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ORIGINAL ARTICLE

PTK7 regulates radioresistance through nuclear factor-kappa B in esophageal squamous cell carcinoma

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Abstract Tumor radioresistance is a major reason for decreased efficiency of cancer radiation therapy. Although a number of factors involved in radioresistance have been identified, the molecular mechanisms underlying radioresistance of esophageal squamous cell carcinoma (ESCC) have not been elucidated. In this study, we investigated the role of oncogenic protein tyrosine kinase 7 (PTK7) in the resistance of ESCC to radiation therapy. ESCC cell lines with high PTK7 expression were more refractive to radiation than those with low PTK7 levels. In radioresistant ESCC cells, PTK7 knockdown by specific siRNAs decreased the survival of irradiated cells and increased radiation-induced apoptosis, while in radiosensitive ESCC cells, PTK7 overexpression promoted cell survival and inhibited radiation-induced apoptosis. We hypothesized that PTK7 could regulate the activation of transcription factor NF-kB known for its role in cancer radioresistance. Our results indicated that the inhibition of PTK7 suppressed nuclear translocation of NF-kB subunit p65 induced by radiation, suggesting relevance of PTK7 expression with NF-kB activation in radioresistant ESCC. Furthermore, the levels of inhibitor of apoptosis proteins (IAPs), XIAP, and survivin, encoded by NF-kB-regulated genes, were induced in irradiated radioresistant cells but not in radiosensitive cells, while PTK7 knockdown downregulated IAP expression. Our findings revealed a novel mechanism underlying radioresistance in ESCC, which is associated with PTK7 and NF-kB-dependent apoptosis. These results suggest that the manipulation of PTK7 expression can be instrumental in enhancing ESCC response to radiotherapy. This study demonstrates that PTK7 plays a significant role in ESCC radioresistance via the NF-kB pathway.

Keywords PTK7 · Radioresistance · NF-kB · Apoptosis · ESCC

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Introduction

Esophageal squamous cell carcinoma (ESCC), one of the main pathological subtypes in esophageal carcinoma, has high incidence in East Asian countries. Although remarkable progress has been achieved in ESCC diagnostics and therapy, the prognosis of invasive metastatic-stage ESCC remains poor [1, 2]. One of the recommended treatments for ESCC is radiotherapy; however, therapeutic outcomes are not satisfactory because of tumor radioresistance due to multiple inherent and induced adaptive mechanisms [3–5]. Accumulating evidence suggests that diverse signaling pathways involved in DNA repair and cell apoptosis may play a role in regulating the response of ESCC to radiation therapy; however, their specific contribution remains unknown [6–8]. Elucidation of these molecular processes may be instrumental in identifying potential molecular targets which should be selectively manipulated to ensure the success and/or predict the outcome of radiation therapy.

Previous studies have identified a number of oncogenes involved in increased survival of irradiated cancer cells. Thus, overexpression and activation of such oncogenes as Ras, Raf, Sis, and Src boosted cancer radioresistance [9–11]. A similar role in inducing radioresistance has been reported for various receptor tyrosine kinases known for their oncogenic potential in many cancers. For example, vascular endothelial growth factor receptor 1 (VEGFR1/FLT1) has been identified as an important regulator of cell survival and modulator of radioresistance through autocrine production of its ligands VEGFA and VEGFB [12]. It has been shown that p21-activated Ser/Thr kinase 1 (PAK1) was translocated to the nucleus of irradiated cells, where it promoted the expression of epithelial-to-mesenchymal transition markers, inducing radioresistance [13]. One of the candidate tyrosine kinases with potential involvement in radioresistance is protein tyrosine kinase 7 (PTK7). Several studies have reported that PTK7 is overexpressed and has oncogenic potential in many types of cancer, including ESCC as evidenced by the fact that PTK7 downregulation inhibited ESCC cell proliferation, migration, and invasion in vitro [14]. Furthermore, PTK7 has been shown to decrease sensitivity to anthracycline chemotherapy in acute myeloid leukemia [15] and breast cancer [16], suggesting its association with drug resistance of cancer cells. However, the role of PTK7 in radioresistance has not been investigated.

Nuclear factor-kappa B (NF-kB) is a transcription factor complex activated by cellular stresses, including radiation and chemotherapeutic agents; upon induction, NF-kB translocates to the nucleus where it binds to specific DNA sequences in target genes involved in carcinogenesis, cell survival, and growth regulation [17, 18]. The genes regulated by NF-kB encode Bcl-2, X-linked inhibitor of apoptosis protein (XIAP), survivin, and AKT kinase which control chemo- and radioresistance via inhibition of apoptotic pathways in various tumor cells [19–21]. There are reports indicating that NF-kB is a central player in the molecular mechanisms underlying radioresistance in various cancers. In breast cancer, NF-kB caused transactivation of β1-integrin conferring radioresistance to cancer cells [22], while in lung cancer cells, phosphorylation and dissociation of the ribosomal protein S3-TRAF2 complex induced radioresistance mediated by NF-kB activation [23]. However, the cooperative mechanisms of radioresistance controlled by NF-kB in ESCC remain to be elucidated.

In this study, we aimed to investigate the relationship between NF-kB and PTK7 in radioresistant ESCC cells. We showed that PTK7 expression positively affected the survival of irradiated ESCC by inhibiting NF-kB-regulated apoptosis, suggesting that PTK7 has a potential as a candidate target for improving the efficiency of radiotherapy in ESCC.

Material and methods

Cell lines and culture conditions

Ten human ESCC cell lines (TE-1, TE-4, TE-5, TE-6, TE-8, TE-9, TE-10, TE-11, TE-14, and TE-15) were purchased from RIKEN. All ESCC cells were cultured in RPMI-1640 (Corning) supplemented with 10 % FBS (Hyclone) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin, Corning) at 37 °C in the atmosphere of 5 % CO_2/95 % air.

Irradiation and cell survival

ESCC cells were plated at 5×10^2 cells (TE-8 and TE10) or 2×10^3 cells (TE-4 and TE-14) per well in six-well culture plates for 24 h and exposed to different radiation doses (0, 2, 4, 6, and 8 Gy) using a ^{137}Cs γ-ray source (Atomic Energy of Canada Ltd.) at a dose rate of 3.81 Gy/min. After 14 days of culture, cells were fixed, stained with 2 % crystal violet, and cell colonies were photographed and counted using the ImageJ software.

Transfection

4×10^5 cells were seeded on six-well plates 1 day before transfection to reach 60–80 % confluence the next day. Then, cells were rinsed with medium and transfected in Opti-MEM medium with the PTK7 expression plasmid (pcDNA3-PTK7) or vehicle (pcDNA3) [24], or two PTK7-specific siRNAs (Bioneer; Cat. No. 100481 and 100483) or control siRNA (Bioneer; Cat. No. SN1003) using Lipofecatimine 2000 (Invitrogen). After 6 h, cells were changed to complete growth medium. On the third day after transfection, cells were harvested and managed for further experiments.

Antibodies and reagents

An anti-PTK7 antibody was purchased from R&D Systems, while PARP, caspase-3, p65, XIAP, and survivin antibodies were obtained from Cell Signaling Technology, and lamin B antibody and NF-kB-specific inhibitory peptide SN50 (sc-3060) were from Santa Cruz Biotechnology. β-Actin and α-tubulin antibodies were purchased from Sigma and Millipore, respectively.

Western blotting

For western blot analysis, cells were washed with cold PBS and lysed in RIPA buffer (Thermo Fisher Scientific). Protein concentration was quantified using the Bradford method, and equal amounts of protein were resolved by SDS-PAGE and analyzed by western blotting as previously described [25]. Primary antibodies were applied overnight at 4 °C, and a

secondary antibody (Santa Cruz Biotechnology) was applied for 1 h at RT. Proteins were visualized using enhanced chemiluminescence (ECL; Thermo Fisher Scientific), and relative protein expression was calculated after normalization to β-actin used as loading control.

Apoptosis assay

Irradiated cells were stained with fluorescein isothiocyanate (FITC)-labeled annexin V/propidium iodide (PI) using the Apoptosis Detection kit (BD Pharmingen) according to the manufacturer's instructions and analyzed by flow cytometry (BD Biosciences).

Cell fractionation

Cellular nuclear and cytoplasmic fractions were separated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) according to the manufacturer's instructions. α-Tubulin and lamin B were used as cytoplasmic and nuclear markers, respectively.

Immunofluorescence

Cells were seeded at a density of 5×10^2 cells per chamber in eight-well glass chamber slides (Labtek). Cells were rinsed with PBS three times, fixed with 4 % paraformaldehyde for 15 min, and permeabilized with 0.2 % Triton X-100 in PBS. Cells were blocked with 5 % BSA in TBS for 1 h at RT and incubated with primary antibodies against PTK7 (500:1) and p65 (200:1) overnight at 4 °C and then Alexa-488 and Alexa-568 (500:1)-conjugated secondary antibody for 50 min (Invitrogen) at RT. Cell images were acquired by a Zeiss LSM 710 inverted laser scanning confocal microscope.

Statistical analysis

The data obtained from at three experiments were expressed as the means ± standard deviation. Statistical significance of the difference was analyzed by Student's t test, and $P < 0.05$ was considered significant.

Results

Esophageal cancer cell response to radiation depends on PTK7 expression

Our previous results indicate that PTK7 is one of the oncogenic kinases activated in ESCC [14], suggesting that it could be associated with radioresistance. Western blotting analysis of PTK7 expression in ESCC cells showed that among 10 ESCC cell lines, eight and two had high and low PTK7 levels, respectively (Fig. 1a). To determine whether PTK7 expression was associated with ESCC sensitivity to radiation, we exposed cells with high and low PTK7 expression to various radiation doses and analyzed cell survival using the colony formation assay. The results indicated that cells with high PTK7 levels (TE-8 and TE-10) exhibited more colonies and had better survival rates than those with low PTK7 expression (TE-4 and TE-14) (Fig. 1b). To confirm the role of PTK7 in radioresistance, endogenous PTK7 was knocked down in TE-10 cells using two specific siRNAs and irradiated cells were analyzed for survival. PTK7 depletion in combination with radiation reduced clonogenic growth of TE-10 cells compared with that of control siRNA-treated cells (Fig. 2a). On the other hand, PTK7 overexpression in irradiated TE-14 cells resulted in the induction of clonogenic growth (Fig. 2b). These results suggest that PTK7 regulated radioresistance in ESCC cells.

PTK7 expression is related to radiation-induced apoptosis

Because TE-10 and TE-14 cells exhibited different responses to radiation, we used them as models for radioresistant and radiosensitive ESCC, respectively. To evaluate radiation effects on apoptosis in TE-10 and TE-14 cells, they were analyzed by FACS after annexin/PI staining. The level of apoptosis in irradiated TE-14 cells with low PTK7 expression (42.7 %) was significantly higher than that in TE-10 cells with high PTK7 expression (23.5 %). We also examined the presence of cleaved PARP and caspase-3 used as indicators of apoptosis. The results showed that irradiated TE-14 cells demonstrated higher levels of cleaved PARP and especially of cleaved caspase-3 than irradiated TE-10 cells, and the effect was radiation dose-dependent (Fig. 3a). To further examine the association of PTK7 with apoptosis in irradiated ESCC cells, PTK7 expression was reduced in TE-10 cells and induced in TE-14 cells using specific siRNAs or the PTK7 expression plasmid, respectively. PTK7 knockdown resulted in the increased apoptosis rate compared with the control group after irradiation (22.8 % and 19.7 % vs 15.5 %, respectively), which corresponded to the increased cleavage of PARP and caspase-3 (Fig. 3b). On the other hand, PTK7 overexpression in TE-14 cells decreased apoptosis from 48.1 to 35.7 % after irradiation and downregulated the cleavage of PARP and caspase-3 (Fig. 3c). These results suggest that PTK7 regulates radiation-induced apoptosis in ESCC cells.

PTK7 mediates radiation-induced nuclear translocation of p65

NF-kB pathway is known to be involved in radioresistance in many types of cancers as evidenced by nuclear

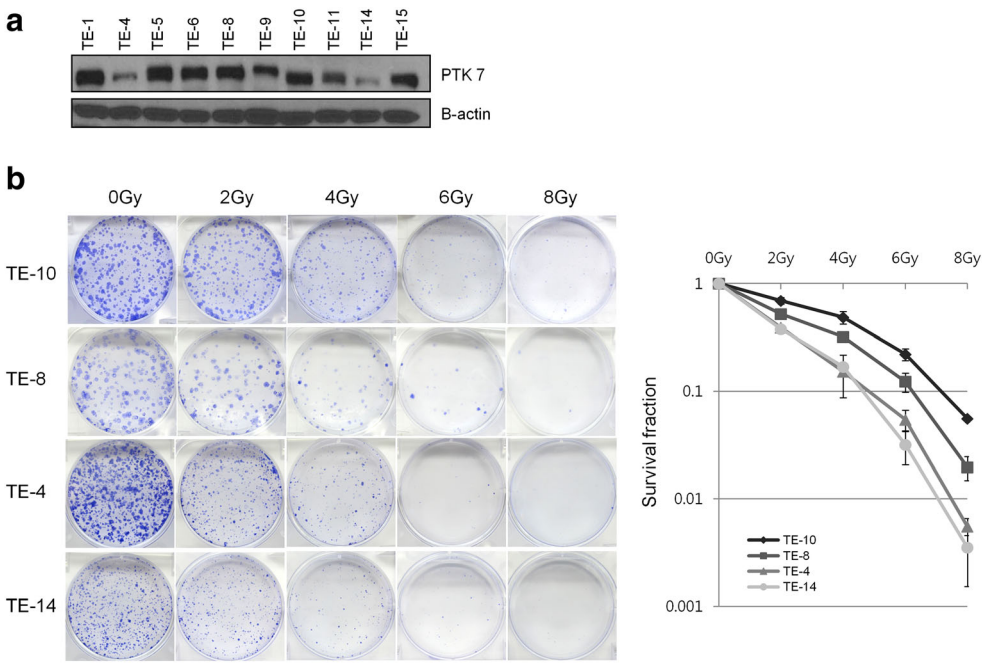

Fig. 1 ESCC cell lines with high PTK7 expression have enhanced resistance to radiation. **a** PTK7 expression in 10 ESCC cell lines examined by western blotting. **b** ESCC cells were seeded on six-well plates and subjected to the indicated doses of radiation. Cell colonies were counted on day 14 after irradiation using the ImageJ software, and the fraction of surviving cells was calculated. The data are presented as the means ± SD of triplicate measurements from a single experiment

translocation of NF-kB subunit p65 in cancer cells resistant to radiation-induced apoptosis [22, 26]. Therefore, we examined the involvement of NF-kB signaling in PTK7-associated radioresistance of ESCC using inhibiting p65 nuclear translocation with cell-permeable synthetic peptide SN50 in TE-10 cells. FACS analysis indicated that in SN50-treated irradiated cells, apoptosis was induced compared with SN50-untreated irradiated cells and negative

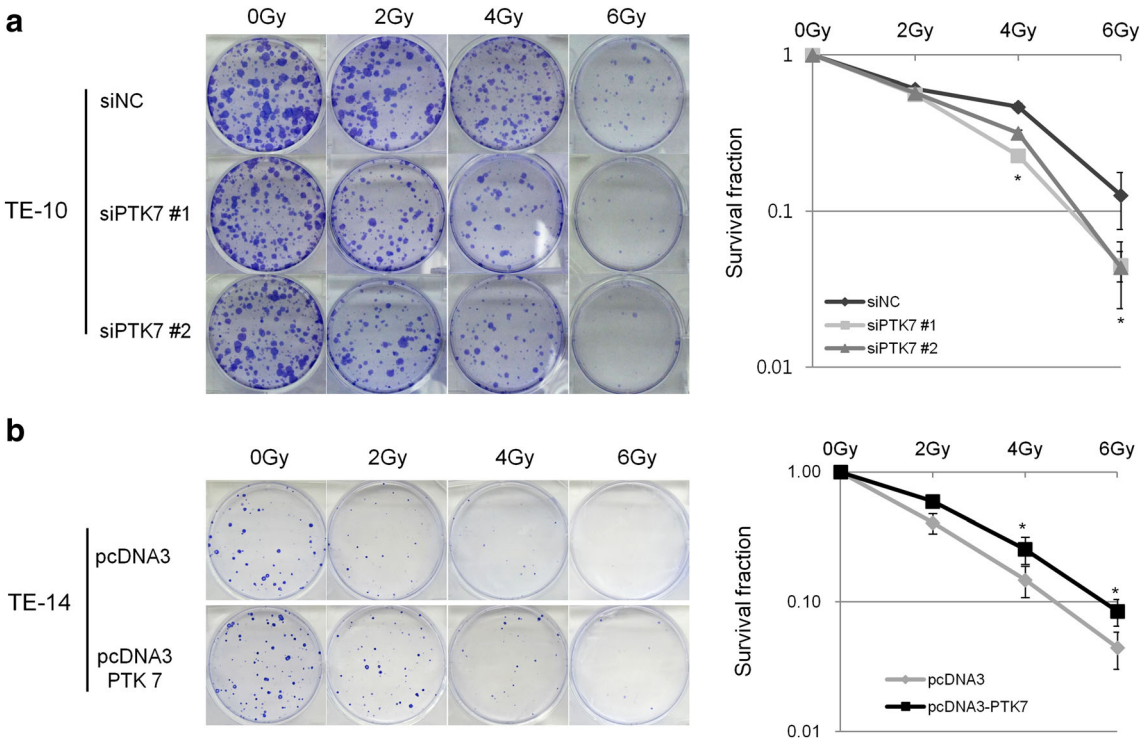

Fig. 2 PTK7 controls radiosensitivity of ESCC cells. **a** Radioresistant TE-10 cells were transfected with the control (siNC) or PTK7-specific siRNAs (siPTK7) and subjected to the indicated doses of radiation. **b** Radiosensitive TE-14 cells were transfected with the control plasmid (pcDNA3) or PTK7-expressing plasmid (pcDNA3-PTK7) and subjected to the indicated doses of radiation. Non-irradiated cells (0 Gy) were used as control. Cell colonies were counted on day 14 after irradiation using the ImageJ software, and the fraction of surviving cells was calculated. The data are presented as the means ± SD of triplicate measurements from a single experiment. *$P < 0.05$ versus control

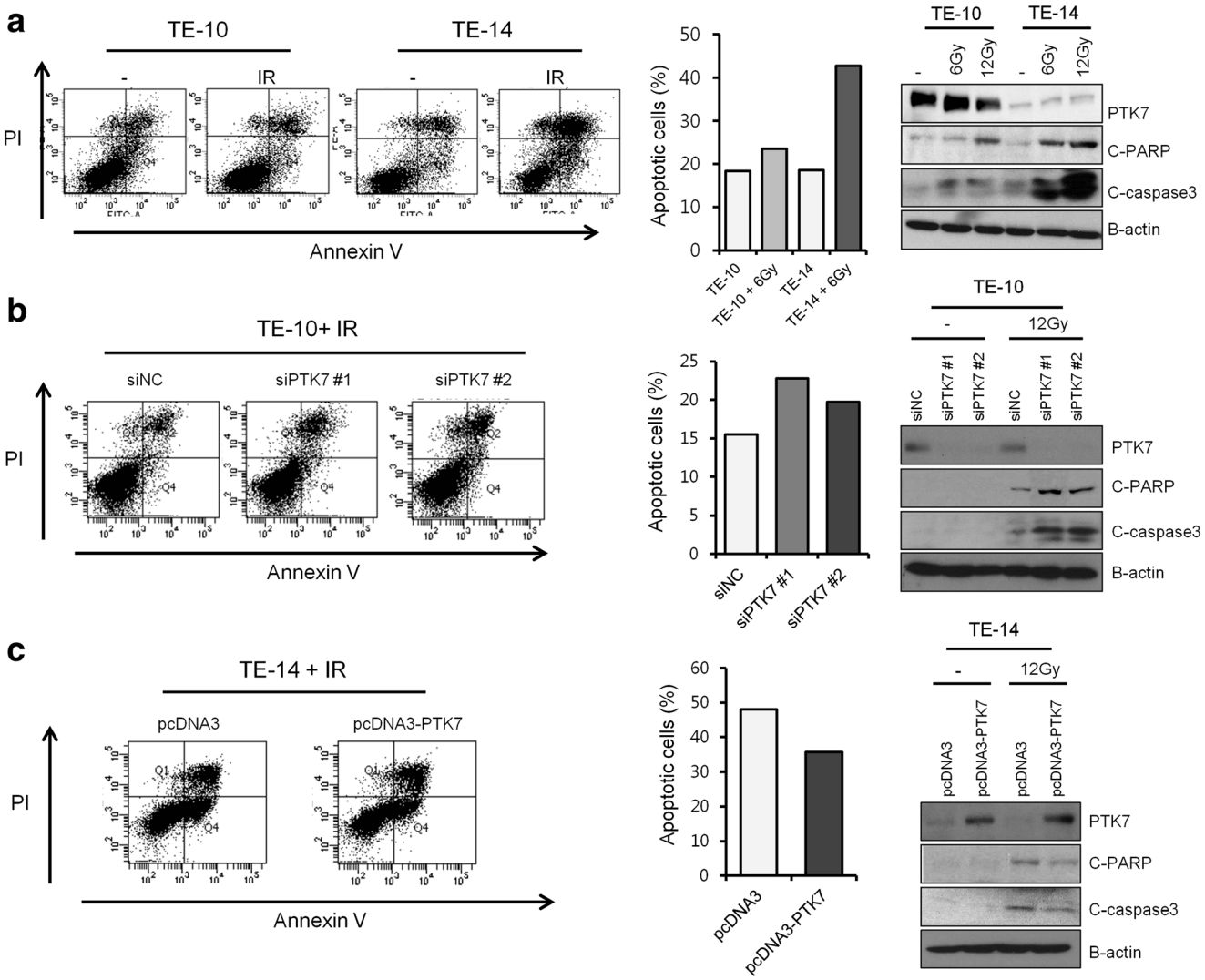

Fig. 3 PTK7 regulates radiation-induced apoptosis. **a** Radioresistant TE-10 cells and radiosensitive TE-14 cells were treated with the indicated doses of radiation and analyzed for the expression of cleaved (C) caspase-3 and PARP by western blotting. **b** TE-10 cells were transfected with the control (siNC) or PTK7-specific siRNAs (siPTK7), irradiated (12 Gy), and analyzed for the proportion of apoptotic cells by flow cytometry and for the expression of C-caspase-3 and C-PARP by western blotting. β-Actin was used as the loading control. **c** TE-14 cells were transfected with the control plasmid (pcDNA3) or PTK7-expressing plasmid (pcDNA3-PTK7), irradiated (12Gy), and analyzed for apoptosis and expression of C-caspase-3 and C-PARP. *PI* propidium iodide, *IR* ionizing radiation

control (25.7 % vs 17.7 % and 9.3 %, respectively), which corresponded to the increase in PARP and caspase-3 cleavage (Fig. 4a). These results indicate that the inhibition of p65 nuclear translocation increased radiation-induced apoptosis in radioresistant ESCC cells. To determine, whether PTK7 was involved in the regulation of p65 nuclear translocation in irradiated cells, we assessed p65 levels in the nuclear fraction of TE-10 cells transfected with PTK7-specific siRNAs by western blotting and found that p65 nuclear levels were significantly reduced compared to the wild-type cells (Fig. 4b). Similar results were obtained using immunofluorescence (Fig. 4c). Taken together, these findings indicated that radiation-induced nuclear translocation of p65 was regulated by PTK7.

PTK7 controls the expression of inhibitor of apoptosis proteins (IAPs) in radioresistant cells

NF-kB activation triggered by radiation induces the transcription of many genes involved in cell growth, inflammation, and apoptosis [17]. Therefore, we examined the expression of IAPs, survivin, and XIAP known to be regulated by NF-kB and to block apoptosis via cooperative interactions with other partners [27, 28]. As shown in Fig. 5a, radiation-induced nuclear translocation of p65 was observed only in radioresistant TE-10 cells but not in radiosensitive TE-14 cells. Similarly, radiation upregulated the expression of IAPs only in TE-10 cells (Fig. 5a); however, the increasing IAPs were reduced by

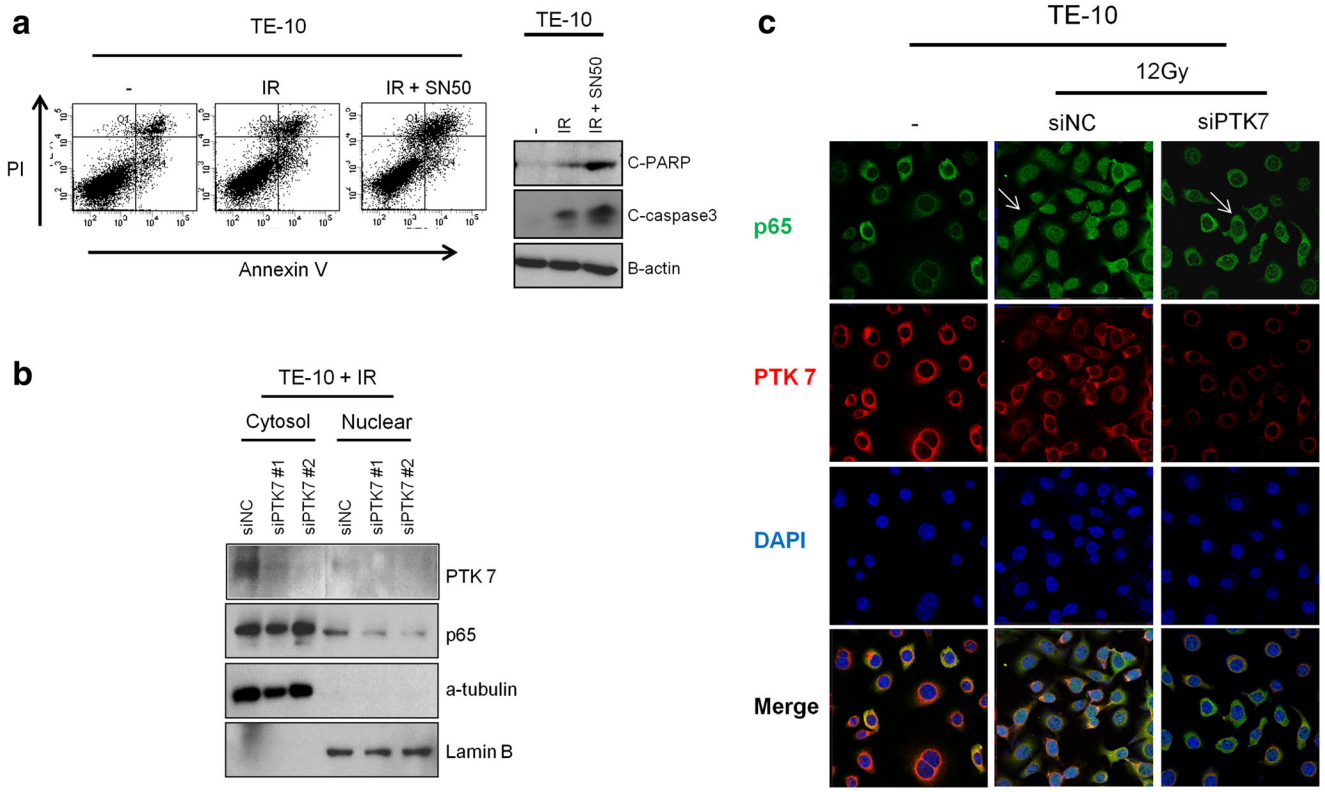

Fig. 4 Inhibition of PTK7 suppresses nuclear translocation of p65. **a** TE-10 cells were treated with synthetic NF-kB inhibitory peptide SN50 (40 uM) for 1 h, exposed to radiation (12 Gy), and analyzed for apoptosis by flow cytometry and for cleaved (C) caspase-3 and PARP by western blotting after 3 days. **b** TE-10 cells were transfected with the control (siNC) or PTK7-specific siRNAs (siPTK7) and analyzed for p65 levels in the cytosolic and nuclear fractions by western blotting. α-Tubulin and lamin B were used as cytosolic and nuclear markers, respectively. **c** TE-10 cells transfected as above were irradiated (12 Gy) and analyzed for the expression of p65 (*green*) and PTK7 (*red*) by immunofluorescence. *Arrows* indicate nuclear p65

PTK7 inhibition with specific siRNAs (Fig. 5b). These results suggest that PTK7 regulates radiation-induced nuclear translocation of p65 and the expression of IAPs in radioresistant cell.

Discussion

Radiotherapy plays an important role in the treatment of esophageal cancers as a part of combined-modality therapy

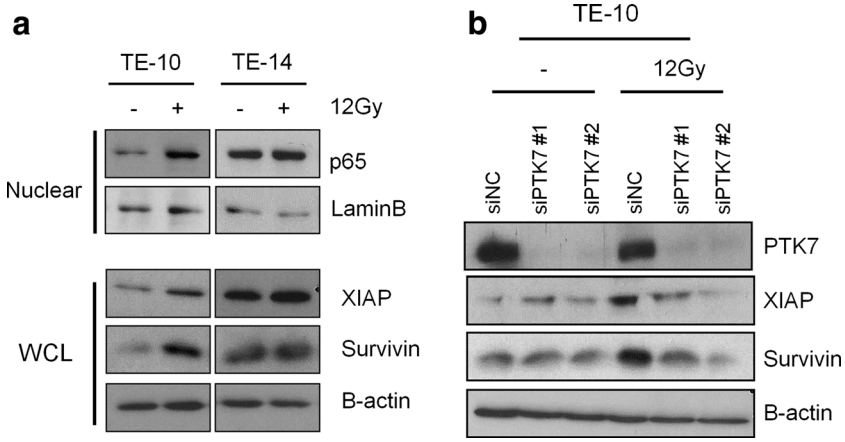

Fig. 5 Radiation-induced expression of inhibitors of apoptosis in radioresistant ESCC cells is regulated by PTK7. **a** Radioresistant TE-10 cells and radiosensitive TE-14 cells were subjected to irradiation with 12 Gy and analyzed for the presence of p65 in the nuclear fraction (Nuclear) and in the whole cell lysate (WCL) by western blotting. **b** TE-10 cells were transfected with the control (siNC) or PTK7-specific siRNAs (siPTK7), irradiated (12 Gy), and analyzed for PTK7, XIAP, and survivin expression by western blotting. β-Actin was used as the loading control

together with chemotherapy and surgery. This treatment approach has improved local tumor control and survival rates [29, 30]; however, tumor recurrence due to radioresistance still occurs in a high proportion of patients, emphasizing the need to develop therapeutic strategies to improve the response to radiation in esophageal cancers [31]. Here, we described a novel mechanism of radioresistance in ESCC involving PTK7 and NF-kB. Our results demonstrate that (1) ESCC cell lines have different sensitivity to radiation according to PTK7 expression levels; (2) PTK7 inhibition promotes radiation-induced apoptosis by blocking nuclear translocation of p65; and (3) radiation induces p65 nuclear translocation and upregulates IAPs only in radioresistant ESCC cells with high PTK7 expression.

Here, we revealed, for the first time, a potential role of PTK7 in the radioresistance of ESCC, showing that ESCC cells with high PTK7 levels are more refractory to radiation treatment than those with low PTK7 expression and respond to radiation by increased nuclear translocation of p65 and upregulation of IAPs. At present, there are no molecular targets for radiosensitization in ESCC and our results indicate a potential for PTK7 to serve as such, considering that siRNA-mediated PTK7 knockdown promoted radiation-induced apoptosis. Further experiments using in vivo models are required to validate the role of PTK7 in the radiosensitivity of ESCC.

PTK7 is upregulated in many human cancers, including colon cancer, breast cancer, acute myeloid leukemia, and esophageal cancer [14, 15, 32, 33]. Previous studies have shown that PTK7 inhibition reduced ESCC cell proliferation, migration, and invasion by regulating the MAPK and PI3K pathways [14] and induced caspase-10-dependent apoptosis in colon cancer [34]. These signaling pathways are known to control radiosensitivity in different cancers [35, 36], so PTK7 could induce radioresistance in ESCC through regulation of MAPK, PI3K, and apoptosis signaling.

Our data indicate that PTK7 may trigger transcriptional activation of NF-kB, which results in the upregulation of XIAP and survivin and inhibition of apoptosis in ESCC. A previous study has reported that PTK7 enhanced NF-kB-dependent expression of VEGFR2 [37] shown to promote the growth of malignant astrocytoma and radioresistance in glioblastoma [38]. Targeting of the VEGF-VEGFR2 pathway leads to the inhibition of its downstream pro-survival signaling and reduces radioresistance in non-small cell lung cancer lines [39]. These findings suggest that PTK7 may also contribute to ESCC radioresistance through NF-kB-mediated upregulation of VEGFR expression. Future studies are required to test this hypothesis.

It has also been suggested that PTK7 may play a role in cancer chemoresistance [15, 16]; however, the underlying mechanisms have not been clarified. Given that NF-kB signaling is known to also inhibit chemotherapy-induced apoptosis and contribute to drug resistance in various cancers [40, 41], it is possible that PTK7 may act through the NF-kB network in enhancing both chemo- and radioresistance in cancer.

To the best of our knowledge, this is the first report of PTK7 inducing radioresistance in cancer cells. This study demonstrates that in ESCC cells, PTK7 inhibits radiation-induced apoptosis via NF-kB pathway and upregulation of XIAP and survivin, suggesting PTK7 as a potential therapeutic target for improving the response of ESCC patients to radiation therapy.

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Compliance with ethical standards

Conflicts of interest None

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