibits proliferation in A549 and NCI-H460 cells. (A) A549 cells were transfected with the indicated siRNAs (100 nM) for 72 h, and the protein levels of Hakai were detected by western blotting. (B) A549 and (C) NCI-H460 cells in the three groups (non-transfected control, NC RNAi and Hakai RNAi) were examined by a trypan blue exclusion assay to determine the proliferation abilities. Upper panels indicate the expression levels of Hakai protein in the three groups, while lower panels indicate the results of trypan blue exclusion assay (two-way ANOVA). (D) Flat plate clone formation assay was used to detect the clonogenic activity of A549 and NCI-H460 cells at 48 h after transfection with NC or Hakai-specific siRNA. The left panel shows representative images, and the right panel shows the quantification of the number of colonies with >50 cells from three separate experiments (one-way ANOVA). *P<0.05, **P<0.01 and ***P<0.0001, vs. NC RNAi group. Con, non-transfected control; RNAi, RNA interference; NC, negative control; siRNA, small interfering RNA.
the current study explored whether downregulation of Hakai was able to regulate the expression levels of E-cadherin and N-cadherin in NSCLC cells. The results of western blot analysis demonstrated that Hakai depletion upregulated E-cadherin and downregulated N-cadherin in both A549 and NCI-H460 cells (Fig. 3C), which may contribute to the inhibitory effect of Hakai decrease on cell migration and invasion. These results indicate that Hakai influences cell migration and invasion in NSCLC cells.

Silencing of Hakai expression suppresses AKT activity in NSCLC cells. A growing body of evidence suggests that AKT perturbations serve an important role in human malignancy (20). Consistently, high activation of AKT has been reported in various types of human cancer, including NSCLC (21,22). Thus, whether Hakai inhibition regulated the activity of the AKT signaling pathway was examined in NSCLC cells. As shown in Fig. 4A and B, the western blot results revealed that downregulation of Hakai decreased AKT phosphorylation at the Ser-473 site in both A549 and NCI-H460 cells (Fig. 3C), which may contribute to the inhibitory effect of Hakai decrease on cell migration and invasion. These results indicate that Hakai influences cell migration and invasion in NSCLC cells.

Downregulation of Hakai sensitizes NSCLC cells to cisplatin treatment. Cisplatin resistance was associated with AKT overexpression and gene amplification in human lung cancer cells that acquired drug resistance (23). Next, the current study tested whether Hakai was involved in the sensitivity of NSCLC cells to cisplatin. The results demonstrated that silencing of Hakai potentiated the susceptibility to cisplatin in A549 and NCI-H460 cells (P<0.05; Fig. 5A and B). This finding indicates that the level of Hakai expression influences the sensitivity to cisplatin in NSCLC cells.
Discussion

Hakai was initially identified as a RING finger type E3 ubiquitin ligase for E-cadherin complex in 2002 (13). Since then, researchers have highlighted its crucial role in cell adhesion and invasion during carcinogenesis. Emerging evidence has also supported the critical role of Hakai in cell proliferation. Figueroa et al (15,16) reported that overexpression of Hakai increased the proliferation of MDCK stable cell lines, while transient knockdown of Hakai expression in McF-7 and HEK293 cells significantly decreased cell proliferation. The authors also demonstrated that Hakai was upregulated in colon and gastric cancer (16). In accordance with these observations, the present study found a significantly increased expression of Hakai in NSCLC cell lines, compared with that in normal bronchial epithelial cells 16HBE and Beas-2B. To further investigate the oncogenic potential of Hakai, the expression of Hakai was downregulated in vitro. The results revealed that the proliferation of NSCLC cells was inhibited by silencing the expression of Hakai. These findings suggest that Hakai may function as an oncoprotein in the development and progression of NSCLC.

The poor outcome of NSCLC is crucially correlated to the onset of tumor metastasis (24). Therefore, reducing cell invasiveness is a potential therapeutic strategy for attenuating the progression of NSCLC. Epithelial-mesenchymal transition (EMT) is a crucial step for cell metastasis, and has been reported to be associated with a poor clinical outcome in NSCLC (25,26). A characteristic of cells that undergo EMT is an increase of E-cadherin expression and a loss of N-cadherin expression (27,28). It was reported that Hakai overexpression enhances invasiveness and is implicated in several processes that often occur during EMT (14). In the present study, knockdown of Hakai was found to suppress NSCLC cell migration and invasion, accompanied by an E-cadherin increase and an N-cadherin decrease, indicating that Hakai regulates the invasive and metastatic ability of NSCLC cells, partially through regulation of EMT.

The molecular mechanisms involved in the oncogenic role of Hakai are largely unknown. Studies reported that there were two proteins influencing proliferation through Hakai, namely PSF and cyclin D1, which may function independently (15,16). Hakai can affect the oncogenic phenotype by increasing the ability of PSF to bind to RNAs that promote cancer-associated gene expression. Knockdown of Hakai specifically inhibited the expression of cyclin D1 in MCF-7, HEK293 and MDA-MB231 cells (16). In the current study, it was reported that Hakai knockdown decreased the pAKT (Ser^{473}) levels in A549 and NCI-H460 cells. Hyperactivation of AKT is detected in the majority of NSCLC cell lines, and certain studies have reported growth inhibition due to blockade of constitutive AKT activity in NSCLC cells (21,29,30). The current study has established a functional link between the pathways of Hakai and AKT, and provides a possible explanation for the oncogenic activity of Hakai in NSCLC. Further investigation is warranted to elucidate the molecular mechanism on how Hakai regulates the AKT signaling pathway.

Cisplatin is the cornerstone of lung cancer therapy; however, its efficacy is limited due to the development of drug resistance in cancer cells. Increasing studies have suggested AKT phosphorylation as a novel mechanism in promoting the resistance of tumor cells against cisplatin (21,23,31,32). As the current study results demonstrated that the protein levels of pAKT (Ser^{473}) were reduced by Hakai depletion, the therapeutic role of Hakai silencing in combination with cisplatin was then explored. To the best of our knowledge, it was revealed for the first time that Hakai downregulation sensitized NSCLC cells to cisplatin. The present data suggest that cisplatin chemotherapy may be more effective in combination with knockdown of Hakai for NSCLC therapy.

In conclusion, the present study reported that Hakai serves an important role in NSCLC progression by regulating the growth, migration and invasion of NSCLC cells. Mechanistically, the results revealed that the oncogenic profile of Hakai may be partially mediated through AKT-associated pathways.

Acknowledgements

Not applicable.
Funding

This study was supported by grants from the National Natural Science Foundation of China (grant nos. 81402511 and 81201577) and the Student Research Training Program of Anhui University of Technology (grant nos. 20150360171 and 20150242).

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LM designed the study. ZL and YW performed the experiments. LM and ZT analyzed the data. LM and ZL wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References