



Aberrantly Upregulated EF4 Is Crucial for The Proliferation and Migration of Bladder Urothelial Carcinoma Cells *via* Orchestration of Mitochondrial Oxidative Phosphorylation*

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Abstract Elongation factor 4 (EF4) is a non-conventional elongation factor which regulates protein synthesis in mitochondria. In this study, we explored its function in bladder urothelial carcinoma. By analyzing the expression of EF4 in bladder urothelial carcinoma and adjacent normal tissues, we found that EF4 was aberrantly elevated in multiple cohorts of bladder cancer patients. Notably, the upregulation of EF4 was positively associated with tumor progression. By manipulating EF4 expression in HTB-9 and T-24 bladder cancer cells, the effects of upregulated EF4 was investigated. Knockdown of EF4 suppressed the proliferation and colony formation in bladder cancer cells; the ability of cells to migrate *in vitro* was also retarded. Knockdown of EF4 down-regulated the expression of mitochondrial DNA-encoded subunits of electron transfer chain complexes, and resulted in the dysfunction of mitochondrial oxidative phosphorylation. These results define a tumor-supportive role for EF4 by maintaining the protein synthesis within the mitochondria, which may serve as a potential therapeutic target in bladder urothelial carcinoma.

Key words EF4, tumorigenesis, mitochondrial translation, mitochondrial oxidative phosphorylation, bladder urothelial carcinoma

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Bladder cancer is the ninth most frequently-diagnosed cancer worldwide and its mortality ranks the top of urinary system^[1-2]. In China, the incidence of bladder cancer has increased in the past decades^[3]. Tobacco is the main risk factor of bladder cancer worldwide, whilst infection with *Schistosoma haematobium* and occupational exposure to toxic chemicals are also involved in selected populations^[1, 4]. Approximately 70% of bladder cancer lesions are non-muscle-invasive bladder urothelial carcinoma, and surgical resection combined with postoperative chemotherapy drug infusion is widely used in clinical treatment^[5]. However, patients with deep muscle invasion would be treated with

cystectomy or radical cystectomy followed by radiation and chemotherapy, whereas micro-metastasized disease should be treated with neoadjuvant chemotherapy^[6]. Although the bladder urothelial carcinoma is effectively managed under

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surveillance, postoperative relapses and metastasis are still threaten of the prognosis in some patients. More than 50% of patients still suffer from metastasis^[7]. Thus, there is an urgent need to explore novel target for combating metastatic bladder cancer.

It has been well-accepted that mitochondria are of crucial during cancer metastasis^[8-9]. Mitochondria are highly dynamic organelles, function as power plants and hubs for cell signaling^[10]. Mitochondrial electron transfer chain complexes are encoded by both the nuclear and mitochondrial DNA (mtDNA). In mammalian cells, 13 polypeptides of the core set of oxidative phosphorylation (OXPHOS) complexes are encoded by mtDNA and are synthesized in the mitochondrial matrix by the 55S ribosome system^[11]. Accumulating evidence suggested that mitochondrial translation plays a crucial role in the assembly of respiration machinery, and is important for the maintenance of mitochondrial quality control^[12]. Recently we demonstrated that EF4, a mitochondrial ribosome elongation factor involved in mitochondrial translation, is up-regulated in several types of cancers^[13], but the potential relationship between EF4 expression and tumorigenesis/metastasis remains to be explored.

In this paper, we continued to investigate the potential role of EF4 in bladder cancer. We profiled EF4 expression in tumor tissues as well as adjacent non-cancerous tissues from patients diagnosed with bladder urothelial carcinoma, and analyzed the correlation between EF4 levels with tumor grade. By manipulating EF4 expression in HTB-9 and T-24 bladder cancer cells, we investigated the effects of EF4 on mitochondrial oxidative phosphorylation, cell proliferation and cell migration. We hope our results will define a tumor-supportive role for EF4 in bladder cancer and will unravel additional mechanisms of bladder cancer metastasis.

1 Materials and methods

1.1 Materials

The antibodies used in this paper are as follows: anti- β -actin (Protein Tech, #66009-1-Ig); anti-VDAC1 (voltage-dependent anion-selective channel protein 1) (Protein Tech, #55259-1-AP); anti-CDK4 (cyclin-dependent kinase 4) (Protein Tech, #11026-1-AP); anti-CDK6 (cyclin-dependent kinase 6) (Protein Tech, #14052-1-AP); anti-EF4 (Abcam, #ab171161); anti-

SNAI2 (Protein snail homolog 2) (Cell Signaling Technology, #9585); anti-VIM (vimentin) (Cell Signaling Technology, #5741); Mito-Profile antibody cocktails (Abcam, #ab110411); HRP-conjugated anti-rabbit (ZSGB BIO, #ZB-2301) or anti-mouse secondary antibody (ZSGB BIO, #ZB-2305). Plasmids are as follows: pLKO.1 puro (Addgene, #8453), pCMV-VSV-G (Addgen, #8454), pCMV-dR8.2 vpvr (Addgen, #8455). Restriction enzymes: *Age*I (MBI, #FD1464), *Eco*RI (MBI, #FD0274).

1.2 Patients and tissue samples collection

The present investigation was approved by the Ethics Committee of Wenzhou Medical University, China. All tissue samples were obtained from patients diagnosed with bladder urothelial carcinoma, at the First Affiliated Hospital and the Second Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) between 2005 and 2013, with written informed consent in accordance with the Declaration of Helsinki. Cancer tissues and adjacent non-cancerous tissue were collected from the same patient without radiotherapy or chemotherapy before the operation.

A total of 34 formalin-fixed, paraffin-embedded bladder cancer specimens (5 μ m) were included in Table 1. Immunohistochemical assay was performed using an anti-EF4 antibody (dilution rate, 1 : 50). EF4 immunopositivity was graded in one to three tumor scores for each patient based on the intensity of the immunoreactivity in the cancer cells: 3 (+++) was strong, 2 (++) moderate, 1 (+) weak, and 0 negative. The scoring of immune reactivity was evaluated as described^[14].

1.3 Western blot

A total of 16 pairs of bladder cancer and adjacent non-cancerous tissue specimens were collected immediately after surgical resection, grounded with homogenizer, and suspended in RIPA buffer (50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS; complete protease inhibitor cocktail was added before using). Protein concentration was quantified with BCA (bicinchoninic acid) protein assay kit (Pierce, #23225). For Western blot assay, samples containing 20 μ g protein were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and blotted with proper primary antibodies. They were then incubated with appropriate peroxidase-conjugated secondary antibodies, and visualized using a

chemiluminescent substrate (Pierce, #32209).

1.4 Cell culture and RNAi

Human bladder cancer cell lines HTB-9 and T-24 were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were cultured in RPMI-1640 medium (Gibco, # 11875093) containing 10% fetal bovine serum (Gibco, # 10099141), 100 U/ml penicillin, and 100 g/L streptomycin.

The silencing of EF4 expression was carried out by lentivirus infection-induced RNAi. The sequence of shRNAs are: shCtrl, AACGACTAGTTAGGCG-ATGTA; shEF4#1, GGTATTTGCAGGAATGTAT-CCT; shEF4#2, GAAGAGCAGTTCAGAAGAATA. HTB-9 and T-24 cells were infected with lentivirus for 24 h and then selected with 2 mg/L puromycin for additional 72 h. The efficacy of EF4 silencing was confirmed by Western blot.

1.5 Assays for cell proliferation, colony formation and cell migration

For cell proliferation assay, 1 000 cells were seeded in 96-well plates. 1, 3, and 5 d after seeding, the number of cells was quantified. For colony formation assay, 100 cells were diluted into 10 ml culture medium and then 100 μ l of culture medium was added to 96-well plates. Ten days after seeding, the diameters of the single-cell clones were measured.

The ability of cells to migrate *in vitro* was assessed by both the wound healing assay and the Transwell assay. For wound healing experiment, 1×10^6 cells were seeded in 6-well plates and cultured for 24 h. Then the medium was replaced by serum-free RPMI-1640 and wounds were caused by 10 μ l pipet tips. The migration was evaluated after another 24 h. For the Transwell migration assay, 5×10^4 cells were suspended in 200 μ l serum-free medium and seeded into the upper chamber. The lower chamber contained 10% serum. Cells were cultured for 36 h to allow the cells to migrate through the membrane pores. Cells that migrated from the upper chamber were fixed with methanol and stained with 1% crystal violet solution. Images were captured by microscope, and cell numbers were counted.

1.6 Mitochondrial oxidative phosphorylation assay

Mitochondrial oxidative phosphorylation in HTB-9 cells was measured by detecting the oxygen consumption rate (OCR) with Seahorse XF24

extracellular flux analyzer. 3×10^4 cell was seeded in the wells of cell culture plate, and the OCR assay was implemented 24 h later. For measuring OCR, the inhibitors of mitochondrial electron transfer chain and ATP synthetase used in this experiment were oligomycin A (ATP synthase inhibitor; 1 μ mol/L), carbonylcyanide m-chlorophenylhydrazone (FCCP; mitochondrial uncoupler; 1 μ mol/L), antimycin A (complex III inhibitor; 1 μ mol/L) and rotenone (complex I inhibitor; 1 μ mol/L). Experiments were performed in culture medium containing 25 mmol/L glucose and 2 mmol/L sodium pyruvate, and measured with a standard cycling program.

1.7 Statistics

Data were presented as the mean $\pm$ SEM unless noted otherwise. Student's *T*-test was used to evaluate the difference between groups. The relationship between EF4 expression and clinic pathological features was checked by using the χ^2 test. *P* value less than 0.05 indicates statistical significance. Significance is represented as **P*<0.05, ***P*<0.01, ****P*<0.001.

2 Results

2.1 EF4 is aberrantly upregulated in bladder cancer

We started the present investigation by analyzing the expression of EF4 in bladder urothelial carcinoma and adjacent normal tissue with an online tool UALCAN (<http://ualcan.path.uab.edu/index.html>)^[15]. In comparison with non-cancerous bladder tissues, EF4 mRNA was upregulated in bladder cancer tissues (TCGA-BLCA), and this finding is in consistent with published online data^[16] (GSE133624) (Figure 1a). To further confirm the up-regulation of EF4 in bladder cancer, we collected 16 pairs of clinical tissue samples from patients, and profiled EF4 protein level by Western blot. The results indicated that EF4 was significantly upregulated in 15 out of 16 bladder cancer tissues (Figure 1b).

2.2 EF4 expression is related to bladder cancer tumor stages

Given the fact that EF4 was upregulated in bladder cancer, we then applied correlation analysis between EF4 expression and various clinic-pathological parameters. EF4 abundance was calculated according to the median protein expression of immunohistochemical assay. The result showed

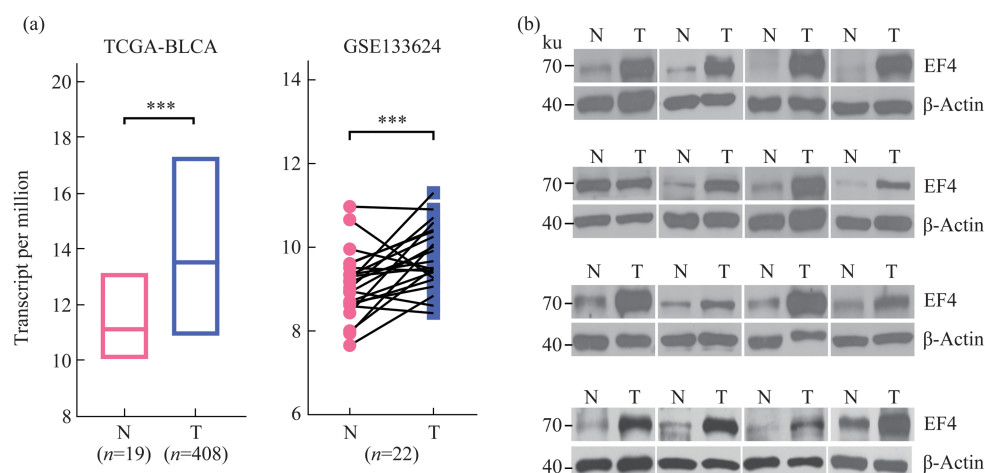


Fig. 1 EF4 is aberrantly upregulated in bladder urothelial carcinoma

(a) EF4 mRNA expression in bladder cancer and adjacent non-cancerous tissues. Data were from TCGA-BLCA or NCBI GSE133624 DataSets. (b) EF4 protein expression in bladder cancer tissues and paired adjacent non-cancerous tissues. N means adjacent non-cancerous tissue; T means tumor tissue. *** $P < 0.001$.

that EF4 expression was independent of gender and age, but was positively correlated with bladder cancer T stage. While the average expression of EF4 was 1.48 in tumor T1–T2 stage, its expression abundance was 2.99 in T3–T4 stage with P value 0.03 (Table 1). These data suggested that EF4 expression was correlated with bladder cancer tumor stages.

2.3 Knockdown of EF4 represses cell proliferation and colony formation

To investigate the possible involvement of EF4 in tumorigenesis, we used short hairpin RNA (shRNA) to interfere EF4 expression in HTB-9 and T-24 bladder cancer cells and determined the impact of EF4 on cell proliferation and colony formation. Knockdown of EF4 decreased the proliferation of HTB-9 and T-24 bladder cancer cells *in vitro* (Figure 2a), which might be due to the arrested cell cycle in G1 phase (Figure 2b). Since cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) are key factors for G1/S transition during cell cycle^[17-18], we then analyzed the expression of CDK4 and CDK6 and found that CDK6 was downregulated upon EF4 knockdown in HTB-9 and T-24 bladder cancer cells (Figure 2c).

To further determine the importance of EF4, we compared the ability of cells to form colony with EF4 knockdown and control cells. The results showed that the ability of single cell to form colony was reduced

Table1 Correlation between EF4 expression and various clinic-pathological parameters in bladder cancer tissues

Characteristic	EF4 expression		P value
	Patients (n)	Median (P25–P75)	
Gender			0.390
Male	26	2.50 (1.46–3.95)	
Female	8	2.18 (1.31–2.68)	
Age			0.801
<69	12	2.23 (1.81–2.91)	
≥69	22	2.44 (1.28–3.83)	
T stage			0.030*
T1–2	14	1.48 (1.21–2.64)	
T3–4	20	2.99 (2.08–4.13)	
N stage			0.240
N0	30	2.63 (1.37–3.70)	
N≥1	4	1.78 (1.58–1.94)	
M stage			0.878
M0	33	2.30 (1.35–3.34)	
M1	1		
TNM stage			0.316
I–II	31	2.60 (1.39–3.58)	
III–IV	3	1.85 (1.53–2.02)	
Grade			0.334
Low	18	2.61 (1.87–3.86)	
High	16	1.78 (1.24–2.81)	

χ^2 test is used in correlation assay, P values less than 0.05 indicate statistical significance.

by EF4 knockdown (Figure 2d), suggesting that EF4 is essential for the proliferation and colony formation of bladder cancer cells.

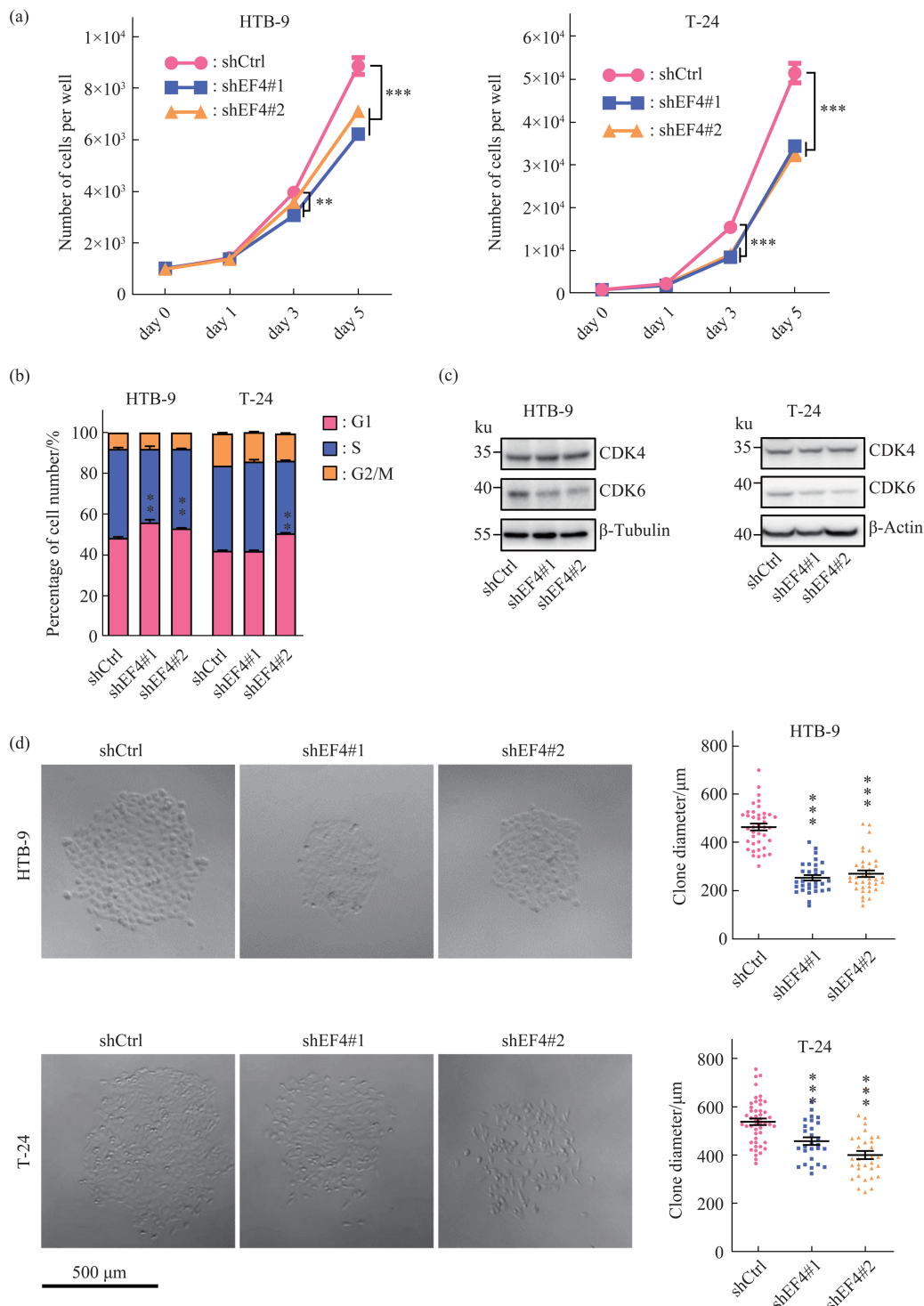


Fig. 2 Knockdown of EF4 represses cell proliferation and colony formation in bladder cancer cells

(a) Control and EF4 knockdown cells were seeded and the number of cells were quantified ($n=8-12$). (b) Cell cycle of control versus EF4 knockdown cells in HTB-9 and T-24. The DNA content of 3×10^4 cells were analyzed by flow cytometry ($n=3$). (c) Western blot analysis of expression of CDK4 and CDK6 ($n=4$). (d) Colony formation ability of control and EF4 knockdown cells. The diameters of single colonies were calculated. $**P < 0.01$, $***P < 0.001$.

2.4 Knockdown of EF4 represses cell migration

Metastasis is the main cause of death in bladder cancer patients. Since EF4 is overexpressed in patients with advanced tumor stages, we next investigated the influence of EF4 on cell migration *in vitro*. The results of both the wound healing (Figure 3a) and Transwell (Figure 3b) assays indicated the marked blockade of cell migration in EF4 knockdown cells. It should be noted that the inhibition of cell proliferation contributed only a small portion in the blockage of cell migration because EF4 knockdown

caused moderate cell growth retardation but significant cell migration arrest. Thus, there might be other mechanisms involved in the suppression of cell migration in EF4 knockdown cells. Since epithelial-to-mesenchymal transition (EMT) plays essential roles in metastasis, we further detected the expression of EMT-related proteins by Western blot. The results showed that vimentin (VIM) and protein snail homolog 2 (SNAI2) were downregulated in HTB-9 and T-24 cells transfected with EF4-targeting shRNA (Figure 3c), suggesting the inhibition of EMT as a result of

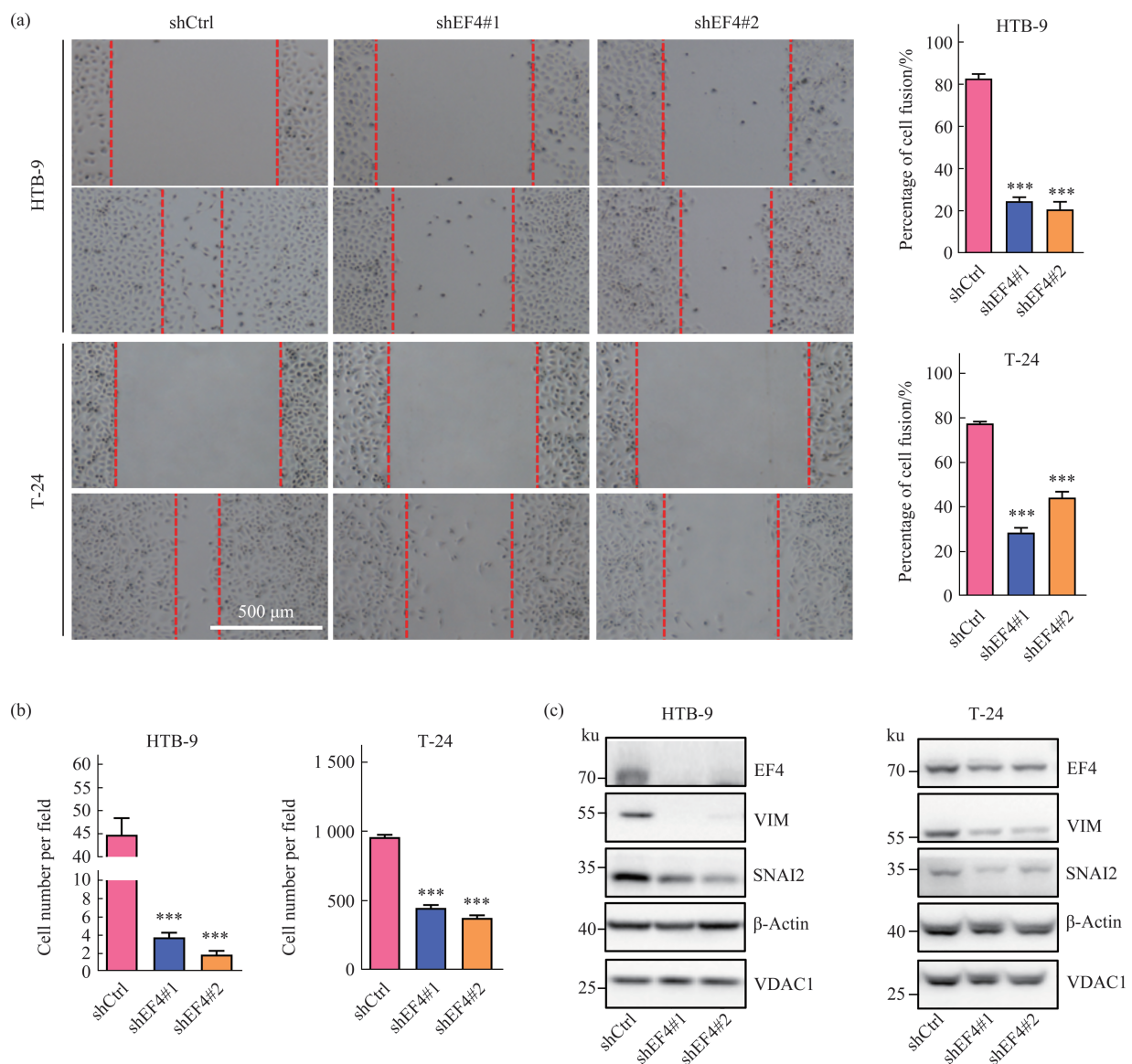


Fig. 3 Knockdown of EF4 represses cell migration *in vitro*

(a) Wound healing assessment of cell migration. HTB-9 and T-24 cells (1×10^6) were seeded in 6-well cell culture plates. Wounds were caused by 10 μ l pipet tips, and cells were cultured in serum-free RPMI-1640 medium for 24 h ($n=6$). (b) Transwell assessment of cell migration. HTB-9 and T-24 cells (5×10^4 cells) were seeded into the upper chamber of the Transwell inserts. The lower chamber contained 10 % serum. 36 h after seeding, the cell was fixed and the number of migrated cells were counted ($n=3$). (c) The expression of EMT marker proteins VIM and SNAI2 in control and EF4 knockdown cells ($n=3-4$). *** $P < 0.001$.

EF4 knockdown. Both the repression of EMT process and the inhibition of cell proliferation contributed to the impaired migration ability.

2.5 Mitochondrial phosphorylation is defected in EF4 knockdown cells

The above data indicated that EF4 is correlated with the proliferation and migration of bladder cancer cells, which need abundant amounts of ATP. As a mitochondrial ribosome protein which orchestrates the synthesis of subunits of mitochondrial complexes, the cellular function of EF4 might be closely related with mitochondrial oxidative phosphorylation (OXPHOS). We then quantified mitochondrial OXPHOS in EF4 knockdown cells by measuring the oxygen consumption rate (OCR) *in situ* with the Seahorse analyzer (Agilent)^[13]. As shown in Figure 4a

and 4b, knockdown of EF4 in HTB-9 cells caused a rapid decrease in the basal and maximal OXPHOS levels.

To address the molecular mechanism underlying the suppression of OXPHOS in EF4 knockdown cells, the expression of core subunits of mitochondrial complexes was analyzed, and the results indicated that the level of a mtDNA-encoded subunit, cytochrome c oxidase subunit 2 (MTCO2), was down-regulated in EF4 knockdown cells (Figure 4c, d). Interestingly, the nuclear DNA-encoded subunits, cytochrome b-c1 complex subunit 2 (UQCRC2) and NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8 (NDUFB8), were also down-regulated, suggesting a possible coordinated translation of nuclear and mtDNA-encoded mitochondrial complexes.

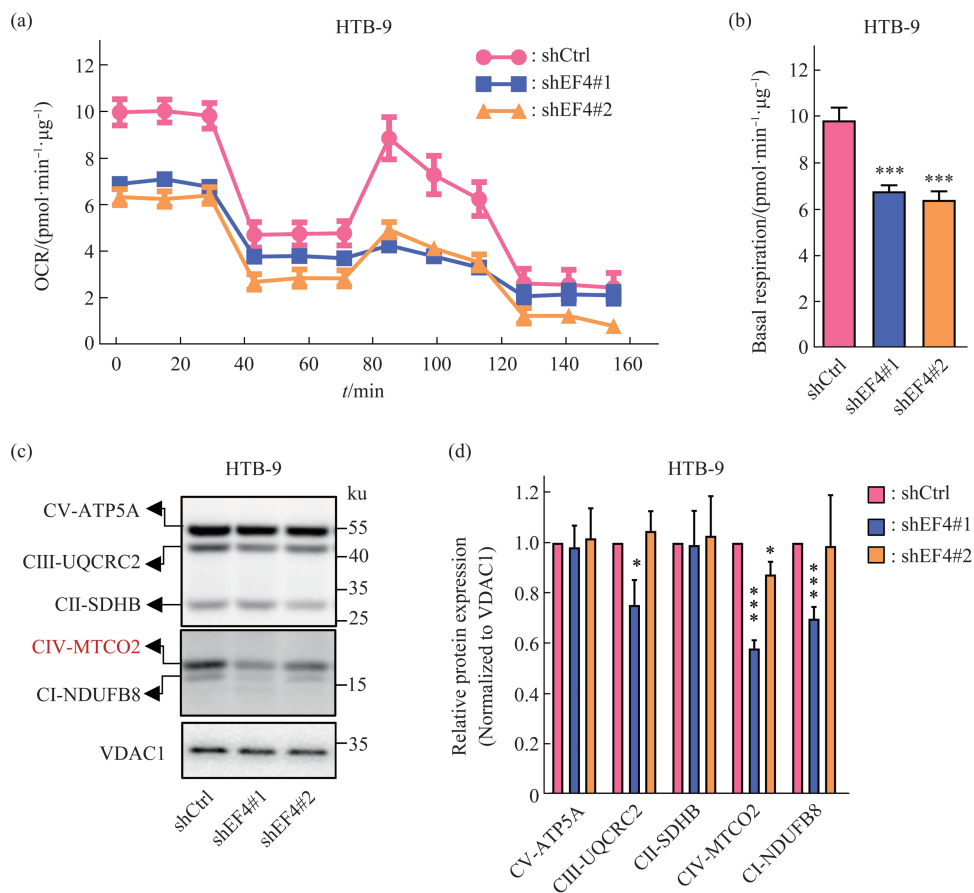


Fig.4 EF4 knockdown defects mitochondrial oxidative phosphorylation

(a) Oxygen consumption rate of control and EF4 knockdown cells. HTB-9 cells (3×10⁴) were seeded in cell culture plate of Seahorse XF-24 analyzer and OCR was detected according to the stand Mito-Stress assay protocol. (b) Basal respiration was calculated from the dynamic curve of OCR (n=6). (c) Western blot analysis of the expression of mitochondria respiration complex subunits. (d) Quantitative analysis of the expression of mitochondria respiration complex subunits(n=7). * P< 0.05, *** P< 0.001.

3 Discussion

In this study, we demonstrated that EF4 was overexpressed in bladder urothelial carcinoma. EF4 knockdown impaired the translation and the assembly of mitochondrial respiratory complexes, down-regulated mitochondrial respiratory, and suppressed the proliferation of bladder cancer cells. The ability of cancer cells to migrate was also abolished by EF4 knockdown, possibly *via* the inhibition of epithelial-to-mesenchymal transition (EMT). These evidences linked the aberrantly upregulated EF4 with the proliferation and migration of bladder cancer cells.

EF4 was first identified in the 1980s in *Escherichia coli*^[19-20] and was reported to play important roles in protein translation^[21], as a molecule orchestrating ribosome back-translocation^[22] and ribosome biogenesis^[23] in prokaryotic cells. In eukaryotic cells, EF4 is a mitochondrial translation elongation factor which controls the synthesis of mtDNA-encoded peptides^[24-25], especially under stressed conditions^[26]. Recently we found that EF4 is overexpressed in several types of cancers and is related with tumorigenesis, probably by modulating the mitochondrial translation^[13]. However, the correlation between EF4 expression and the progression of tumor remains elusive. We hereby provide direct evidence that EF4 is overexpressed in bladder cancers; its expression is positively correlated with tumor stages.

Cancer cells face a considerable metabolic challenge as they divide and grow uncontrollably, thus resulting in a drastic adjustment of many metabolic pathways. Different from normal cells which show active mitochondrial respiratory function and use oxidative phosphorylation (OXPHOS) as the main route to generate ATP, cancer cells often reprogram their core metabolic pathways and exhibit an increased rate of glycolysis, even in the presence of high O₂ concentrations^[27]. This phenomenon of “active aerobic glycolysis” is known as Warburg effect^[27-29], and is regarded as an important hallmark of cancer^[30-32]. However, mitochondrial function is also important for certain types of cancer cells, particularly metastatic solid tumors^[31, 33-36]. For instance, mitochondrial dynamics drives liver cancer metastasis by reprogramming glucose metabolism^[37], and PGC-1 α promotes breast cancer metastasis *via*

mediating mitochondrial biogenesis^[38]. Mitochondria not only govern energetic functions, but also support building blocks for tumor cell proliferation, maintain cell internal redox balance, control calcium homeostasis, and regulate cell survival^[39-40].

Mitochondrial homeostasis has been reviewed as a considerable target for bladder cancer therapy^[41-42]. Mitochondria are semi-autonomous organelles with their own genome (DNA) and protein synthesis machineries (ribosomes). The mitochondrial ribosomes are responsible for the synthesis of mtDNA-encoded peptides, which are core sets of mitochondrial complexes^[43]. In the last twenty years, the importance of mitochondrial ribosome proteins in programmed cell death and cell proliferation had been revealed, and mitochondrial ribosome proteins have been regarded as a therapeutic target for OXPHOS-dependent cancers^[43]. There is a cross-talk between cytoplasmic mTOR-regulated protein synthesis pathway and EF4 dependent mitochondrial quality control, by which the cell will activate the mTOR to increase the cytoplasmic translation to remedy mitochondrial translation^[44]. We confirmed in the present investigation that the nuclear DNA-encoded protein UQCRC2 and NDUFB8 protein expression was also suppressed in EF4-knockdown bladder cancer cells. This was consistent with our previous study that mitochondrial complex activity was improved in EF4 overexpressed cells and decreased in EF4 knock down cells^[13], suggesting that nuclear DNA-encoded mitochondrial complex subunit stability could be influenced by mitochondria ribosome mediated peptide translation. These results indicated that mitochondrial homeostasis was defected in EF4 knockdown cell.

Our findings in this and previous study^[13] demonstrated that EF4, a key component in regulating mitochondria translational machinery, is essential for the proliferation and migration of cancer cells, probably by mediating the synthesis and assembly of mitochondrial electron transport chain complexes, and maintaining the mitochondrial homeostasis. The results of our study and future work may offer a feasible therapeutic target for bladder cancers.

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EF4影响线粒体氧化磷酸化并调控膀胱癌细胞增殖及迁移*

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摘要 延伸因子4 (EF4) 是一种非传统的线粒体延伸因子, 参与调控线粒体蛋白质合成过程. 在本研究中, 我们进一步探索了其在膀胱癌中的作用机制. 通过检测 EF4 在膀胱癌及邻近正常组织中的表达, 发现 EF4 在膀胱癌患者肿瘤组织中异常升高, 并在 T 分期较高的肿瘤中高表达. 随后, 通过在 HTB-9 和 T-24 膀胱癌细胞中敲低 EF4 的表达, 进一步探索了 EF4 在膀胱癌发生及发展中的作用. 研究结果表明, 通过 RNAi 敲低 EF4 表达不仅可以抑制膀胱癌细胞 HTB-9 和 T-24 在体外的增殖和集落形成, 还显著降低了其迁移能力, 这一结果主要归因于下调 EF4 表达阻碍了线粒体 DNA 编码的呼吸链复合物亚基的翻译, 影响呼吸链复合物的组装, 并最终导致线粒体氧化磷酸化功能障碍. 以上结果表明 EF4 通过维持线粒体重要复合物的翻译而影响膀胱癌细胞的细胞增殖迁移及膀胱癌发生发展, 并提示 EF4 可能是治疗膀胱癌的潜在目标分子.

关键词 EF4, 肿瘤发生, 线粒体翻译, 线粒体氧化磷酸化, 膀胱癌

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