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Original Article

The inhibition of EGFR nuclear translocation attenuates radioresistance through decreasing the expression of p-DNA-PK in cervical cancer cells

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Abstract: Objective: As the leading factor for female mortality, cervical cancer with high incidence has brought huge burden to both the patients' family and the whole society. So far radiotherapy is the general treatment for cervical cancer. However, radioresistance and metastasis at the advanced stage have become the common cause for its poor prognosis and high mortality. This study aimed to elucidate the role of epidermal growth factor receptor (EGFR) nuclear translocation in radioresistance, and its correlation with the DNA damage repair pathway in cervical cancer cells. Method: In this study, the dynamic expression of EGFR, DNA-dependent protein kinase (DNA-PK) and their phosphorylation level in irradiated cervical cancer cell line CaSki were tested by western blotting. In addition, nuclear localization signal (NLS) peptide inhibitor was synthesized to elucidate the correlation between EGFR translocation and DNA damage repair. Results: The expression of EGFR, protein kinase N1 (PKN1), and DNA-PK in the nucleus increased after irradiation in CaSki cells. Furthermore, irradiation also enhanced the phosphorylation level of EGFR at Thr654, PKN1 at T774 and DNA-PK at T2609. The inhibition of EGFR nuclear translocation decreased the expression level of EGFR and DNA-PK in the nucleus, and attenuated their phosphorylation process. Conclusion: EGFR nuclear translocation promoted DNA damage repair in irradiated cervical cancer cells. This work facilitated the investigators to understand the possible molecular mechanism of the resistance to irradiation in the treatment of cervical cancer, which provided a potentially compelling clinical method for cancer therapy.

Keywords: CaSki, EGFR, nuclear translocation, DNA-PK, pEGFR-T654, pDNA-PK-T2609

Introduction

Cervical cancer is a common gynecological malignancy, and it's the leading cause of cancer-related mortality among females worldwide [1-3]. There are approximately more than 520,000 new patients diagnosed with cervical cancer annually. The most popular treatment for cervical cancer is radiation therapy [4]. However, despite the numerous advances in cervical cancer treatment in recent years, the prognosis of advanced/recurrent cervical cancer is still poor. Generally, radiation resistance is an indication of poor prognosis [5].

Recently, it was reported that mutation in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) could dramatically improve therapeutic outcomes for cervical cancer patients [6]. EGFR, a member of ErbB family is a transmembrane receptor protein [7]. The activation of EGFR leads to autophosphorylation, and activation of several downstream signal transduction molecules and cascades [8]. Nuclear EGFR (nEGFR) plays a key role in resistance to cancer therapeutics [9, 10]. Increased nuclear localization of EGFR has enhanced tumor resistance and DNA repair after radiation and chemotherapy. Therefore, EGFR has been considered to be a potential biomarker that represents the response to various therapies in cancers.

Structurally, EFGR contains a juxtamembrane nuclear localization signal (NLS) [11]. The NLS domain in EGFR is essential for its correct

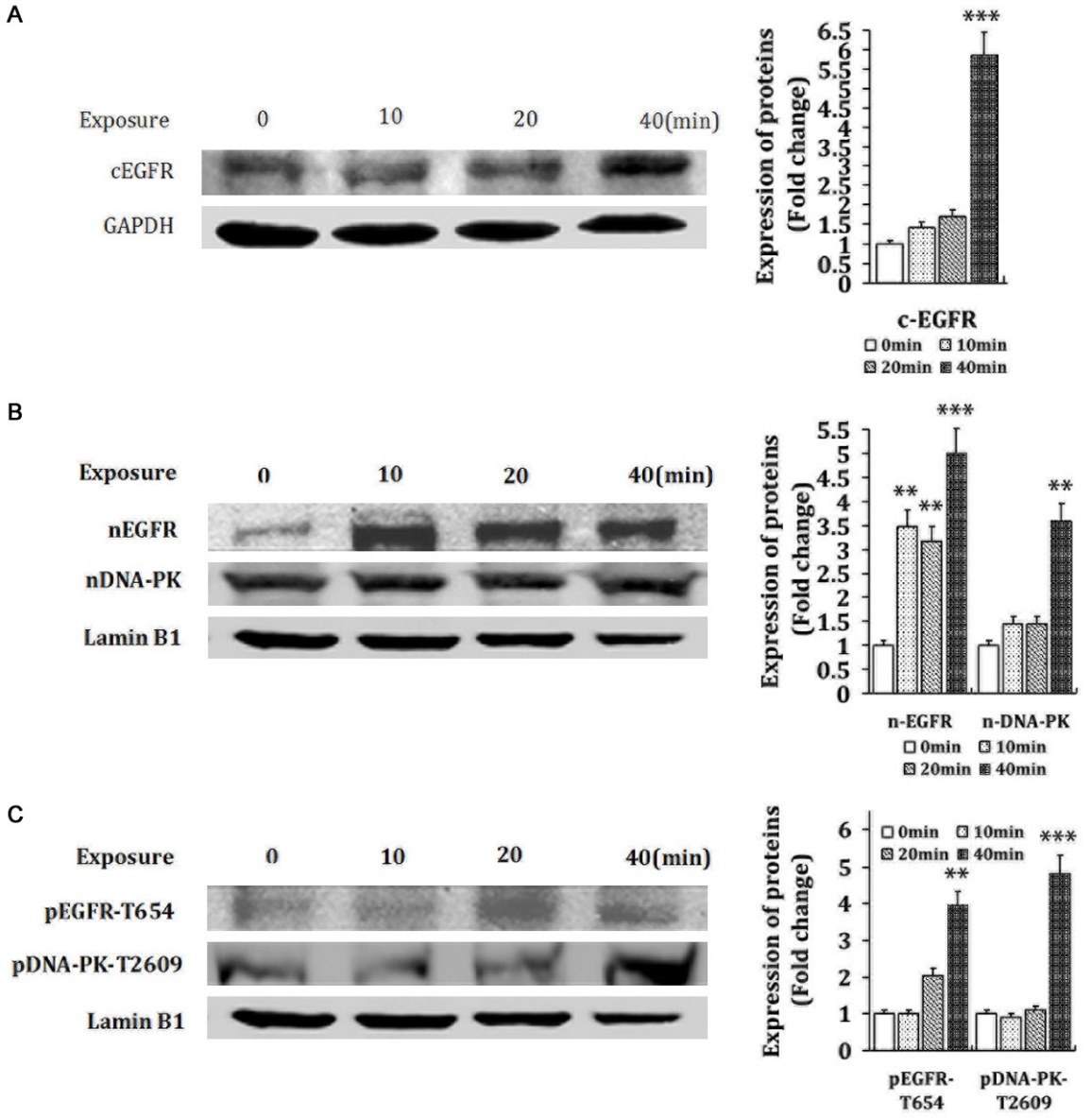

Figure 1. A. Expression level alterations of cytosolic EFGR (c-EGFR), c-PDK1 and c-PKN1 in CaSki cells after exposure to irradiation. B. Dynamic expression levels of nuclear EGFR (n-EGFR), n-PDK1, n-PKN1 and n-DNA-PK in CaSki cells after irradiation. C. Expression level changes of phosphorylated EGFR at Thr654 (pEGFR-T654), pPDK1-Ser241, pPKN1-Thr774, and pDNA-PK-Thr2609 after irradiation. **P*<0.05, ***P*<0.01, ****P*<0.001, compared with untreated cells (group at zero-minute).

translocation, and the residue Thr654 in NLS has been proven to play a central role in nuclear transport [12]. The deletion of NLS in EGFR-decreased DNA repair of radiation induces double strand breaks and increases the sensitivity to chemotherapy [13]. With DNA-dependent protein kinase (DNA-PK), DNA repair after therapies can come true [14, 15]. After radiation, DNA-PK and nuclear EGFR could form a complex in the nucleus to enhance DNA-PK activity and DNA repair [16]. In order to understand the molecular mechanism of nuclear EGFR transport, Dittmann *et al.* demonstrated that threonine at position 654 in EGFR was phosphorylated when radiation-induced nuclear transport and DNA damage repair was initiated [17].

Though there are some studies about EGFR in cervical cancer, studies focusing on the role of EGFR nuclear translocation in radioresistance for cervical cancer and the relationship between EGFR nuclear translocation and DNA-PK

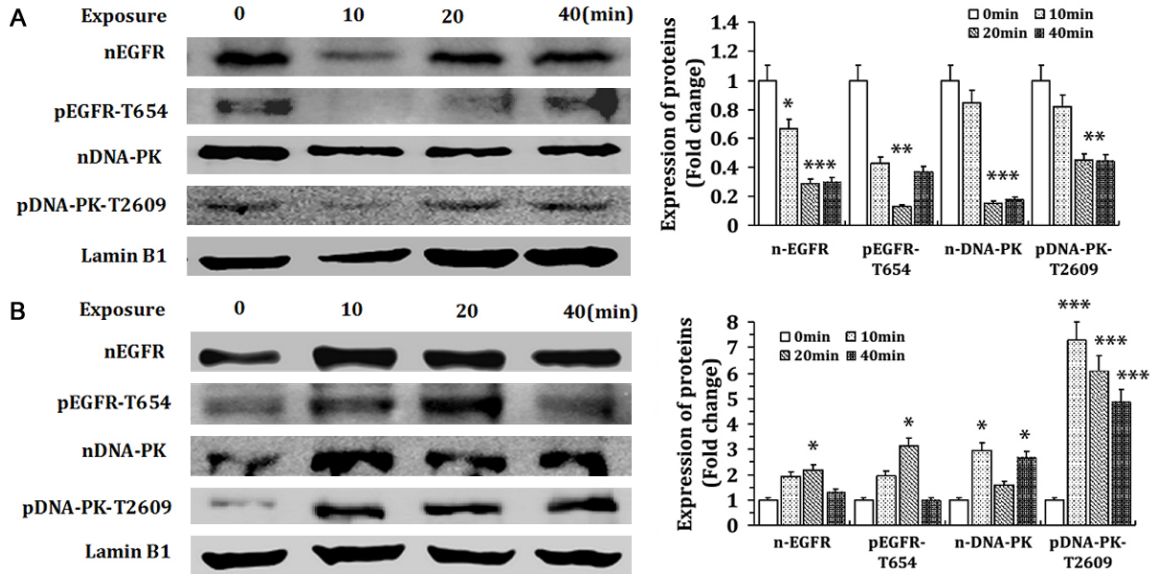

Figure 2. A. Dynamic expression levels of n-EGFR, pEGFR-T654, n-DNA-PK and pDNA-PK-T2609 after irradiation exposure in the presence of the NLS peptide inhibitor. B. Expression alterations of n-EGFR, pEGFR-T654, n-DNA-PK and pDNA-PK-T2609 after irradiation with the treatment of the control peptide. **P*<0.05, ***P*<0.01, ****P*<0.001, compared with untreated cells (group at zero-minute).

are still very few. In this study, the expression pattern and translocation of EGFR in irradiation-exposed CaSki cells were detected. The involvement of critical factors, such as DNA-PK [15], pEGFR-T654 [12] and pDNA-PK-T2609 [18] in irradiation-evoked automatic DNA repair in cervical cancer cells, were also studied. This study may provide a promising insight to understand the molecular mechanism of radioresistance in the treatment of cervical cancer, and may improve the efficiency of cancer therapies, especially for patients at the advanced stage.

Materials and methods

Cell culture and irradiation

Human cervical cancer cell line CaSki was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 units/ml of penicillin and gentamicin at 37°C with 5% CO_2. Cells were exposed to a single dose of X-rays of 4 Gy using the linear accelerator at a dose rate of 450 cGy/min.

NLS peptide inhibitor

EGFR NLS peptide inhibitor (RKRT (PO_3H_2) LRRLK) and control peptide (KKALRRQEAVNAL) were synthesized by GL Biochem (Shanghai, China). Cells were treated with 5 μM of peptide for 16 hours. The function of the inhibitor and control peptides was confirmed in a previous report [12]. The inhibitor peptide with phosphorylated T654 (corresponding to the EGFR NLS) impaired the nuclear EGFR transport, while, in order not to affect the EGFR translocation, the control peptide did not compete with EGFR phosphorylated at T654.

Western blotting

According to manufacturer's instructions (Life Technologies), cytosolic and nuclear proteins were extracted at 10, 20, or 40 minutes after irradiation with a cytosolic and nuclear protein extract kit. Then, 2 μg of cell lysates were loaded on each lane of 10% polyacrylamide gel, and blotted onto a polyvinylidene difluoride (PVDF) membrane. After blocking with a phosphate buffered saline Tween-20 (PBST) containing 5% nonfat dry milk, the membrane was incubated with antibodies against EGFR (BD Biosciences), pEGFR-Thr654 (Millipore), DNA-PK (Epitmics), or pDNA-PKcsThr2609 (Abcam) overnight at 4°C. Peroxidase-linked anti rabbit or mouse IgG (Life Technologies) were used as secondary antibodies. LaminB1 and GAPDH (Epitmics) served as the loading controls. Proteins were detected using an ECL western blotting detection kit (Amersham Biosciences). All protein

bands were visualized through the FluorChem MI imaging system (Alpha Innotech Santa Clara, CA).

Statistical analysis

The relative density of the protein expression level was analyzed by ImageJ. One-way ANOVA was used to determine significant differences by GraphPad Prism software 6.0. *P*-value <0.05 was considered statistically significant. All calculation was conducted by SPSS 18.0.

Results

Expression of EGFR in the nucleus and cytoplasm increased after irradiation

It was found that the expression level of cEGFR gradually increased with the extension of exposure time (**Figure 1A**). Notably, the expression of cEGFR significantly increased at 40 min after irradiation, compared to untreated cells ($P<0.001$). However, there was no significant change in cEGFR at 10 or 20 minutes after irradiation.

Similarly, the expression level of nEGFR in CaSki cells was found to be increased after irradiation. The expression level of nEGFR gradually increased and reached its maximum at 40 minutes after irradiation (**Figure 1B**, $P<0.001$). However, the level of cEGFR significantly increased at 10 and 20 minutes after irradiation, compared to the unexposed cells ($P<0.01$). These results indicated that the accumulation of EGFR was faster in the nucleus than that in the cytoplasm of irradiation-exposed human cervical cancer cells.

Irradiation enhanced the phosphorylation level of EGFR at Thr654

In the present study, it was found that irradiation induced the phosphorylation of EGFR at Thr654, a key amino acid residue responsible for the transport of EGFR into the nucleus. The phosphorylation of EGFR at Thr654 drastically increased at 20 ($P<0.05$) and 40 ($P<0.01$) minutes after exposure (**Figure 1C**), indicating the initiation of the nuclear transport of EGFR. This finding was consistent with the elevated expression level of nEGFR immediately after irradiation (**Figure 1B**). DNA repair in cervical cancer cells was promoted after irradiation.

Resistance to irradiation is a critical issue in the treatment of cervical cancer. After irradiation for 40 minutes, the significant increasing expression level of nuclear DNA-dependent kinase (DNA-PK) was observed (**Figure 1B**, $P<0.01$). However, no increased expression was found at 10 or 20 minutes after exposure. These results demonstrated that long term exposure to irradiation induced automatic DNA repair, which was likely to be a key factor that causes the resistance to irradiation. Furthermore, it was also found that the expression level of the phosphorylation of DNA-PK at T2609 was elevated at 40 minutes after irradiation (**Figure 1C**, $P<0.001$).

The inhibition of EGFR nuclear translocation attenuated self-repair in CaSki cells

Then, the effect of EGFR nuclear translocation inhibition on the self-repair process of cervical cancer cells after irradiation was determined. With the treatment of NLS peptide inhibitor, the expression level of EGFR in the nucleus gradually decreased with the extension of exposure to irradiation (**Figure 2A**). The expression of nEGFR was significantly lower at 10 ($P<0.05$), 20, or 40 ($P<0.001$) minutes after irradiation, compared to untreated cells, which indicated the failure in EGFR translocation into the nucleus. This conclusion was further confirmed by the observation that pEGFR-T654 levels also decreased when CaSki cells were exposed to irradiation (**Figure 2A**, $P<0.01$). Furthermore, the ability to repair DNA damage was impaired due to the lower expression of n-DNA-PK after irradiation in the presence of the NLS peptide inhibitor. In addiction, the expression level of DNA-PK significantly decreased at 20 or 40 minutes when cells were exposed to irradiation (**Figure 2A**, $P<0.001$). The phosphorylation level of DNA-PK at Thr2609 also decreased due to radiation exposure (**Figure 2A**, $P<0.01$), indicating that the EGFR-DNA-PK complex-mediated DNA repair was weakened. In order to further prove the specificity of the effect of the NLS peptide inhibitor, we designed a control peptide. In contrast to the peptide inhibitor, it was found that treatment with the control peptide did not reversely affect the expression level of nEGFR, pEGFR-T654, n-DNA-PK, or pDNA-PK-T2609 (**Figure 2B**). However, a decreasing expression level was observed when exposure time was prolonged to 40 minutes. This result

was probably due to the cytotoxic effect of the control peptide. Collectively, these data revealed that phosphorylation at T654 was closely correlated with EGFR nuclear translocation induced by irradiation, and the inhibition of EGFR nuclear translocation specifically counteracted the DNA repair in irradiated cervical cancer cells.

Discussion

Cervical cancer is the leading cause of mortality for females worldwide. Though the widely use of radiotherapy contributes a lot to the treatment of cervical cancer, it shows poor prognosis and high mortality mainly due to resistance to irradiation and metastasis, especially for the advanced stage. In the present study, we determined the role of EGFR nuclear translocation in the radiosensitivity of cervical cancer cells by detecting the expression pattern and translocation of EGFR in irradiation-exposed CaSki cells and we also determined the involvement of critical factors such as DNA-PK, pEGFR-T654 and pDNA-PK-T2609 in irradiation-evoked automatic DNA repair in cervical cancer cells.

First, we studied the expression of EGFR in the nucleus and cytoplasm increased after irradiation. Results showed that irradiation could promote EGFR nuclear translocation in CaSki cells, along with the elevated expression of DNA-PK, a key kinase responsible for DNA repair. EGFR was phosphorylated at different residues following irradiation such as Y992/Y1173 [19] and Y845/Y1068 [20]. Previous reports have shown that the phosphorylation of EGFR at Tyrosine 654 is vital to nuclear transport and DNA damage repair in radiated human bronchial carcinoma cells [12, 17]. A similar observation was found in cervical cancer cells in our study.

Secondly, we found that the irradiation could promote the phosphorylation of EGFR at T654 and DNA-PK at T2609, which were two critical molecular mechanisms of EGFR nuclear transport and DNA damage repair. This probably resulted from the activation of PKCε and the modulation of other kinase activities [21]. Accordingly, the signaling transductions responsible for Thr654 phosphorylation needs to be further explored, potentially including kinase activity regulation, the blockage of EGFR degradation, and tyrosine kinase activation [22, 23].

DNA-dependent protein kinase (DNA-PK) is a serine/threonine kinase that has been believed to play a major role in repairing double strand DNA breaks [24, 25]. High expression levels of DNA-PK have been found in multiple cancers [26]. In contrast, decreased expression levels of DNA-PK have been reported in ovarian cancers, which were associated with tumor progression and metastasis [26]. This paradox for DNA-PK was likely to attribute to tissue specificity. From the present study, high expression levels of DNA-PK were detected in cervical cancer cells after long exposure to irradiation, and failure in EGFR nuclear translocation led to the decreased expression level of DNA-PK, indicating its stimulative role in cervical tumor progression. Threonine 2609 was responsible for DNA-PK activity. Failure in the phosphorylation of DNA-PK-T2609 made it lose its activity, and decrease survival after irradiation in bronchial and breast cancer cells [14, 24]. Consistent with the above findings, our study correlated the EGFR nuclear translocation with the expression of pDNA-PK-T2609, which demonstrated that EGFP translocation was an important regulator to the radiosensitivity of cervical cancer cells. This was probably due to the formation of the EGFR/DNA-PK complex [16], as well as the increasing level of nucleus EGFR, which led to the promotion of cell proliferation and angiogenesis, and the activation and stabilization of proliferating cell nuclear antigens [27]. A previous study reported that the phosphorylation of DNA-PK at T2609 was an outcome of the EGFR-DNA-PK binding in the nucleus, repairing the damaged DNA in cancer cells [28]. This study may further prove that DNA repair in cervical cancer cells was promoted after irradiation evoked by EGFR nuclear translocation.

At last, we demonstrated that the inhibition of NLS, which is essential for the correct nuclear translocation, prohibited EGFR transport to the nucleus and DNA repair. The NLS sequence of EGFR is comprised with 13 amino acids (645 to 657, RRRHIVRKLLRR) [29]. NLS is indispensable for receptor activation [30]. In addition, we also found that NLS is critical to EGFR nuclear transport and the activation of DNA-PK after irradiation in cervical cancer cells. This is consistent with the dysfunction of EGFR mutants (at the NLS region) in nucleus translocation and DNA repair after chemotherapy in NIH3T3 mouse fibroblast cells [28].

In conclusion, we conducted a study to elucidate the correlation between EGFR translocation and its sensitivity to irradiation. Results suggested that nuclear EGFR and its translocation played a significant role in sensitivity to irradiation in cervical cancer cells and the NLS of EGFR was a critical factor in the phosphrylation of EFGR and DNA-PK when exposed to irradiation. These results may provide deeper insights for radioresistance in the treatment of cervical cancer and may give a potential target to the treatment of cervical cancer in the future.

Disclosure of conflict of interest

None.

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