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Original Article

Clinical significance of focal adhesion kinase (FAK) in cervical cancer progression and metastasis

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Abstract: Focal adhesion kinase is a non-receptor, tyrosine kinase of cells whose key functions are cell adhesion, migration, and invasion. Aberrant expression and regulation of FAK-mediated intracellular signaling pathways has been reported in several cancers and they are involved in cancer cell migration and apoptosis resistance. By RT-PCR, we found that cervical cancer cells showed a 4-fold increase of relative mRNA expression of FAK compared to control cells. In parallel, the FAK protein expression level was also elevated in cervical cancer cells. Interestingly, knockdown of FAK in cervical cancer cells showed attenuated cell proliferation and migration. Further, the FAK RNAi cells became more sensitive to chemotherapeutic drugs such as 5-FU and docetaxel and therefore the rate of cell survival is declined. The significant over-expression of FAK in cervical cancer cells might involve in cervical carcinogenesis and prolonged cell survival. This FAK overexpression might be a potential target for anti-cancer drugs to attenuate rapid cell proliferation and invasion by inducing apoptosis.

Keywords: Human papilloma virus, oncoproteins, focal adhesion kinase, cervical cancer, metastasis

Introduction

Infections of human papilloma virus (HPV) are associated with progression of cervical cancer and it is a major health concern in world-wide. HPV belongs to the small, heterogeneous group of non-enveloped, double strand circular DNA viruses, with the size ranges between 50-55 nm in diameter [1]. The high risk and most prevalent type of HPVs are HPV-16 (>50%) and HPV-18 (<20%), which accounts for more than 60% cervical cancer tumorigenesis. Globally, cervical cancer is third most common cause of cancer-allied deaths in women [2]. Although the persistence of HPV infection and mortality rate are continuously increasing worldwide, the rate of incidence is different between the developing and developed countries. The malignant transformation activity of HPV mainly relies on two oncoproteins such as E6 and E7 [2, 3]. Extensive studies revealed that oncoproteins (E6 and E7) interfering the normal host cellular and signal transduction pathways, targets and downregulates the tumor suppressor proteins such as p53 and pRb. Thus, oncoproteins are crucial for uncontrolled cell cycle, decreased apoptosis, genomic instability, and cell immortalization [4-7]. Cancer metastasis is a process where the cancer cells exhibit the secondary growth at different sites, which are the major implications of the cancer therapy. Reports demonstrated that therapeutics targeted at molecular level considerably improved the patient survival by decreased metastasis [8, 9]. Hence, there is an urge to understand and elucidate the molecular mechanism of cancer progression in detail.

Focal adhesion kinase (FAK) is a non-receptor cytoplasmic tyrosine kinase plays crucial role in signal transduction pathways, which are stimulated by integrins and several growth factors. In response to such signals, tyrosine kinase is phosphorylated ($pp125^{FAK}$), localized at focal adhesion sites and thus contributes their role in cell adhesion, cell spreading and migration [10]. Several studies showed that FAK over expressed in malignant tumors, involved in cancer invasion, migration and metastasis [10, 11]. Meanwhile, inhibition of FAK or attenuation

FAK signaling such as MMPs and Src-FAK mediated pathways significantly reduces cancer cell proliferation, migration and survival [12]. In the current study, we have evaluated the relative mRNA and protein expression of FAK between control and cervical cancer cells. One step further, we have determined the effect FAK depletion (by RNAi approach) on cell migration and apoptosis activation in cervical cancer cells.

Materials and methods

Tissue collection and culturing

A total number of 15 patients (n=15) were subjected to cervical cancer surgery and the specialized gynecological oncologists classified the cancer stage. The biopsies were collected in agreement with the patients and also according to the ethical rules of the hospital. We have also collected normal tissues (n=15) as a control study. Patient details: Age- 55-60; Grade-High; Differentiation-moderate to well differentiated squamous cell carcinoma (WDSCC). Histopathological examinations were performed for both normal and cancer tissues as described previously [13].

The tissues were washed extensively in PBS and incubated overnight in DMEM/F12 (GIBCO) with penicillin (500 U/ml) and streptomycin (500 µg/ml). Subsequently, enzymatic digestion was performed for 1 hour in PBS solution with 1.5 mg/mL of collagenase and 20 µg/mL of hyaluronidase. The obtained cell monolayers were subjected to serial passaging and further culturing was performed for various experiments. Cells were cultured in corning petri dishes (Thermo Fisher scientific) in DMEM together with 10% FBS and antibiotics (penicillin/streptomycin) supplied by humidified 5% CO_2 & 95% air atmosphere at 37°C.

Immunohistostaining

Immunohistostaining was carried out as described previously [14]. The non-specific binding of primary antibodies is prevented by using blocking solution. The primary antibodies used: Rabbit polyclonal of E6 and E7 (from Biorbyt; 0.5 mg/mL) incubated for 1 hour. Followed by secondary antibody incubation, the color detection was performed as per the product protocol. In last step, hemalaun was used to counterstain the slides, followed by ethanol dehydration and subjected to microscopic analysis.

RT-PCR analysis

Extraction of cellular RNA was performed as per the guided protocol by Qiagen RNA Kit. The Biorad iCycler protocol was followed to perform RT-PCR. The primer sequences for FAK and GAPDH were used as mentioned previously [15]. The PCR parameters: Initial denaturation -94°C 50 sec; denaturation -94°C for 5 min. followed by annealing at 60°C for 30 sec and extension at 72°C for 20 s. Final extension was executed at 72°C for 10 min and repeated for 35 cycles. The relative mRNA expression values are adjusted with the housekeeping gene, GAPDH. The average values from three independent experiments were represented as a quantification graph.

Cell transfection

Cells were cultured in 6 well plates (app 2×10^6 cells/well) for 24 hours. The *FAK* small interfering RNA (siRNA) of 100 nM mixed in in Opti-MEM (In Vitrogen) with the 6 µL of transfection reagent of Lipofectamine® RNAiMAX (Thermofishers). Followed by incubation for 25 minutes at room temperature, the reaction mix was added to the cells. After 24 hours of incubation, cells were subjected to different analysis. The *siFAK* oligo sequence used was mentioned previously [16].

Western blot analysis

Extraction of total cellular protein and separation by SDS-page electrophoresis were performed according to the protocol in the literature [17]. Primary antibody used: Rabbit monoclonal anti-FAK of 1:100 (Cell Signaling) and Mouse-monoclonal anti-GAPDH of 1:1000 (ProteinTech). ECL (Enhanced Chemilumiscence reagent) kit from Biorad was used to detect the protein signal.

In vitro cell culture assays cervical

The *in vitro* cell proliferation [18] and matrigel invasion assays [19] were performed as mentioned previously. Similarly, the chemoresistance assay was executed by following the protocol in the literature [18]. The concentration of 5-FU (10 µg/mL) and Docetaxel (10 µg/mL) were used.

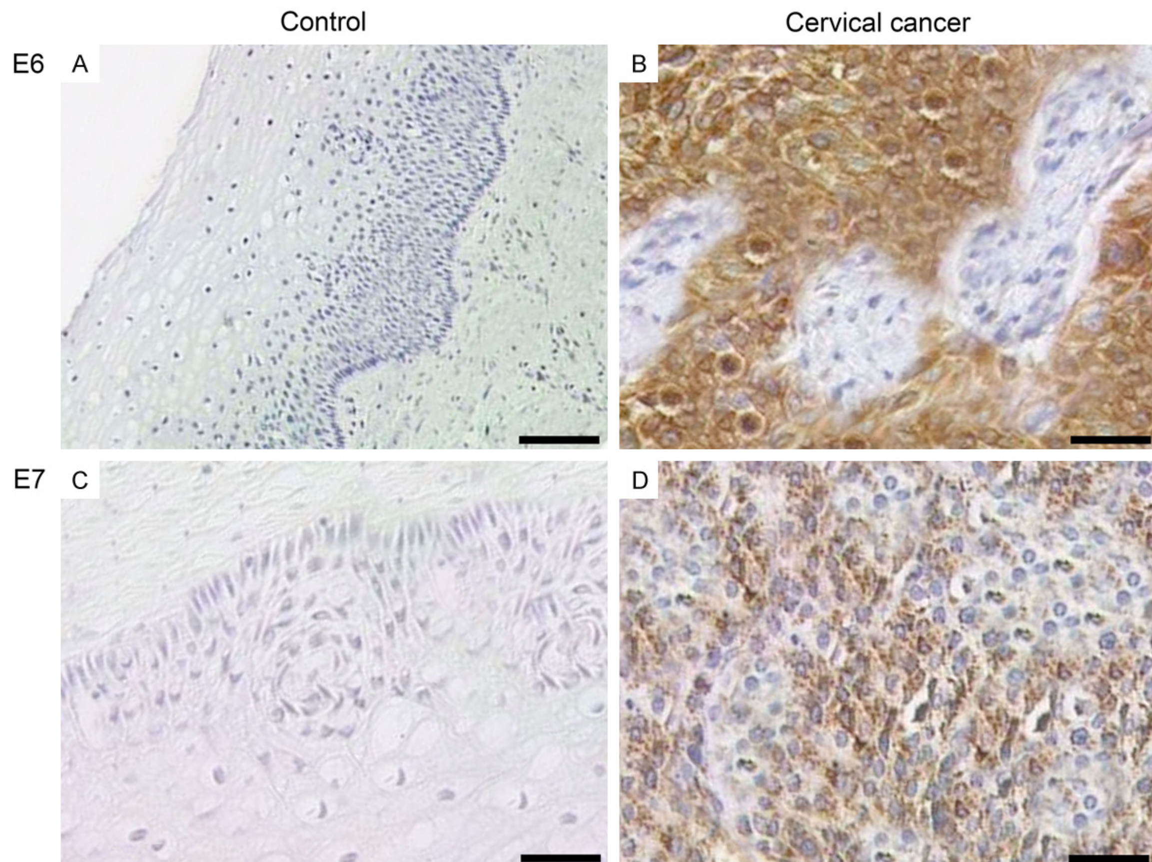


Figure 1. Immunohistostaining of HPV-16 oncoproteins E6 (A, B) and E7 (C, D). Cervical cancer tissues showed strong positive and intensive staining for oncoproteins E6 (B) and E7 (D), whereas there is no positive staining observed in control tissues (A, C). Scale Bar: 50 m.

Statistical analysis

The t-test was used to compare between the groups and a multi-factor Q-test was employed between multi-groups. The values defined were *, P<0.05 and **, P<0.01 significant.

Results

E6 and E7 immunohistochemistry

All cervical cancer samples showed HPV-16 by PCR amplification (data not shown). The HPV-16 positive samples and the adjacent control tissues were further subjected to immunohistostaining of oncoproteins. By fluorescence microscopic analysis, the E6 expression intensively stained the cytoplasm of the cancer tissues (**Figure 1A**), whereas there was no significant staining was observed in control tissues (**Figure 1B**). Similarly, the E7 immunostaining evaluation showed intensive staining in cervical cancer tissues (**Figure 1C**) and there was no E7 expression in control tissues (**Figure 1D**). Thus, our data suggest that HPV-16 infected cervical cancer samples pose high expression of oncoproteins, which are crucial for rapid cell proliferation and reduced apoptosis.

FAK expression profile in cervical cancer cells

We have investigated the expression profile of FAK by RT-PCR and western blot analysis. We found that the relative mRNA expression of FAK was found to be significantly upregulated in cervical cancer cells rather than control cells (**Figure 2A**). Concomitantly, the focal adhesion kinase protein level was significantly enhanced in cervical cancer cells (**Figure 2B**). Therefore, the cervical cancer at metastatic and recurrence stage have overexpression of FAK and thus may plays an important role in cancer metastasis.

Effect of FAK knockdown on cancer migration and survival

Next we have determined the upshot of FAK knock down on cervical cancer cells. By RNAi

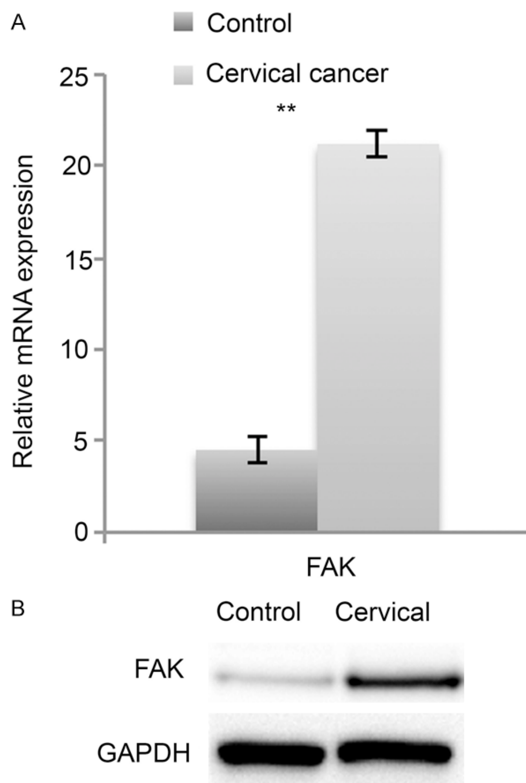


Figure 2. *FAK* expression profile in cervical cancer cells. RT-PCR (A) and western blot analysis (B) showed FAK upregulation in cervical cancer cells. The error bar represents standard deviation. *, P<0.02; **, P<0.01.

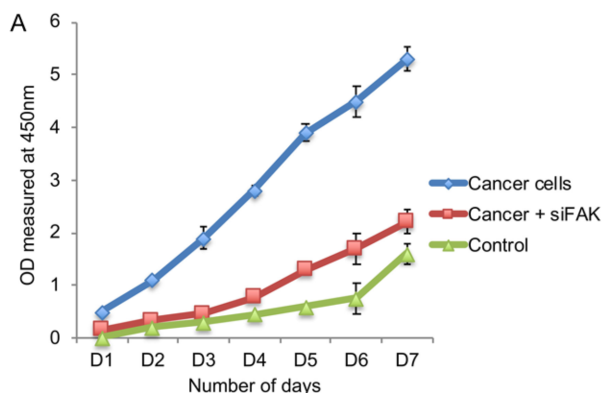
approach, gene silencing of *FAK* was executed and cells were further analysed by *in vitro* assays namely cell proliferation, invasion, and chemoresistance assays. **Figure 3A** displayed a reduced rate of cell proliferation in si*FAK* cervical cancer cells, when compared to the controls. Similarly, the number of cancer cells invaded through the matrigel was significantly lowered in the *FAK* knock down cancer cells (**Figure 3B**). Finally, the *FAK* knockdown made the cervical cancer cells more sensitive towards the chemotherapeutic drugs such as 5-FU and docetaxel and therefore the survival rate is deceased (**Figure 3C**). Hence, these data suggest that down regulation of *FAK* compromise the rapid proliferation, migration, and survival of cervical cancer cells.

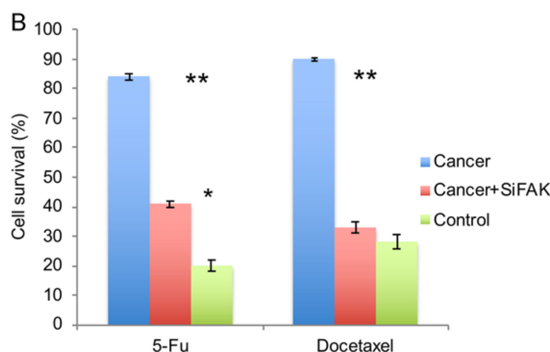
Discussion

In the present study, we have demonstrated that obtained cervical cancer samples displayed strong intensive staining of oncoproteins E6 and E7, which are crucial for HPV-16 mediated carcinogenesis. These oncoprotein E6 and E7 disrupts the regulated cell cycle by targeting the tumor suppressor pathways such as p53 and pRb within the host cells, respectively [20]. Further, E6 and E7 are the crucial players for angiogenesis, cancer invasion, metastasis, and uncontrolled telomerase activity through the downregulation of apoptosis. Accordingly, several studies reported that absence of oncoproteins sensitize the cancer cells to undergo apoptosis and cell death [21, 22], thus proving the absolute requirement of E6 and E7 for persistence of HPV-mediated cancer.

The co-ordination of cell adhesion to the extracellular matrix is essential for cell proliferation, migration, and survival [16]. Activation of focal adhesion sites is crucial for integrating the signals essential for cytoskeletal organization, cell adhesion, and cell survival. The major finding from our study is the overexpression of FAK in cervical cancer cells. Studies have previously shown that overexpression of FAK in different cancers (breast, thyroid, oral, ovarian and head and neck cancers) increases cancer invasion and migration and thus results in poor survival of patients [10, 16, 23-25]. We have also demonstrated that the silencing of FAK reduced tumorigenesis potential of cancer cells and therefore tumor metastasis was prevented. Meanwhile, it has been shown that overexpressed FAK provide protection to cancer cells from stress factors, UV radiation, and chemotherapy treatments [26]. However, the molecular mechanism behind FAK mediated cancer cell prolonged survival and inhibited apoptosis are not clearly understood so far. One such signalling mechanism elucidated in breast cancer cells is FAK/phosphatidylinositol 3-kinase/Akt signaling which inhibits apoptosis efficiently and therefore they are highly chemoresistant [27]. Similarly, FAK affecting Src and PTEN activity, which promotes cancer cell survival [28].

Interestingly, the chemotherapy efficacy was improved in FAK RNAi cells. Upon treatment with 5-FU and docetaxel, the FAK knockdown cells were more susceptible to the drug treatment and therefore apoptosis is stimulated. Similarly, it was reported that inhibition of FAK expression at focal adhesions by expression





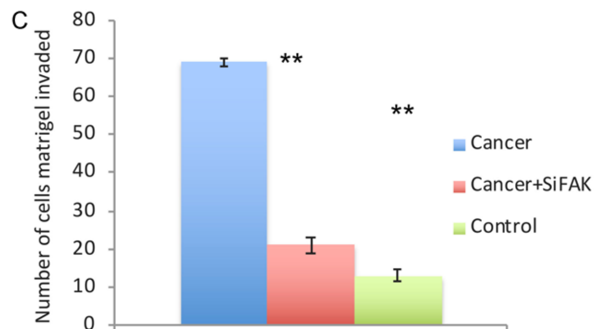


Figure 3. Effect of *FAK* knockdown in cervical cancer cells. (A) In vitro proliferation assay showing slowed down cell proliferation in *siFAK* cells. Similarly, in matrigel invasion assay (B) the invasion potential of *siFAK* cervical cancer cells was significantly reduced. (C) Chemoresistance assay -*siFAK* cervical cancer cells showed more sensitivity to 5-FU and docetaxel treatment. The error bar represents standard deviation. *, P<0.02; **, P<0.01.

showed increased sensitivity to apoptosis-inducing agents [29]. One possible explanation could be that FAK contains caspase-3 cleavage sites, separates the kinase domain from FAK at the targeting sequence of COOH- terminal, and that may induce apoptosis of cancer cells. Thus, FAK cleavage correlates with the absence of FAK at focal adhesions and therefore FAK is redistributed to the apoptotic membrane structures. On the other hand, the therapeutic drugs might also involve in cell detachment through caspase-mediated FAK degradation, resulting in cell detachment from cytoskeletal organization and induced apoptosis [16]. Taken together, our preliminary data suggest that the determination of anti-cancer drugs targeting FAK-protein interactions and FAK mediated signaling pathways would be certainly beneficial to overcome cancer cell proliferation, metastasis, and multi-drug/apoptosis resistance properties.

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Disclosure of conflict of interest

None.

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