JBUON 2018; 23(3): 782-786
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Effects of human enhancer of filamentation 1 (HEF1) gene on the proliferation, invasion and metastasis of bladder cancer cells

Zhiling Tang

Department of Urology, Jiaxing Second Hospital, Zhejiang, China

Summary

Purpose: *To investigate the effects of human enhancer of filamentation 1 (HEF1) gene on the proliferation, invasion and metastasis of bladder cancer cells.*

Methods: *Three human bladder cancer cell lines (T24, EJ and BIU-87) were selected to extract total RNA at logarithmic growth phase. The relative expression level of HEF1 in these cell lines was detected by semi-quantitative reverse transcription PCR (RT-PCR), and the cell lines with relatively high expression and relatively low expression level of HEF1 cells were identified. HEF1 overexpression recombinant adenovirus was transfected into the bladder cancer cells with low expression level of HEF1, and HEF1 siRNA was transfected into the bladder cancer cells with high expression level of HEF1. MTT assay, migration assay and invasion assay were performed to detect the proliferation, migration and invasion of bladder cancer cells.*

Results: *The relative expression level of HEF1 mRNA in T24 cell line was significantly higher than that in EJ and BIU-87 cells, and BIU-87 cell line showed the lowest expression level ($p<0.05$). After transfection with HEF1 overexpression recombinant adenovirus, the proliferation, migration and invasion of BIU-87 cells were significantly improved ($p<0.05$). After siRNA silencing, the proliferation, migration and invasion of T24 cells were significantly inhibited ($p<0.05$).*

Conclusion: *High expression level of HEF1 gene can promote the proliferation, invasion and metastasis of bladder cancer cells.*

Key words: *bladder cancer cell, human enhancer of filamentation 1, invasion, migration, proliferation*

Introduction

Bladder cancer is the most common type of malignant tumor of the urinary system with high recurrence rate. About 10% of patients with superficial bladder cancer will develop invasive carcinoma or metastases [1]. At present, the most common treatment for bladder cancer is radical surgery-based comprehensive therapy, but this method failed to significantly improve the survival of patients [2]. The main causes of death of bladder cancer patients is metastasis and tumor recurrence [3], which are closely correlated with multiple genes, and multi-level cell signal network disorders [4].

HEF1, also known as Cas-L, is a cytoskeleton protein belonging to CAS (Crk-associated substrate) family. HEF1 plays an important role in cell signaling, which can connect external stimulating signals with downstream effector molecules, including molecules that can promote tumor invasion and metastasis [5]. In one of our previous studies, expression of HEF1 in 180 cases of bladder cancer tissues was detected using tissue microarray, and we found that HEF1 overexpression was significantly associated with tumor invasion depth and distant metastasis [6]. In addition, HEF1

Correspondence to: Zhiling Tang, PhD. Department of Urology, Jiaxing Second Hospital, 1518 Huanchengbei Road, Nanhu District, Zhejiang, 314000 China.
Tel: +86 13567323406, E-mail: tcq4gs@163.com
Received: 04/12/2017; Accepted: 21/12/2017

gene plays an important role in the development and progression of various malignant tumors such as hepatocellular carcinoma [7], colon cancer [8] and melanoma [9]. Based on this information, this study aimed to analyze the role of HEF1 gene in the regulation of bladder cancer cell proliferation, invasion and metastasis at both protein and cell levels.

Methods

Experimental materials

Three human bladder cancer cell lines (T24, EJ and BIU-87) and normal human bladder epithelium cell line SV-HUC-1 were purchased from the cell bank of Chinese Academy of Sciences. After routine resuscitation, primary and subcultures cells were collected during the logarithmic growth phase. Human embryonic kidney 293 cell line (HEK293) was purchased from Sangon (Shanghai, China) and adenoviral vectors were purchased from Sigma (St. Louis, MO, USA).

Main reagents and instruments purchased

RIPM1640 medium containing 10% fetal bovine serum (FBS) (Beyotime Biotechnology, Nanjing, China), Trizol reagent (Sigma, St. Louis, MO, USA), Reverse transcription kit (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.), Perv polymerase (Invitorgen, USA), Adenopure kit (Sigma, St. Louis, MO, USA), MTT and DMSO solutions (Hyclone, Logan, Utah, USA), CO_2 incubator (Beijing Liuyi Instrument Factory), Pipettes (GE, Fairfield, Connecticut, USA), 96-well cell culture plates and Transwell chambers (Bio-Rad Corporation, Hercules, California, USA), UV spectrophotometer (Applied Biosystems, City Foster, California, USA), PCR amplification and electrophoresis equipment (R&D, Minneapolis, MN, USA), microplate reader (Applied Biosystems, City Foster, California, USA).

Research methods

The relative expression level of HEF1 in these cell lines was detected by semi-quantitative RT-PCR to screen the cell lines with relatively high expression level and relatively low expression level of HEF1. Bladder cancer cells with low expression level of HEF1 were transfected with HEF1 overexpression recombinant adenovirus, and bladder cancer cells with high expression level of HEF1 were transfected with HEF1 siRNA. MTT assay, migration assay and invasion assay were performed to detect the proliferation, migration and invasion of bladder cancer cells.

RT-PCR

Total RNA was extracted using Trizol reagent and quality of RNA samples was determined by UV spectrophotometer. Reverse transcription kit was used to synthesize cDNA. Primers used in PCR reaction were: 5'-CGCGAGAAGATGACCCAGAT-3'(forward) and 5'-GCACTGTGTTGGCGTACAGG-3' (reverse) for HEF1, and 5'-CATACCCGCCAGATGCTAAA-3' (forward) and 5'-CCGGGTGCTGCCTGTACT-3'(reverse) for β-actin. Reaction system was: 2 μl of cDNA + 3 μl of each primer + 0.5 μl of Pfu polymerase + 1 μl of dNTPs + 3 μl of $MgCl_2$ + 2 μl of 10 × Buffer + 5.5 μl of ddH_2O. Reaction conditions were: 95°C for 5min, followed by 30 cycles of 95°C for 30s, 58°C for 30s, and 72°C for 60s, and 72°C for 10min. PCR products were separated by 1% agarose gel electrophoresis, and the results were detected using a UV spectroscopy gel imaging analysis system. Image J 5.0 software was used for gray value analysis with β-actin as endogenous control.

Construction of HEF1 recombinant adenovirus

1) Total RNA was extracted and cDNA was synthesized by reverse transcription; 2) HEF1 open reading frame was amplified by PCR using cDNA as template; 3) HEF1 open reading frame was inserted into adenovirus shuttle vector, and was sequenced and identified; 4) the vector was co-transfected with adenovirus vector into HEK293 cells, and recombinant adenovirus was packaged in HEK293 cells. Then recombinant adenovirus was amplified in HEK293 cells; 5) Adenopure kit was used to purify adenovirus according to the instructions; 6) TCID50 method was used to determine the titer of adenovirus.

MTT assay

1) Cells were inoculated into 96-well cell plates and cultured overnight until cell adhesion; 2) Bladder cancer cells with low expression level of HEF1 were transfected with HEF1 overexpression recombinant adenovirus, and bladder cancer cells with high expression level of HEF1 were transfected with HEF1 siRNA. HEF1 siRNA was purchased from Santa Cruz Corporation (USA), and transfection of HEF1 siRNA into bladder cancer cells with high expression level of HEF1 was performed using lipofectin 2000 (Invitrogen, USA) according to the instructions; 3) Cells were cultured for 24 hrs, and then were digested with trypsin. Cells were collected and the cell concentration was adjusted, followed by inoculation into 96-well plates; 4) MTT solution (50 μl) was added 24, 48 and 96 hrs later; 5) After incubation for another 4 hrs, culture medium was discarded and DMSO (50 μl) was added. After shaking, the absorbance was measured at 490 nm by a microplate reader. Cell proliferation rate = (1- optical density (OD) value of experimental group/OD value of control group) × 100%.

Migration and invasion assay

1) Cells were inoculated into 96-well cell plates and cultured overnight to reach cell adhesion; 2) After incubation for 24 hrs, cells were digested with trypsin, and were then collected and washed three times with serum-free medium; 3) Cell density was adjusted and cells were transferred to the upper chamber, while the lower chamber was filled with complete medium containing serum; 4) After incubation for 24 hrs, membranes were fixed by ethanol. After Giemsa staining, cells were counted under a microscope.

Statistics

Statistical analyses were performed using SPSS20.0 software (SPSS Inc., Chicago, IL, USA). Quantitative data were expressed as mean±standard deviation. Comparisons between two groups were performed by independent sample t-test, and comparisons among different time points were performed by Repeated Measures Analysis of Variance. P<0.05 was considered to be statistically significant.

Results

Comparison of relative expression levels of HEF1 in three cell lines

The relative expression level of HEF1 mRNA in bladder cancer cell lines T24, EJ and BIU-87 was significantly higher than that in normal bladder epithelium cell line SV-HUC-1 (p<0.05). The relative expression level of HEF1 mRNA in T24 was significantly higher than that in EJ cell line, and the relative expression level of HEF1 mRNA in BIU-87 cell line was significantly lower than that in EJ cell line (p<0.05; Figure 1).

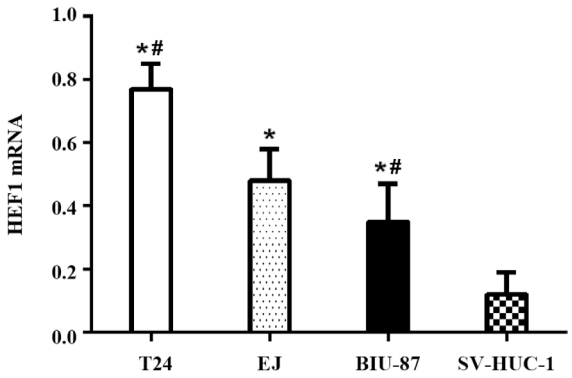

Figure 1. Relative expression level of HEF1 mRNA in three bladder cancer cell lines detected by RT-PCR. *p<0.05 compared with normal bladder epithelium cell line SV-HUC-1. #p<0.05 compared with BIU-87 cell line.

Comparison of cell proliferation rates

After transfection with HEF1 overexpression recombinant adenovirus, the proliferation rate of BIU-87 cells was significantly increased (p<0.05). After siRNA silencing, the proliferation rate of T24 cells was significantly reduced (p<0.05; Figures 2 and 3).

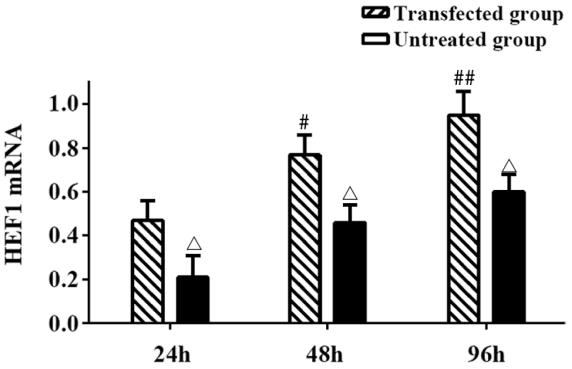

Figure 2. Cell proliferation rate of BIU-87 cells with HEF1 recombinant adenovirus transfection detected by MTT assay. #p<0.05 compared with those at 24 hrs. ##p<0.05 compared with that of the untreated group. △p <0.05 compared with that of untreated group.

Comparison of cell migration rate

After transfection with HEF1 overexpression recombinant adenovirus, the migration distance of BIU-87 cells was significantly increased (1865±568 vs 1025±454, t=5.625, p=0.05). After siRNA silencing, the migration distance of T24 cells were significantly decreased (565±154 vs 958±322 μm, t=5.425, p=0.009).

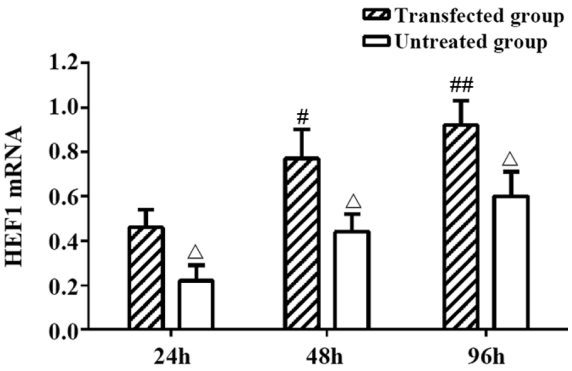

Figure 3. Cell proliferation rate of T24 cells with HEF1 recombinant adenovirus transfection detected by MTT assay. #p<0.05 compared with those at 24 hrs. ##p<0.05 compared with those at 48 hrs. △p<0.05 compared with that of transfected group at the same time point.

Comparison of cell invasion ability

After transfection with HEF1 overexpression recombinant adenovirus, the number of invaded BIU-87 cells was significantly increased (55±13 vs 35±9, t=5.325, p=0.015). After siRNA silencing, the number of invaded T24 cells was significantly decreased (15±6 vs 28±9, t=5.245, p=0.021).

Discussion

Studies have found that HEF1 protein is mainly located in the cytoplasm, and is composed of 843 amino acids. HEF1 gene is highly conserved in vertebrates, and is located on human chromosome 6p25-24 [10]. Studies have confirmed that HEF1 is a downstream effector of distant metastasis of glioma, and is also an important gene for melanoma metastasis. HEF1 expression is decreased in

breast cancer cell lines, so HEF1 may play different roles in different tumors [10].

Several authors have shown that the HEF1 plays an important role in the molecular signaling pathway of tumor metastasis, playing a role like a router that connects the upstream input signal and the downstream effector molecules [11,12]. Kim et al. [13] found that HEF1 was a gene that promoted melanoma tumor metastasis, and decreasing the expression of HEF1 by RNAi reduced the ability of melanoma metastasis. Natarajan et al. [14] found that HEF1 is a necessary and specific downstream effector molecule for FAK to promote the migration of malignant glioma cells, and reduced HEF1 expression by siRNA can significantly inhibit the migration of malignant glioma cells. Chang et al. [15] found that HEF1 expression was upregulated in lung adenocarcinoma tissue, and its expression level was closely correlated with tumor size, clinical stage and grade of differentiation. Li et al. [16] found that HEF1 overexpression is closely related to lymph node metastasis and tumor differentiation of colon cancer. HEF1 plays an important role in the proliferation, migration, invasion and metastasis of colon cancer through Wnt/β-catenin signaling pathway, so it may serve as a novel target for clinical treatment of colon cancer. Lucas et al. [17] have shown that the interaction between HEF1 and FAK is an important initiation, and event for cell migration and invasion, and it may be related to the positive regulation of Src-FAK-Crk migration switches by HEF1. In addition, HEF1 interacts with a number of different signaling pathways that promote cell migration and invasion, such as BCAR3, AND-34, SHEP2, Nsp2 and CHAT-H/SHEP1 [18,19]. Overexpression of HEF1 can lead to the activation of downstream molecules, including mitogen-activated protein kinase (MAPK) [20], extracellular related kinase (Erk1/2) [21], and so on. More studies are needed to identify the specific target molecules of HEF1 signal pathway.

This study showed that the expression level of HEF1 mRNA in T24 cell line was significantly higher than that in EJ and BIU-87 cell lines, and BIU-87 showed the lowest expression level, indicating the different expression levels of HEF1 in different bladder cancer cell lines. After transfection with HEF1 overexpression recombinant adenovirus, the proliferation, migration and invasion abilities of BIU-87 cells were significantly increased. In contrast, after siRNA silencing, the proliferation, migration and invasion abilities of T24 cells were significantly decreased, indicating that high expression level of HEF1 gene can promote the proliferation, invasion and metastasis of bladder cancer cells. It is of great clinical significance to find out the molecular mechanism of the occurrence, development invasion and metastasis of bladder cancer, so as to aid early diagnosis and potentially new treatments of bladder cancer.

Acknowledgement

This work was supported by Science and Technology Project Plan of Jiaxing (No. 2015AY23024).

Conflict of interests

The authors declare no conflict of interests.

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