Abstract. Feline sarcoma-related protein (Fer) is a type of nuclear and cytoplasmic non-receptor protein tyrosine kinase, which is associated with the progression of numerous types of cancer. Previously, we identified that Fer is associated with the migration and invasion of bladder cancer. The present study aimed to investigate the role of Fer in bladder cancer cell viability and apoptosis. Reverse transcription-quantitative polymerase chain reaction and western blot analysis were performed to detect the expression levels of Fer; short interference RNA (siRNA) and overexpression vectors were used to downregulate or upregulate Fer expression, respectively. The effects on cell proliferation ability and cell apoptosis were then tested by MTT assay and flow cytometry. The results revealed that Fer expression was upregulated in bladder cancer cell lines. Downregulation of Fer expression by siRNA significantly suppressed T24 cell viability and induced apoptosis, as well as inducing cell cycle arrest. Conversely, Fer overexpression in 5637 cells significantly promoted cell viability and cell cycle progression, but inhibited cell apoptosis. Furthermore, the suppression and overexpression of Fer significantly altered the expression of cleaved caspase-3 and Bcl-2, and dysregulated the P38 mitogen-activated protein kinase signaling pathway. The findings of the present study indicate a possible molecular mechanism of Fer in bladder cancer and may be considered as a potential target in the treatment of this disease.

Introduction

Bladder cancer is one of the most common malignant tumors of the genitourinary system worldwide (1). Researchers have identified that 75-85% of patients with bladder cancer have non-muscle-invasive bladder cancer (NMIBC), while 15-25% of cases progressed to muscle-invasive bladder cancer (MIBC) (1). At present, radical cystectomy with urinary diversion is the standard treatment for patients with NMIBC and MIBC (2); however, ~50% of patients with MIBC develop metastatic disease, which is likely to be fatal. The prognosis following recurrence after cystectomy is poor (3). Therefore, in order to gain a comprehensive understanding of the pathogenesis of bladder cancer, the molecular mechanisms underlying the occurrence and development of this disease must be identified. This may provide insight into novel and effective treatment strategies for the treatment of bladder cancer.

Feline sarcoma-related protein (Fer) is a unique Src homology 2 non-receptor tyrosine kinase, which is expressed in certain mammalian cell subpopulations, and resides in the cytoplasm and nucleus (4). Fer is highly expressed in numerous types of cancer, including lung (5), hepatic (6), prostate (7), breast (8) and bladder cancer (9). Previous studies have demonstrated that Fer expression is associated with the proliferation of certain cancer cell lines cultures; the poor prognosis of cancer has been associated with increased Fer expression levels (10-12). In addition, Fer is involved in the signaling downstream of the receptor systems of cell proliferation and invasion in several cell types (13). These studies indicate a potential function of Fer in the progression of cancer; however, the exact roles and underlying mechanisms of Fer in the proliferation and apoptosis of bladder cancer remain to be fully elucidated. To the best of our knowledge, the present study is the first to determine the role of Fer in the viability and apoptosis of bladder cancer cells.

In the present study, the effects of transfection of bladder cancer cells with short interfering RNA against Fer (Fer-siRNA) and Fer overexpression vector (Vector-Fer) on the expression of Fer mRNA and protein. Furthermore, the underlying mechanism of Fer in the proliferation and apoptosis of bladder cancer cells was investigated. The results of this study demonstrated that Fer serves a role in development of the bladder cancer.
Materials and methods

Cell line culture and maintenance. Bladder cancer cell lines T24, 5637 and an immortalized normal human epithelial cell line SV-HUC-1 were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). T24 and 5637 cell lines were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C under 5% CO2 and 95% humidified air. SV-HUC-1 cells were cultured under an atmosphere of 5% CO2 at 37°C in F12k medium (F12K; WISENT Inc., Saint-Jean-Baptiste, QC, Canada) with 10% fetal bovine serum (PAAB Laboratories; GE Healthcare, Chicago, IL, USA), 100 U/ml penicillin, and 100 lg/ml streptomycin.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from T24, 5637 and SV-HUC-1 cells (2x106 cells/ml) by RNAiso Plus (Takara Bio, Inc., Osu, Japan), according to the manufacturer's protocols, and 5 µg of each sample was reverse-transcribed using the M-MLV First-strand Synthesis System (Promega Corporation, Madison, WI, USA) as follows: 37°C for 25 min, followed by incubation at 85°C for 5 sec in 20 µl of reaction volume. All reactions were performed in triplicate using the MJ Real-Time PCR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). qPCR was performed using the Power SYBR Green Master Mix (Takara Bio, Inc.) and an ABI 7300 real-time PCR detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the following primers (all primers were synthesized by Invitrogen; Thermo Fisher Scientific, Inc.): Fer, forward 5'-TTCGAGGGCACTGGGTCTTCTC-3', reverse 5'-TTCCCT TCAGCCATTAATCTCTC-3'; GAPDH, forward 5'-GGTGAA GTCTGGAGTCAACGGA-3', reverse 5'-GAGGAGTCTCCG TCCTGGAAPGA-3'; GAPDH served as an internal control. qPCR was performed under the following thermocycling conditions: 96°C for 2 min; followed by 21 cycles of 96°C for 30 sec, 55°C for 30 sec, 68°C for 30 sec, and a final elongation at 68°C for 30 sec. The relative levels of individual mRNA in each sample were normalized to GAPDH and calculated using the 2-∆∆cq method (14).

Western blotting. Cells (5x105 cells/ml) were harvested 72 h following infection and lysed in Radioimmunoprecipitation Assay buffer (Fermentas; Thermo Fisher Scientific, Inc.) supplemented with 1% protease inhibitors (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) on ice, followed by centrifugation at 12,000 x g for 15 min at 4°C. Protein concentrations were determined with the Bicinchoninic Protein assay (Pierce; Thermo Fisher Scientific, Inc.). Subsequently, proteins were diluted to equal concentrations (20 or 30 mg), boiled for 5 min and separated by 7.5-10% SDS-PAGE, followed by transblotting to an Immobilon-Blot polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc.). The membranes were blocked with 5% defatted milk in TBS + 0.1% Tween-20 and probed with primary antibodies overnight at 4°C. Membranes were subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. Protein bands were visualized using an Enhanced Chemiluminescence Detection kit (GE Healthcare). The following rabbit monoclonal antibodies were used: Anti-Fer (1:500; catalog no. ab191060; Abcam, Cambridge, UK); anti-Bcl-2 monoclonal antibody [1:500; catalog no. 4223; Cell Signaling Technology (CST), Inc., Danvers, MA, USA]; CyclinD1 (1:500; catalog no. 2978; CST); p21(1:500; catalog no. 2947; CST); cleaved Caspase-3 (1:500; catalog no. 9661; CST); phosphorylated (p)-p38 MAPK (Thr180/Tyr182) (1:500; catalog no. 4511; CST); Rabbit anti-GAPDH polyclonal antibody (1:500; catalog no. Ab9485; Abcam) was used as an internal control protein. Densitometric analysis to quantify protein expression levels was performed using ImageJ software v1.46 (National Institutes of Health, Bethesda, MD, USA).

Small interfering RNA (siRNA) transfection. siRNAs targeting Fer and a negative control (NC) siRNA were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The targeting sequences of three Fer-siRNAs (siRNA1, 5'-AAAA GAAATTTATGGCCTTAG-3'; siRNA2, 5'-CATAGATAG CCTAGTACAGAA-3'; siRNA3, 5'-AACTACGGTTCCTGG AGACAG-3') and one NC-siRNA (5'-UUUCUCCGAACGU GCACGU-3') were designed using an RNAi algorithm available online at The RNAi Web (http://www.rnaieweb.com). For transfection, the siRNAs (100 nM/l) were transfected into T24 using Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. T24 cells (4x105 cells/cm2) were grown in regular medium for 72 h at 37°C and subsequently transfected with siRNAs, control cells were transfected with the NC siRNA. Cells were transfected at 37°C for 48 and 72 h at which time they were harvested for RNA and protein extraction, respectively.

Generation of plasmid constructs and establishment of Fer overexpression cell lines. To generate Fer overexpression vectors, Fer coding sequences were obtained by RT-PCR (primers: Forward 5'-TTCGAGGGCACTGGGTCTTCTC-3', reverse 5'-TTCCCT TCAGCCATTAATCTCTC-3'; vector: Fer coding sequence was obtained by rT-Pcr (Takara Bio, Inc.) and was cloned into the pEGFP-N1 plasmid (Clontech Laboratories, Inc., Mountainview, CA, USA). The resulting plasmid was designated as pEGFP-N1-Fer and was transfected into 5637 bladder cancer cells to induce Fer overexpression. 5637 cells were plated in regular medium at a density of 1x104 cells/cm2 for 72 h at 37°C and then transfected with pEGFP-N1-Fer; pEGFP-N1 empty vector was used as a control, and the resulting cell lines were designated as 5637/pEGFP-N1-Fer and 5637/pEGFP-N1, respectively. After 24 h post-transfection, G418 solution was added to cells for the selection of stable clones, which were then cultured in medium containing G418. Both 5637/pEGFP-N1-Fer and 5637/pEGFP-N1 cells were maintained in fresh regular medium for 2 days and then harvested for cell number counting.

MTT assay. Bladder cancer cells were seeding (5x103 cells/well) in flat-bottomed 96-well plates. After 24 h, cells were transfected for 1, 2, 3 or 4 days, as aforementioned. Following culture, 10 µl MTT (5 mg/ml) was added to each well and plates were incubated at 37°C for 4 h. The medium was removed and 100 µl dimethyl sulfoxide solution was added to each well to dissolve the purple formazan crystals. Absorbance was
measured at 490 nm using a microplate reader to determine cell viability; three replicate wells were analyzed per assay and each experiment was repeated three times.

**Cell cycle and apoptosis assays.** Transfected cells (3x10⁵ cells/well) were harvested using 0.25% trypsin at 37°C for 30 min, and were subsequently added to 1 ml of 70% cold ethanol overnight at 4°C. The next day, cells were centrifuged at 12,000 x g for 5 min at room temperature. The medium was removed, and cells were washed once in PBS. Cells were resuspended in 500 µl annexin V binding buffer, and 5 µl annexin V-FITC and 10 µl PI were added; cells were incubated for 15 min at room temperature in the dark and analyzed by flow cytometry (Beckman Coulter, Inc., Brea CA, USA) with Cell Quest software v5.1 (BD Biosciences, San Jose, CA, USA).

Annexin V-fluorescein isothiocyanate (FITC)/PI Apoptosis Detection kit (catalog no. KGA108; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) at 4°C for 30 min. The percentage of cells in G0/G1, S and G2/M phase was determined by DNA flow cytometry with Cell Quest software v5.1 (BD Biosciences, San Jose, CA, USA).

**Statistical analysis.** SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analysis. All the experiments were repeated three times independently and data are presented as the mean ± standard deviation. Statistical significance was compared between the treatment and controls groups using the one-way analysis of variance followed by a
Results

**Fer expression in bladder cancer cell lines.** The mRNA and protein expression levels of Fer in SV-HUC-1, 5637 and T24 cells were examined by RT-qPCR and western blot analysis, respectively. The expression levels of Fer mRNA and protein were significantly upregulated in the two bladder cancer cell lines (5637 and T24) compared with expression in the SV-Huc-1 normal bladder epithelium cell line (Fig. 1a and B; \( P<0.05 \)). Furthermore, the expression of Fer in the highly invasive and mesenchymal-like bladder cancer T24 cells was significantly higher compared with in 5637 cells, and these two bladder cancer cell lines were used in subsequent experiments to determine the biological roles of Fer.

**Efficacy of siRNA and overexpression plasmid transfection of Fer in bladder cancer cells.** To investigate the biological function of Fer in the progression of bladder cancer, T24 cells were transfected with one of three siRNAs against Fer (siRNA1, siRNA2 or siRNA3), whereas 5637 cells were transfected with a Fer overexpression plasmid; NC-siRNA and empty vectors were used as the respective negative controls. RT-qPCR results demonstrated that the relative Fer mRNA expression levels in the Fer-siRNA1 and Fer-siRNA2 groups were significantly decreased compared with the NC-siRNA group (\( P<0.01 \); Fig. 1C); similar results were observed for Fer protein levels (\( P<0.01 \); Fig. 1D). However, no significant differences in expression levels were observed in the Fer-siRNA3-transfected cells compared with the NC-siRNA group. Conversely, Fer overexpression in 5637 cells by plasmid transfection. As demonstrated by RT-qPCR and western blot analysis, the expression of Fer was significantly increased compared with the untreated control and the empty vector group (\( P<0.05 \); Fig. 1E and F).

**Effects of down- or upregulation of Fer on cell viability and the cell cycle.** To investigate the role of Fer on the viability of bladder cancer cells, the viability of T24 and 5637 cells transfected with Fer-siRNAs or Vector-Fer was determined by an MTT assay at days 1, 2, 3 and 4 post-transfection. The results demonstrated that the viability of Fer-siRNA-transfected T24 cells was significantly decreased compared with that of NC-siRNA-transfected cells (\( P<0.01 \); Fig. 2A); the viability of
Vector-Fer-transfected 5637 cells was significantly increased compared with that of the control and empty vector groups at day 4 (P<0.01; Fig. 2B). Furthermore, flow cytometric analysis was conducted to determine the potential mechanism underlying the effects of Fer on bladder cancer cell viability and cell cycle following Fer knockdown and overexpression. As presented in Fig. 2C and D, Fer-siRNA significantly increased the proportion of cells in G0/G1 phase and reduced the proportion in S phase compared with n c-sirna-transfected cells. Conversely, treatment with Vector-Fer significantly reduced the proportion of cells in G0/G1 phase and elevated the number of cells in G2/M phase compared with the controls. Furthermore, the proportion of Vector-Fer-transfected cells at S phase was significantly decreased compared with the control and empty vector groups (P<0.05). These results indicated that Fer may be closely associated with cell viability and the cell cycle in bladder cancer cells.

**Effects of down- or upregulation of Fer on cell apoptosis.** To further study the effects of Fer on the apoptosis of bladder cancer cells following transfection an Annexin V/FITC kit and flow cytometry were employed. As presented in Fig. 3A and B, the apoptotic rate of T24 cells in the Fer-siRNA group was significantly higher compared with the Nc-siRNA group (P<0.05). However, the apoptotic rate of the Vector-Fer group of 5637 cells was significantly lower compared with the control and empty vector groups (P<0.05). The results indicated that downregulation of Fer promoted the apoptosis of T24 cells, while overexpression of Fer inhibited 5637 cell apoptosis.

Bcl-2 family proteins regulate cell apoptosis through promoters or inhibitors (15). Cleaved caspase-3, as a prognostic predictor involved in the ‘execution’ phase of apoptosis, is a key regulator of promoting tumor repopulation induced by dying cells (16). Therefore, the expression of Bcl-2 and cleaved caspase-3, key mediators of apoptosis, was investigated. As presented in Fig. 3C and D, downregulated Fer decreased the expression of Bcl-2 and increased the expression of cleaved caspase-3 in T24 cells. GAPDH was used as a loading control. All experiments were repeated three times; n=3; *P<0.05 vs. NC-siRNA, Empty vector and/or Control. Bcl-2, B-cell lymphoma 2; Fer, feline sarcoma-related protein; FITC, fluorescein isothiocyanate; PI, propidium iodide; NC, negative control; siRNA, small interfering RNA; Vector-Fer, Fer overexpression plasmid.
and downregulated that of cleaved capsase-2 compared with the controls.

**Effects of down- or upregulation of Fer on P38 MAPK phosphorylation.** P38 MAPK signaling is one of the main pathways undertaken by MAPK, and serves a key role in regulating cell apoptosis, growth, differentiation and oncogenic transformation (17). Therefore, the effects of Fer down- or upregulation on the P38 MAPK signaling pathway were investigated. As presented in Fig. 4A and B, a significant decrease in the expression of p-P38 MAPK following Fer knockdown in T24 cells compared with in the nc-sirna group was observed. Conversely, overexpression of Fer significantly upregulated the expression of p-P38 MAPK in 5637 cells compared with the controls; however, the levels of total P38 MAPK were markedly unaffected in either bladder cancer cell group. These findings suggested that Fer may regulate cell proliferation and apoptosis via P38 MAPK modulation.

**Discussion**

Previously, several studies have reported that Fer is widely expressed in proliferating mammalian cells (18,19). Overexpression of Fer has also been associated with poor prognosis in various types of human cancer and serves as a prognostic marker (5,7-9); however, the biological role and underlying mechanism of Fer in bladder cancer cell viability and apoptosis require further investigation. In the present study, to reveal the biological effects of Fer on cell viability and apoptosis in bladder cancer, the transfection of bladder cancer cells with sirna or plasmid was conducted to knock down or overexpress Fer, respectively. The effects of downregulation or upregulation of Fer on bladder cancer cell viability and apoptosis were determined. The results revealed that Fer siRNA suppressed the viability and G1/S transition of T24 cells. This resulted in cell cycle arrest at G1, which may explain the inhibition of cell growth induced by Fer knockdown in T24 cells (21). In addition, Annexin FITC/PI staining was used to observe the rate of apoptosis; cell death is the process by which aged or damaged cells are eliminated and serves a key role in carcinogenesis (22). The present study reported that compared with nc-sirna-transfected cells, the fluorescence intensity of Fer‑siRNA cells increased significantly, indicating that Fer knockdown could induce the apoptosis of T24 bladder cancer cells. Overexpression of Fer in 5637 cells exhibited opposing effects on cell viability, cell cycle and apoptosis in vitro. Bcl-2 is a member of the regulatory Bcl-2 protein family (15). Overexpression of Bcl-2 is associated with cell cycle arrest (23), and the inhibitory effects of Bcl-2 could increase the rate of apoptosis (24,25). Bcl-2 also increases the expression levels of activated caspase-3 (cleaved caspase-3), which is a key mediator of programmed cell death (16). Therefore, the present study also investigated the association between the activity of Fer, and the expression of the Bcl-2 and caspase-3. Fer knockdown downregulated tissues (9). These results indicate that Fer may be involved in the progression of bladder cancer.

A recent report has indicated that Fer activation is required in tumorigenesis and the invasiveness of certain cancer cells in which C-Src is upregulated (20); however, the molecular mechanisms of Fer remain unknown. In the present study, to reveal the biological effects of Fer on cell viability and apoptosis in bladder cancer, the transfection of bladder cancer cells with siRNA or plasmid was conducted to knock down or overexpress Fer, respectively. The effects of downregulation or upregulation of Fer on bladder cancer cell viability and apoptosis were determined. The results revealed that Fer siRNA suppressed the viability and G1/S transition of T24 cells. This resulted in cell cycle arrest at G1, which may explain the inhibition of cell growth induced by Fer knockdown in T24 cells (21). In addition, Annexin FITC/PI staining was used to observe the rate of apoptosis; cell death is the process by which aged or damaged cells are eliminated and serves a key role in carcinogenesis (22). The present study reported that compared with nc-sirna-transfected cells, the fluorescence intensity of Fer‑siRNA cells increased significantly, indicating that Fer knockdown could induce the apoptosis of T24 bladder cancer cells. Overexpression of Fer in 5637 cells exhibited opposing effects on cell viability, cell cycle and apoptosis in vitro. Bcl-2 is a member of the regulatory Bcl-2 protein family (15). Overexpression of Bcl-2 is associated with cell cycle arrest (23), and the inhibitory effects of Bcl-2 could increase the rate of apoptosis (24,25). Bcl-2 also increases the expression levels of activated caspase-3 (cleaved caspase-3), which is a key mediator of programmed cell death (16). Therefore, the present study also investigated the association between the activity of Fer, and the expression of the Bcl-2 and caspase-3. Fer knockdown downregulated...
Bcl-2 and upregulated cleaved caspase-3 expression in T24 cells. The overexpression of Fer in 5637 cells had opposing effects, which may explain the inhibition of the cell cycle and apoptosis. Therefore, these findings indicate that Fer serves an important role in the biological behavior of T24 cells, which is mediated by regulating the expression of certain genes.

Additionally, present study reported that knockdown or overexpression of Fer disrupted the MAPK signaling pathway by altering the expression of phosphorylated p38 MAPK, whereas the expression of total p38 MAPK protein did not notably change. Senis et al (26) revealed that many small G protein/MaPK cascades are involved in downstream signal transduction of FPS/FES tyrosine kinase. Craig and Greer (27) also revealed that Fer kinase is required for sustained p38 kinase activation and the maximal chemotaxis of activated mast cells. Of note, p38 MAPK is activated via sequential phosphorylation in the MAPK signaling pathway (28). Therefore, the results of the present study suggest that Fer may affect cell viability and apoptosis via the P38 MAPK signaling pathway.

In conclusion, these findings contribute to the increasing evidence that Fer is involved in the development and progression of cancer. To the best of our knowledge, the present study is the first to report of Fer as a novel regulator of cell viability and apoptosis in bladder cancer. Fer was indicated to exert its effects by regulating the expression of certain genes and inhibiting the p38 MAPK signaling pathway. In addition, the association between Fer, and viability and apoptosis of bladder cancer cells was determined. Future investigation into the association between phosphorylated P38 and apoptosis is under way. Collectively, the results of the present study indicated that Fer serves a role in the biological behavior of bladder cancer cells, and suggest that Fer may be considered as a novel molecular target for the treatment of bladder cancer.

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Availability of data and materials

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

Authors' contributions

XH, DY and CZ conceived and designed the study. ZG, JX and XH performed cell culture and cell transfection. XM and XH performed reverse transcription-quantitative polymerase chain reaction, western blotting, MTT assay and flow cytometry. MB, FJ and XH provided reagents and interpreted the data. XH performed data analysis and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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