Kinesin family member 14 (KIF14) induces tumor cell proliferation in muscle invasive bladder cancer (MIBC)

Type
Research paper

Keywords
cell proliferation, clinical features, therapeutic target, Muscle invasive bladder cancer (MIBC), Kinesin family member 14 (KIF14)

Abstract
Introduction
Bladder cancer ranks the first in the morbidity of urogenital malignancies in China. Bladder cancers are pathologically classified into 2 sub-types, including non-muscle invasion bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC). MIBC is a highly lethal tumor and targeted therapies showed promising prospect for the treatment of MIBC. Novel therapeutic targets are still badly needed to combat this disease. The kinesin family member 14 (KIF14) is an engaging molecular motor and involved in multiple cellular processes such as cell division. Additionally, KIF14 is highly expressed in multiple tumor tissues and participates in the progression of several cancers such as gastric cancer and hepatocellular carcinoma. However, its possible role in the development of bladder cancer remains unclear.

Material and methods
Herein, 107 cases of MIBC tissue specimens were collected and detected by immunohistochemistry assays, and we analyzed the relationship between AKIF14 expression and clinical features. Then we used the cell line T24 and 5637 of bladder cancer into the experimental group transfected shKIF14 plasmid.KIF14, additionally, fascinated tumor growth of MIBC in mice.

Results
We demonstrated the high expression of KIF14 in tumor tissues from patients who underwent MIBC. Furthermore, KIF14 was statistically correlated with clinical feature, such as tumor stage (P = 0.001). Our results further confirmed the impairment of proliferation capacity after KIF14 ablation in vitro and in vivo.

Conclusions
In summary, we revealed KIF14 could serve as a promising therapeutic target for the treatment of MIBC.
Kinesin family member 14 (KIF14) induces tumor cell proliferation in muscle invasive bladder cancer (MIBC)

Hai-Tao Zhang, Zhao-Fei Liu, Heng Wang, Si-Nan Hou, Peng-Fei Mao, Rui-Zhi Nie, Xiang-Jun Xu*.

Department of Urology, Lianyungang TCM Hospital Affiliated to Nanjing University of Chinese Medicine, No.160 Chaoyang Middle Road, Haizhou District, Lianyungang 222000, Jiangsu Province, China. *Corresponding author. Email: xxjnju@qq.com.

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Abstract

Bladder cancer ranks the first in the morbidity of urogenital malignancies in China. Bladder cancers are pathologically classified into 2 sub-types, including non-muscle invasion bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC). MIBC is a highly lethal tumor and targeted therapies showed promising prospect for the treatment of MIBC. Novel therapeutic targets are still badly needed to combat this disease. The kinesin family member 14 (KIF14) is an engaging molecular motor and involved in multiple cellular processes such as cell division. Additionally, KIF14 is highly expressed in multiple tumor tissues and participates in the progression of several cancers such as gastric cancer and hepatocellular carcinoma. However, its possible role in the development of bladder cancer remains unclear. Herein, 107 cases of MIBC tissue specimens were collected and detected by immunohistochemistry assays, and we analyzed the relationship between AKIF14 expression and clinical features. We demonstrated the high expression of KIF14 in tumor tissues from patients who underwent MIBC. Furthermore, KIF14 was statistically correlated with clinical feature, such as tumor stage (P = 0.001). Then we used the cell line T24 and 5637 of bladder cancer into the experimental group transfected shKIF14 plasmid. Our results further confirmed the impairment of proliferation capacity after KIF14 ablation in vitro. KIF14, additionally, fascinated tumor growth of MIBC in mice. In summary, we revealed KIF14 could serve as a promising therapeutic target for the treatment of MIBC.

Key words: Muscle invasive bladder cancer (MIBC), Kinesin family member 14 (KIF14),
Introduction

Bladder cancer ranks the first in the morbidity of urogenital malignancies in China [1, 2]. Bladder cancer is biologically and clinically heterogeneous, and causes approximately 330,000 annual new cases and 130,000 annual deaths worldwide [3, 4]. Bladder cancers are classified into 2 sub-types according to its pathological features: about 80% of bladder cancers are non-muscle invasion bladder cancer (NMIBC), whereas the remaining 20% are muscle invasive bladder cancer (MIBC) [5, 6]. Nowadays, MIBC is still a highly lethal tumor with high recurrence rates [7]. In fact, despite improvement in therapeutic methods such as surgical techniques, the 5-year overall survival rate of MIBC is low [8, 9]. Recently, targeted therapies showed great promise for the treatment of MIBC [10]. A variety of different targeted drugs have shown good therapeutic effects [11, 12]. But given the high heterogeneity and high recurrence rate of MIBC, novel and effective therapeutic targets are still badly needed.

Kinesin proteins are known critical for multiple cellular processes such as cargo transport, microtubule dynamics and mitosis [13, 14]. The kinesin family member 14 (KIF14), as a member of this family and an engaging molecular motor, plays key roles in chromosomal segregation, alignment and cytokinesis during cell division [15, 16]. Interestingly, previous study indicated that KIF14 could tightly bind to microtubules so that to promote cell division [17]. Additionally, KIF14 mutations could lead to primary microcephaly, severe microcephaly and kidney development defects, suggesting its important function in development [18]. In addition to its effect on development, the potential roles of KIF14 on cancer progression has been widely reported [19-26].

Previous studies indicated that KIF14 served an oncogenic role in multiple tumors [19]. KIF14 is highly expressed in tumor tissues of multiple types of cancer, such as prostate cancer, medulloblastoma, and ovarian cancer [20-22]. KIF14 could promote cell proliferation and invasion of several types of cancers including gastric cancer, lung adenocarcinoma, and hepatocellular carcinoma [23-25]. Previous studies also indicated the oncogene effect of KIF14 in
breast cancer through negatively regulating Rap1a-Radil signaling [26]. Although a large number of studies have reported the roles of KIF14 in tumorigenesis and development, nevertheless, its possible role in the progression and metastasis of bladder cancer remains poorly defined.

Here, we assessed the potential role of KIF14 in the progression of MIBC. We revealed that KIF14 was highly expressed in human MIBC tissues and found the potential link between KIF14 expression and tumor stage of patients with MIBC. We also found that depletion of KIF14 markedly blocked MIBC cell proliferation and suppressed tumor growth in mice. According to our results, a novel and promising therapeutic target, KIF14, was provided for the treatment of MIBC.

Materials and methods

Ethics statement

All methods conducted in the present study and all the experimental protocols were approved by the Research Ethics Committee of Lianyungang TCM Hospital Affiliated to Nanjing University of Chinese Medicine (Jiangsu province, China).

Antibodies, primers and shRNA plasmids

Rabbit anti-KIF14 (for IHC assays, 1:200 dilution, for Immunoblot assays, 1:2000 dilution, #ab71155, Abcam, Cambridge, UK), Rabbit anti-Ki67 (for IHC assays, 1:100 dilution, for Immunoblot assays, 1:1000 dilution, #ab16667, Abcam, Cambridge, UK), Rabbit anti-proliferating cell nuclear antigen (PCNA) (1:500 dilution, #ab92552, Abcam, Cambridge, UK), Mice anti-β-actin (1:1000 dilution, #ab8226, Abcam, Cambridge, UK).

The quantitative PCR primer sequences targeted KIF14 were as follows: forward, 5′-CCGACATTACAGATGCACCA-3′ and reverse, 5′-CTTCATTCTAAGCCTACACC-3′; The quantitative PCR primer sequences of GAPDH were as follows: 5′-TGCACCACCACCTGCTTAGC-3′ and 5′-GGCATGGACTGTGGTCA TGAG-3′.

Ready-to-package AAV shRNA plasmids targeted KIF14 was purchased from the Addgene plc. The shRNA sequences targeted KIF14 were as follows: sense, 5′-TTCCTTCTTCCAAAAATAGTTCA-3′.

Human tissue samples and analysis
The 107 human MIBC and corresponding non-tumor tissue samples studied in this study were collected from the patients receiving surgical treatment in our hospital. The clinical–pathological characteristics, such as patient age, gender, and tumor stage, were listed in Table 1.

To explore the possible correlations between the expression levels of KIF14 and MIBC progression, immunohistochemical (IHC) assays were performed. Briefly, sample sections were fixed with 4% PFA for 30 minutes and subsequently blocked with 2% BSA for 30 minutes. Slides were then incubated with KIF14 and Ki67 antibodies at room temperature for 2 hours. Subsequently, the sections were incubated with biotinylated secondary antibody for 1.5 hours, and diaminobenzidine was used as a chromogen substrate.

KIF14 was found located in both the cytoplasm and nucleus of MIBC tissues. The scoring methods were as follows. In brief, the percentage of positive stained cells was graded as follows: 0 = 0% stained cells; 1 = 1–30% stained cells; 2 = 31–60% stained cells; 3 = 61–100% stained cells. The staining intensity was further evaluated on a score of 0 (no staining), 1 (low level staining), 2 (moderate level staining) and 3 (high level staining). The expression levels of KIF14 were examined based on the staining index: score of staining intensity + score of stained cells percentage. Staining index < 3 was considered relative low expression, while staining index 3 or > 3 was thought high expression.

Cell culture and transfection

The human MIBC cell lines, T24 and 5637, were bought from ATCC. T24 and 5637 cells were maintained in Dulbecco’s modified essential medium (DMEM) and RPMI-1640 medium, respectively, supplemented with 10% fetal bovine serum (FBS, Gibico, CA, USA) in a 5% CO₂ incubator.

The KIF14 shRNA plasmids were transfected into both T24 and 5637 cells using lipofectamine 2000 (#11668019, Invitrogen, CA, USA). KIF14 stably depleted-T24 cells was screened by its shRNA lentivirus infection and used for the tumor growth assays in vivo.

Quantitative PCR assay
Trizol (#15596026, Invitrogen, CA, USA) was used to extract total RNA from human MIBC cells. Subsequently the RNA was reverse-transcribed by M-MLV reverse transcriptase (#M1701, Promega, Wisconsin, USA).

Total mRNA was reverse transcribed to produce cDNA by cDNA synthesis system. Quantitative PCR was performed through a SYBR Ex Taq kit (#638319, Takara, Japan), and the KIF14 expression level was normalized to the expression of GAPDH.

**Immunoblot assays**

Tumor cells or tissues of MIBC were lysed in RIPA Buffer (#9800, Cell Signaling, Danvers, MA). Then the total proteins were analyzed by SDS-PAGE assays. Subsequently the polyvinylidene fluoride (PVDF) membranes were blocked with 5% milk buffer and then incubated with the primary antibodies for the detection of KIF14, Ki67, PCNA, and β-actin at room temperature for 2 hours. Then the PVDF membranes were incubated with HRP-conjugate secondary antibodies for 1 hour. Blots were detected with an ECL kit. Image Pro software was used in this assay to calculate the intensity of each blot.

**Colony formation assay**

Approximately 1000 cells were added into a 6-well culture plate and transfected with control or KIF14 shRNA plasmids and cultured at 37 °C. The medium was refreshed with fresh medium every 2 days. After 2 weeks, cells were fixed with PFA for 30 minutes and stained with 0.2% crystal violet at room temperature for 20 minutes and washed with PBS twice. Then the number of colonies was manually counted and analyzed.

**MTT assay**

T24 and 5637 cells were plated into 96-well plates with a density of about 500 cells each well, transfected with control or KIF14 shRNA plasmids and maintained for 48 hours. Cells were then incubated with MTT for 4 hours and removed the medium. Subsequently, cells were washed with PBS. Then 100-µL dimethyl sulfoxide (DMSO) was added into each well to extract the stained cells, and the absorbance value was measured with a microplate reader at 570 nm wave length.

**Tumor growth assays**
All animal assay processes were approved by our Institutional Animal Care and Use Committee (IACUC). Briefly, T24 cells were stably infected with control or KIF14 shRNA lentivirus. Subsequently, about $10^6$ T24 cells were subcutaneously implanted into athymic nude mice. After 14 days, tumors were isolated, photographed every 3 days. After 29 days, the growth curves were calculated and compared.

Statistics

GraphPad 5.0 software was used for statistical analysis in this study. All results were represented as mean ± standard deviation (SD). The correlations between clinical features and KIF14 expression levels were calculated using $\chi^2$ analysis. Student’ s t-test was used for statistical comparisons. * indicates $P<0.05$, which was also considered as a statistically significant difference.

Results

 Significant up-regulated of KIF14 expression was found in human MIBC tumor tissues, and KIF14 WAS correlated with the prognosis of patients with MIBC.

To assess the potential role of KIF14 in the progression of tumor, we first conducted bioinformatic analysis by the use of TCGA database (http://gepia.cancer-pku.cn/detail.php?gene=KIF14): Bioinformatic analysis provided the evidence that KIF23 mRNA level is enhanced in human bladder urothelial carcinoma (BLCA) tissues (n=404) compared to the normal tissues (n=28) (Fig. supplementary material A, $P< 0.05$), and it is correlated with the poor prognosis: KIF14 is associated with the disease-free survival (DFS) rate in samples with different numbers of cases (Fig. supplementary material B, $P=0.019 <0.05$, respectively). Then, to assess the potential role of KIF14 in the progression of tumor in China, KIF14 expression levels in MIBC tissues of patients who underwent surgical resection were examined through IHC assays. We initially found that KIF14 was mainly located in the cytoplasm of MIBC cells (Figure 1A). To further evaluate the effects of KIF14 in MIBC development, we detected the difference of KIF14 expression levels between MIBC tissues and the adjacent tissues.
through IHC assays. Consistent with our expectations, MIBC tissues showed relative high expression levels of KIF14 compared with adjacent tissues (Figure 1A, B).

According to the staining results, a total number of 107 tissue samples taken from MIBC patients who underwent surgical treatment were manually classified into KIF14 low and high expression groups (Figure 1A and table 1). Based on the staining intensity of KIF14 in tumor tissues, 32 patients showed low expression of KIF14, whereas 75 exhibited high KIF14 expression levels (Table 1). We subsequently assessed the clinical significance of KIF14 in MIBC patients. Clinical characteristics including patient age, gender, and tumor size, etc. were analyzed, respectively. However, no clinical significance was found in features such as patient age and gender between these two groups (Table 1). Interestingly, our results revealed that the expression level of KIF14 was markedly related with tumor stage ($P=0.01<0.05$) in MIBC patients (Table 1).

Collectively, the possible significance between KIF14 expression and tumor stage was found in MIBC patients.

**KIF14 contributes to tumor cell proliferation of MIBC in vitro.**

To further evaluate the mechanism underlying KIF14 promoting the progression of MIBC, the KIF14 shRNA plasmids were transfected into 2 types of human MIBC cell lines, T24 and 5637, to suppress its expression levels. Through quantitative PCR assays, we found that the transfection of KIF14 shRNA plasmids effectively blocked its expression in both T24 and 5637 cells (Figure 2A). Similarly, the results of Immunoblot assays further proved the obviously dropped expression levels of KIF14 in both T24 and 5637 cells transfected with its shRNA plasmids (Figure 2B).

Subsequently, colony formation assays were performed to examine the proliferation capacity of MIBC cells. We found that the KIF14 depletion effectively decreased colony number through colony formation assays (Figure 3A). Similarly, an obvious dropped absorbance value at 570 nm was detected in both T24 and 5637 cells through MTT assays (Figure 3B). Meanwhile, the expression levels of Ki67 and PCNA, two bio-markers reflecting proliferate capacity, were detected through Immunoblot assays. We found that knockdown of KIF14 resulted in the
Depletion of KIF14 inhibited tumor growth of MIBC in mice.

We then explored the possibility that KIF14 promoted tumor growth of MIBC through animal assays.

To confirm our hypothesis, T24 cells were infected with KIF14 or control shRNA lentivirus and subcutaneously injected into nude mice. After 2 weeks, the tumor was isolated, photographed, and the volume was measured every 3 days. After 29 days, all tumors are isolated and representative photographs were then shown (Figure 4A). The growth curve was also calculated and shown in Figure 4A. Interestingly, tumor volume in KIF14 depletion groups was markedly smaller than control (Figure 4A).

Further, IHC assays exhibited the effective silencing of KIF14 in tumor tissues from KIF14 shRNA lentivirus infected groups (Figure 4B). We measured the expression levels of both Ki67 and PCNA in tumor tissues from control and KIF14 knockdown groups by IHC assays. Interestingly, the significant decrease of Ki67 expression levels were detected in tumors from KIF14 knockdown groups, suggesting that proliferation capacity of tumor cells was obviously weaker after KIF14 knockdown (Figure 4C). In summary, the results of our in vivo assays indicated the potential involvement of KIF14 in tumor growth of MIBC in mice.

Discussion

In this study, we evaluated the possible correlation of KIF14 expression with pathological and clinical features in bladder cancer. We found that KIF14 was not only highly expressed in human tumor tissues of MIBC, and also correlated with tumor stage. We further showed here that depletion of KIF14 by its shRNA strongly inhibited proliferation of T24 and 5637 cells through colony formation and MTT assays. The potential role of KIF14 as a proliferation bio-marker is also evaluated by the obvious significance was found between KIF14 and Ki67, PCNA expression
levels. Similarly, we confirmed the anti-proliferation effects of KIF14 shRNA lentivirus in mice. Together, these in vitro and in vivo findings suggested that KIF14 could be a very promising target for MIBC therapeutic development, whereas the precise regulatory mechanism of KIF14 in MIBC progression needs further study.

To our knowledge, this is the first link between KIF14 expression and the progression of bladder cancer. As a molecular motor, KIF14 was widely reported in the progression and development of multiple cancers [20-26]. KIF14 expression level is a predictor of the prognosis of breast cancer and ovarian cancer [22, 26]. Another study also indicated that KIF14 contributes to AKT phosphorylation in triple-negative breast cancer (TNBC) [23]. KIF14, notably, promotes tumor cell proliferation and invasion of breast cancer through the Rap1a–Radil signaling pathway [26]. We here reported KIF14 promoted cell proliferation of bladder cancer, and whether through Rap1a–Radil or AKT signaling pathways need study in future. In addition, previous studies also reported that KIF14 suppressed tumor growth and metastasis in lung cancer through recruiting adhesion molecules to cell membrane and modulating cell adhesive and invasive properties [24]. Whether KIF14 induces MIBC metastasis through similar manner is worth further exploration.

As we know, KIF14 plays important roles in cell cycle progression and cell division, and the inhibition of KIF14 expression could lead to a number of human diseases including malignancies [25]. KIF14, as a member of kinesin family, was firstly cloned in 1994 and characterized by C-terminal citron kinase binding domain and N-terminal motor domain, therefore KIF14 has the capacity to bind microtubules and affect cell division [27]. Here we found that KIF14 fascinated cell proliferation of bladder cancer, possibly through its role in cell division. Similarly, there was also study indicating that KIF14 expression obviously varied during the cell cycle, with peak expression in S phase, and its depletion led to distinct phenotypes depending on the degree of ablation of KIF14 [25]. We next should detect the effects of KIF14 depletion on cell cycle, which perhaps explain the proliferation defects caused by KIF14 knockdown.

Except for KIF14, multiple members of kinesin family are involved in tumorigenes and could serve as prognostic predictor of several types of cancers [28]. High expression of KIF22 and KIF15 could be poor prognostic factors in patients with prostate cancer and lung adenocarcinoma, respectively [29, 30]. In addition, KIF20A is correlated with unfavorable clinical outcome and the
progression of epithelial ovarian cancer [31]. KIF26B depletion could suppress cell proliferation, migration and invasion of breast cancer [32]. Recently, we can see the similar published articles [33,34]. These studies, together with our findings in this study, indicate the key roles of kinesins on tumorigenesis and development, and suggest that the development of inhibitors targeted kinesins could be a promising prospect in future.

Collectively, our data indicated the high expression levels of KIF14 in human MIBC tumor tissues. We investigated the possible link between KIF14 expression levels and clinical characteristics of MIBC patients. Also, KIF14 fascinates cell proliferation of cancer in vitro and promoted tumor growth of MIBC in mice. We therefore provided a mechanically exploration of KIF14 in the progression of MIBC and a novel molecular target for MIBC.

References


Figure legends

Figure 1: KIF14 was highly expressed in human MIBC tissues. (A) Immunohistochemical assays were performed, and the representative photographs of KIF14 in human MIBC tissues were taken and shown (100× and 200× magnification, respectively). (B) Immunohistochemical staining showed the expression levels of KIF14 in non-tumor tissues (100× and 200× magnification, respectively).

Figure 2: KIF14 expression levels were significantly blocked in both T24 and 5637 human MIBC cells after KIF14 knockdown. (A) Quantitative PCR assays revealed the obviously decreased expression levels of KIF14 following the transfection of its shRNA plasmids in T24 and 5637 cells, respectively. (B) Immunoblot assays showed the efficiently inhibition of KIF14 expression after the transfection of KIF14 shRNA plasmids in both T24 and 5637 cells. Results are presented as mean ± SD, * P < 0.05.

Figure 3: KIF14 promotes cell proliferation of MIBC in vitro. (A) T24 and 5637 cells transfected with control or KIF14 shRNA plasmids, and colony formation assays were then performed. (B) The results of MTT assays exhibited the decreased cell proliferation capacity caused by KIF14 depletion. (C-D). Immunoblot assays revealed Ki67 and PCNA expression levels in the indicated shRNA treated T24 and 5637 cells, respectively. Results are presented as mean ± SD, * P < 0.05.
Figure 4: KIF14 induces tumor growth of MIBC in mice. (A) T24 cells infected with control or KIF14 shRNA lentivirus were subcutaneously implanted into nude mice. After 2 weeks, tumors were isolated, and the volume of tumor was measured every 3 days. (n=5 in each group). After 29 days, tumor growth curves were calculated and analyzed based on the average volume of 5 tumors in KIF14 knockdown and control groups. (B). IHC assays revealed the expression levels of KIF14 in control or KIF14 depletion tumors isolated from mice. (C). IHC assays showed the expression levels of Ki67 in control or KIF14 ablation tumors isolated from mice. Results are presented as mean ± SD, * P < 0.05.

Figure supplementary material Bioinformatic analysis indicate that KIF14 is enhanced in BLCA patients and correlated with the prognosis. (A) KIF14 expression level in BLCA tissues and normal tissues was analyzed from bioinformatic analysis. (For tumor tissues and normal tissues, n=404 and 28 respectively, from TCGA dataset). (B, C) KIF14 expression was correlated with the DFS of BLCA patients in different numbers of cases. * P < 0.05.
Table 1. Relationships of KIF14 and clinicopathological characteristics in 107 patients with muscular invasive bladder cancer

<table>
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A

Tumor volume (mm³)

control
shRNA

Days after injection

B

KIF14 expression

control shRNA

C

Ki67 expression

control shRNA

* denotes statistically significant difference.