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ORIGINAL ARTICLE

PBX1 Increases the Radiosensitivity of Oesophageal Squamous Cancer by Targeting of STAT3

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Abstract

The radioresistance of oesophageal squamous cell carcinoma (OSCC) is a critical factor leading to a poor prognosis among patients. The expression of PBX1 is abnormally high in a broad range of human tissues, and this gene plays a key role in tumour proliferation. This research intended to explore the radiosensitization of OSCC by silencing PBX1. The OSCC cell lines KYSE450 and KYSE150 were subjected to PBX1 silencing and/or irradiation (IR). Cell proliferation, colony formation, and apoptosis were tested to evaluate the radiosensitization ability of PBX1 silencing. The levels of STAT3 and p-STAT3 in the OSCC cells were tested by Western blotting. Furthermore, KYSE150 cells with or without PBX1 silencing were xenografted into nude mice with or without radiation exposure. Concomitant PBX1 silencing and IR can obviously suppress growth and enhance radiosensitivity in OSCC cells and xenografts. Moreover, the downregulation of PBX1 inhibits the expression of STAT3 and p-STAT3. The downregulation of PBX1 may increase radiosensitivity in OSCC cells and xenografts via the PBX1/STAT3 pathway. Our findings demonstrate that PBX1 may be a potential target for promoting the effect of radiation therapy in OSCC patients.

Keywords Oesophageal squamous cancer · PBX1 · Radiosensitivity · STAT3

Introduction

Oesophageal cancer is a common malignant tumour. Worldwide, there are more than 400,000 new oesophageal cancer patients each year, and the incidence rate and mortality of oesophageal cancer rank sixth and eighth among malignant tumours, respectively, worldwide [1–3]. Pathologically, oesophageal cancer involves the following two main types: oesophageal squamous cell carcinoma (OSCC) and oesophageal adenocarcinoma. China is a high-risk area for oesophageal cancer, and the most prevalent pathological type is OSCC, which accounts for more than 90% of cases [4]. Patients with oesophageal cancer usually present with non-specific symptoms during the early stage, leading to disease progression and a missed opportunity for surgical resection. Therefore, radiation therapy is widely used in patients with advanced oesophageal cancer, especially OSCC [5]. However, oesophageal cancer cells are only moderately sensitive to radiation therapy [6]. Increasing the local radiation dose to improve the therapeutic effect could further damage normal tissues [6]; thus, there is an imperative need to explore key biological targets and molecular mechanisms that can increase the radiosensitivity of oesophageal cancer.

Pre-B cell leukaemia homeobox 1 (PBX1) has been reported in acute B cell leukaemia [7] and identified in the t(1; 19) chromosomal translocation site [8]. In one quarter of children, t(1; 19)(q23; p13) chromosomal translocation is correlated with the poor prognosis of pre-B cell acute lymphoblastic leukaemia due to the rearrangement of PBX1 and E2A [9], which produce transform chimeric DNA-binding proteins [10, 11]. PBX1, which is a 430-amino-acid protein, is a part of the tri-amino acid loop extension (TALE) family of typical

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homologous domain proteins whose members are characterized by the insertion of three residues in the first helix of the homologous domain [12, 13]. PBX1 is expressed in most tissues; for example, in the developing kidney, PBX1 plays a role in signalling the mesenchymal epithelia and regulating mesenchymal function [14, 15]. In addition, PBX1 plays a crucial regulatory role in tumour formation and proliferation. PBX1 can interfere with the proliferation and development of various malignant tumours, such as breast carcinoma, ovarian cancer and non-small cell lung carcinoma [16–22]. Liu Debin et al. recently found that PBX1 can be observed in the nuclei and cytoplasm of OSCC cells; in over half of 56 OSCC tissue samples, PBX1 exhibited light or strong expression [23].

Signal transducer and activator of transcription 3 (STAT3) is a major member of the signal transducer and activator of transcription (STAT) protein family and an important nuclear transcription factor. STAT3 can be regulated by various cytokines, growth factor receptors, such as Janus kinase (JAK) and epidermal growth factor receptor (EGFR), and oncoprotein activation [24, 25]. Under normal physiological conditions, STAT3 activation is rapid, transient and necessary for maintaining the physiological functions of cells and regulating cell proliferation and differentiation [6, 26]. However, if STAT3 is continuously activated, it is involved in the development of various cancers and promotes cell proliferation and survival by directly upregulating the expression of Bcl-2 and Bcl-xL. STAT3 is also recognized as a key gene responsible for increasing radiosensitivity [27, 28]. PBX1 silencing suppresses STAT3 phosphorylation and decreases the transcription of the STAT3 target gene cyclin D1, thereby suppressing malignant cell proliferation, viability and cell cycle progression [22, 29].

In this research, we investigated the increased radiosensitization effect of PBX1 silencing on OSCC in vitro and in vivo. PBX1 silencing efficiently reduces cell proliferation and promotes apoptosis. Our study demonstrated that the downregulation of PBX1 could radiosensitize human OSCC cells by targeting STAT3 signalling.

Materials and Methods

Cell Culture

The human OSCC cell lines KYSE450 and KYSE150 were purchased from Shanghai Institutes for Biological Science, Shanghai, China. The cells were cultured in RPMI-1640 (Gibco, CA, USA) medium supplemented with 10% foetal bovine serum (Gibco, CA, USA) and 1% penicillin/streptomycin (Sigma, St. Louis, USA). The cells were cultured at 37 °C in a humidified incubator with 5% CO_2. The cells and mice were treated with irradiation (IR) as previously described [30]. The cells were exposed to a single dose of 566 cGy/min through an X-ray irradiator (Elekta, Sweden). Additionally, the animals received 6 Gy (2.47 cGy/min) for tumour radiation therapy (RS-2000 Pro Biological Irradiator).

Reagents and Antibodies

The Cell Counting Kit-8 (CCK8), RIPA lysis buffer and BCA kit were acquired from the Beyotime Institute of Biotechnology, Shanghai, China. The following primary antibodies were used: PBX1 (2 μg/ml, Abcam), STAT3, pSTAT3, and GAPDH (1:1000 Cell Signalling Technology, CST). The secondary antibody was goat anti-mouse/rabbit IgG (1:5000 Cell Signalling Technology, CST). The enhanced chemiluminescence detection kit was purchased from Thermo Fisher Scientific.

Transfection

The lentiviral vector (PBX1-Lv105) and Lv105 (empty vector) were purchased from GeneCopoeia and employed to infect the KYSE450 and KYSE150 cells. The sequences of the PBX1-shRNAs were as follows: forward 5′- TAATACGACTCACTATAGGG-3′, reverse 5′- CTGGAATAGCTCAGAGGC-3′; the sequence of the scramble shRNA was GCTTCGCGCCGTAGTCTTA. Stably infected cells were selected using medium including 500 ng/ml puromycin and applied in the subsequent experiments. Knockdown efficiency was assessed by Western blotting.

Cell Proliferation Assay

A CCK8 assay was used to determine the cell viability. Cells receiving different treatments were collected at 24, 48 and 72 h after transfection and irradiation (8Gy X-rays); then, the cells were plated in 96-well plates at a density of 5×10^3 cells per well and cultured at 37 °C overnight. Then, a CCK8 proliferation and cytotoxicity assay kit was applied; 10 μl CCK8 solution was added to each well at different time points, and a cell-free blank group was created using an equal volume of solution. Cells viability was evaluated by a microplate reader (BioTek ELx800, USA) at an absorbance of 450 nm after cultured at 37 °C in an incubator for an additional 2 h.

Clonogenic Survival Assay

Cells in the log phase were trypsinized and seeded in 6-well plates at a specific numbers (0 Gy: 300 cells; 2 Gy: 600 cells; 4 Gy: 1200 cells; 6 Gy: 3000 cells; and 8 Gy: 6000 cells). After an overnight incubation, the cells were subjected to 0, 2, 4, 6, or 8 Gy radiation at room temperature. The cells were incubated at 37 °C for an additional 10 to 12 days, fixed with methanol and stained with Giemsa. Colonies with at least 50 cells were counted under a microscope (Nikon, Tokyo, Japan).

Flow Cytometry Analysis

The cells were seeded in 6-well plates at a defined density (1×10^5 cells per well). After an overnight culture at 37 °C, the cells were exposed to X-rays (8 Gy). After a 24-h culture, based on the manufacturer's instructions, the cells were collected and stained with an Annexin V-FITC Apoptosis Detection Kit (BD Bioscience, Oxford, UK). Apoptosis was detected by flow cytometry using light scatter characteristics.

Western Blot Analysis

The cells were lysed in lysis buffer, and the lysates were centrifuged at 12,000 rpm for 15 min at 4 °C. The total protein concentration in the supernatant of the cell lysates was detected via a BCA kit . The same amounts of proteins were detached on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Schleicher and Schuell BioScience). The membranes were blocked for 1 h, probed with primary antibodies at 4 °C overnight, and incubated with a secondary antibody for 1 h at room temperature. The immunoblotted proteins were tested by enhanced chemiluminescence. The grey value analyses were performed using Imagine Lab 5.2.1 (Bio-Rad Laboratories, CA, USA).

Xenograft Mouse Model

All animal studies were carried out in accordance with the institutional guidelines for the care and use of experimental animals. Male 4- to 6-week-old BALB/c nude mice (Vital River Laboratory Animal Technology, Beijing, China) were injected with the indicated tumour cells (5×10^6 per mouse) and randomly divided into four groups: control group, IR group, knockdown group and IR plus knockdown group. Six nude mice were included in each group. The mice in the IR group and IR plus knockdown group were irradiated with an RS-2000 Pro Biological Irradiator at a dose of 6 Gy (2.47 Gy/min) with X-rays on day 10. The tumour sizes were measured by callipers, and the tumour volumes were computed by applying the following formula: (length×width2)/2. Tumour volume and mouse weight were measured every two days to evaluate the antitumour effect of PBX1 knockdown. For further appraisal of the antitumour effect of the PBX1 knockdown, we analysed the doubling time (the time required for a tumour to grow to twice its size) and the relative tumour proliferation rate (the relative tumour volume of the treatment group divided by that of the control group). Moreover, we calculated the enhancement factor to determine whether PBX1 knockdown can enhance radiation. At the end of the experiments, all animals were euthanized, and the tumours were excised for research.

Statistical Analysis

The data are expressed as the mean ± standard deviation (SD). GraphPad Prism program version 5.0 (Graph Pad Software, San Diego, CA, USA) was applied to analyse the data. One-way ANOVA and independent samples t-test were used to determine the differences between the groups. A p value <0.05 was considered statistically significant. All experiments were repeated at least three times.

Results

PBX1 Silencing Promotes Radiosensitivity in OSCC Cell Lines

To determine whether PBX1 indeed regulates oesophageal cancer radiosensitivity, KYSE450 and KYSE150 cells with a high expression of PBX1 were transfected with shRNA specifically targeting PBX1 (shPBX1) or negative control shRNA (shNC). The knockdown efficiency of PBX1 expression was confirmed by Western blotting (Fig. 1a). The grey values were detected with Imagine Lab 5.2.1 software, and the relative PBX1 expression was calculated (Fig. 1b). The PBX1 knockdown efficiency in KYSE450 and KYSE150 cells was calculated to be 0.35 ± 0.036 and 0.44 ± 0.041, respectively.

To further examine the ability of the PBX1 gene to confer radiosensitivity to OSCC cells, we examined the OD value (absorbance at 450 nm) of the OSCC cell lines at different time points. Then, cell viability was calculated by the formula: $\text{cell viability} = \frac{\text{OD (treatment group)} - \text{OD (blank group)}}{\text{OD (control group)} - \text{OD (blank group)}}$. The results suggested that the cell viability of the treatment group was lower at each timepoint than that of the control group. Especially in the knockdown combined with radiotherapy group, the cell viability was significantly inhibited, suggesting that the low expression of PBX1 has radiosensitizing effects. (Fig. 1b-c).

The clonogenic assay revealed irradiation dose–response survival curves in the KYSE450 and KYSE150 cells with or without PBX1 knockdown (Fig. 1d-e). The results in Tables 1 and 2 show that the SF2 of OSCC cells was 0.55 (KYSE450 cells irradiated alone), 0.38 (KYSE450 cells with irradiation and PBX1 silencing), 0.62 (KYSE150 cells irradiated alone) and 0.44 (KYSE150 cells with irradiation and PBX1 silencing). The OSCC cells with the PBX1 knockdown showed a remarkable increase in radiosensitivity as reflected by the notable decrease in their ability to form colonies.

In addition, the induction of apoptosis is a major mechanism of killing tumour cells. We tested apoptosis in KYSE450

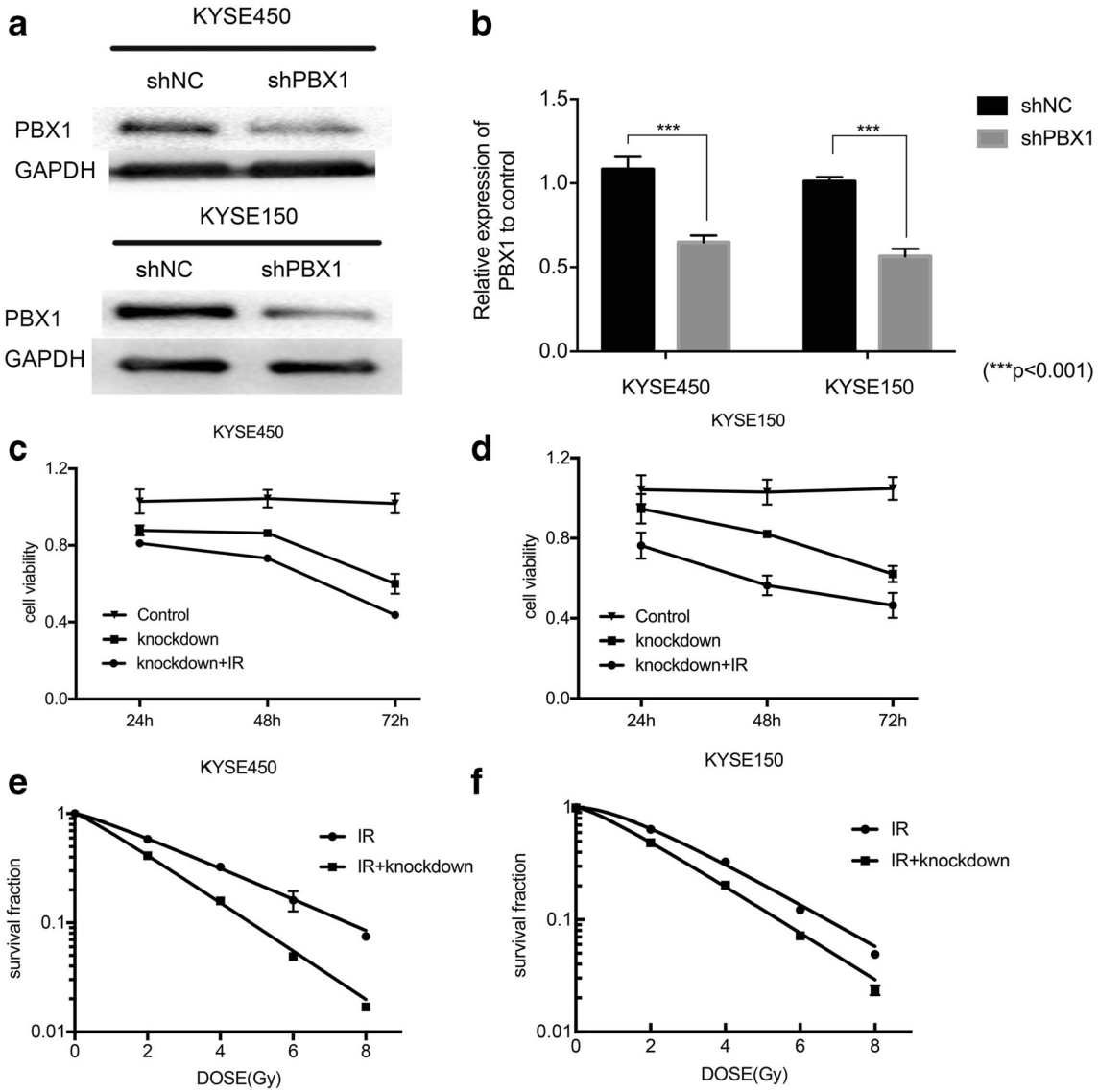

Fig. 1 Pre-B cell leukaemia homeobox 1 (PBX1) silencing sensitizes KYSE450 and KYSE150 cells to IR. (**a**-**b**) Western blotting was used to test the PBX1 protein knockdown efficiency. (**a**) Relative PBX1 protein expression levels in the KYSE450 and KYSE150 cell lines. GAPDH was used as a loading control. (**b**) Transfection efficiency was confirmed by Western blotting. The Data are presented as the mean ± SD of three replicates. ***$p < 0.001$. (**c**-**d**) CCK8 test results show that PBX1 knockdown inhibits OSCC proliferation and synergistically improves radiosensitivity. Cells viability was calculated on the basis of the absorbance at 450 nm.(**d**-**e**) PBX1 silencing suppressed the ability of OSCC cells to form colonies when subjected to 8 Gy irradiation. (**g**-**j**) KYSE450 and KYSE150 cells were subjected to PBX1 silencing, IR, or PBX1 silencing+IR, and apoptosis was tested by flow cytometry. (**g**-**h**) Representative histograms of flow cytometry according to annexin V-FITC staining in a single experiment. X-axis FITC log, Y-axis counts. (**i**-**j**) Three independent flow cytometry analyses were performed to obtain data, which are presented as the mean ± SD ($n = 3$). *$p < 0.05$

and KYSE150 cells treated with PBX1 silencing and/or IR (8 Gy). The apoptosis rates in the KYSE450 and KYSE150 cells with concomitant PBX1 silencing and radiotherapy were approximately 38% and 24%, respectively, which were significantly increased compared with those in cells subjected to IR or PBX1 knockdown alone (Fig. 1f-i). Our data suggest that the downregulation of PBX1 promotes the apoptosis rate in KYSE450 and that KYSE150 cells to enhance the radiosensitivity of OSCC cells.

PBX1 Silencing Inhibits the Activation of STAT3 in OSCC Cells

To probe the molecular mechanism underlying the effect of PBX1 in which sensitivity to IR in increased in OSCC, we detected the protein levels of STAT3 and p-STAT3. The Western blot analysis displayed that the expression of STAT3 and p-STAT3 was the highest in the KYSE450 and KYSE150 cells without IR or PBX1 silencing (Fig. 2). In

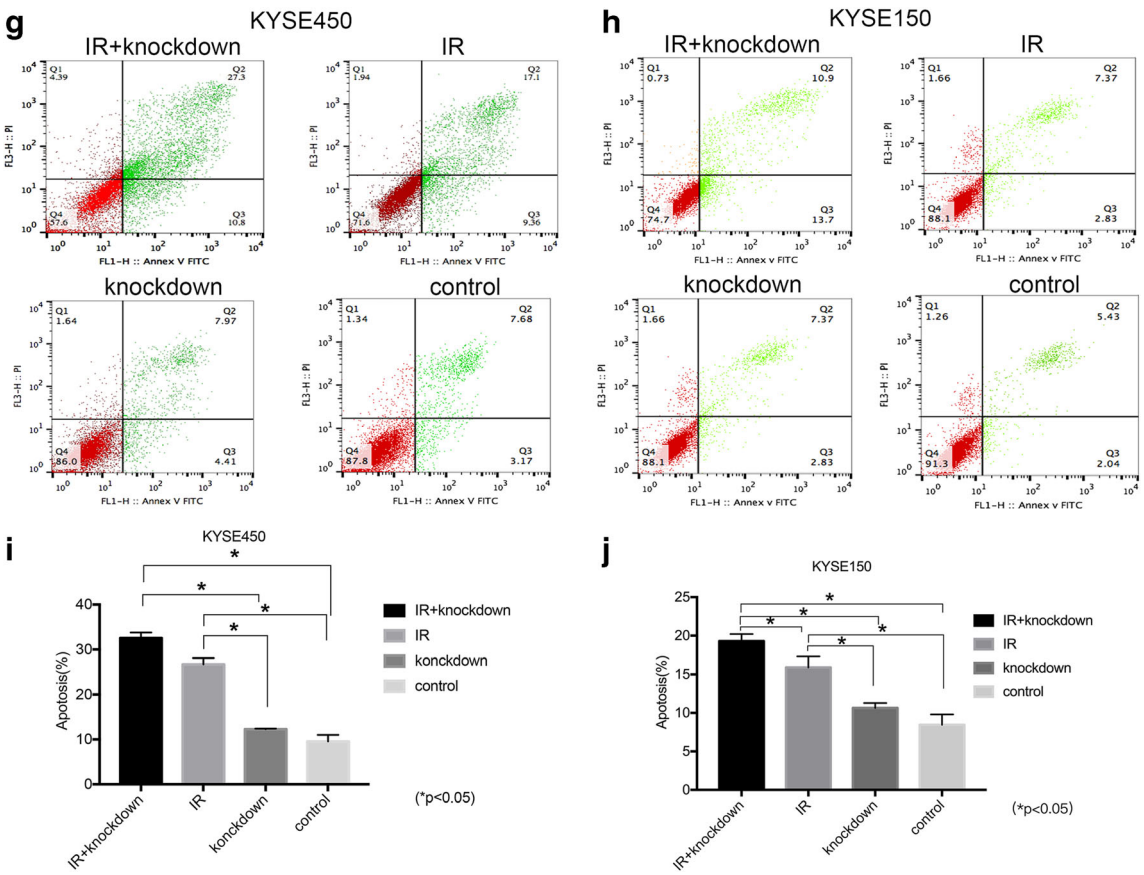

Fig. 1 continued.

addition, the levels of STAT3 and p-STAT3 were significantly decreased in the OSCC cells subjected to PBX1 silencing and/or exposure to IR (8 Gy) (Fig. 2).

PBX1 silencing increases radiosensitivity in KYSE150 OSCC cells in Vivo

To evaluate the effects of PBX1 silencing on radiosensitivity in OSCC cells in vivo, KYSE150 OSCC cells with or without PBX1 silencing were subcutaneously injected into the right armpit of nude mice (5×106/nude mice). The anti-tumour effect of the PBX1 knockdown was dynamically observed by measuring the tumour volume and mouse weight every other day. Compared to the control group, the IR group, knockdown group, and IR combined with knockdown group showed effective suppression of tumour growth and reduced tumour weight (Fig. 3a–c). Compared with the control group, the relative tumour proliferation rate (the relative tumour volume in the treatment group divided by that in the control group) in the other three groups was significantly reduced, especially in the IR combined with knockdown group (Fig. 3d).

Moreover, we analysed the doubling time (DT) required for the tumour to grow to twice its size in the four groups. Table 3 indicates that the DT in the IR group (6.2 ± 0.8 days) and knockdown group (7.3 ± 1.6 days) was significantly reduced compared with that in the IR combined with knockdown group (9.8 ± 1.8 days). However, the DT in the control group was 4.4 ± 0.4 days (Table 3). Based on the calculation of the normalized tumour growth delay in the IR plus knockdown group, we conclude that PBX1 knockdown enhanced the response of the KYSE150 xenografts to IR with an enhancement factor (EF) of 1.4 (Table 3).

Table 1 The radiosensitization of PBX1 in KYSE450 cells

	D0(Gy)	(Gy)	SF2	SER
IR	2.99	0.66	0.55	
IR + knockdown	1.95	0.37	0.38	1.53

Table 2 The radiosensitization of PBX1 in KYSE150 cells

	D0(Gy)	Dq(Gy)	SF2	SER
IR	2.84	1.51	0.62	
IR + knockdown	2.06	0.70	0.44	1.11

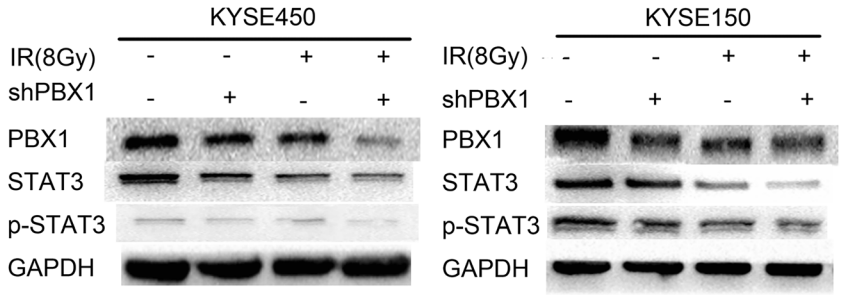

Fig. 2 STAT3 and p-STAT3 were regulated by irradiation and PBX1 knockdown. The downregulation of PBX1 inhibits the activation of STAT3 in OSCC cells. Western blot analysis of PBX1, p-STAT3 and STAT3 levels in KYSE450 and KYSE150 cells with or without IR and/or PBX1 silencing. GAPDH was used as a loading control

Discussion

The PBX family contains the PBX1, PBX2 and PBX3 proteins, which are expressed in a wide range of adult and foetal tissues [31]. Their common feature is an extensive sequence identity within and outside the homeodomain. These proteins constitute a widely expressed family of highly conserved PBX homeodomain proteins with expression patterns and

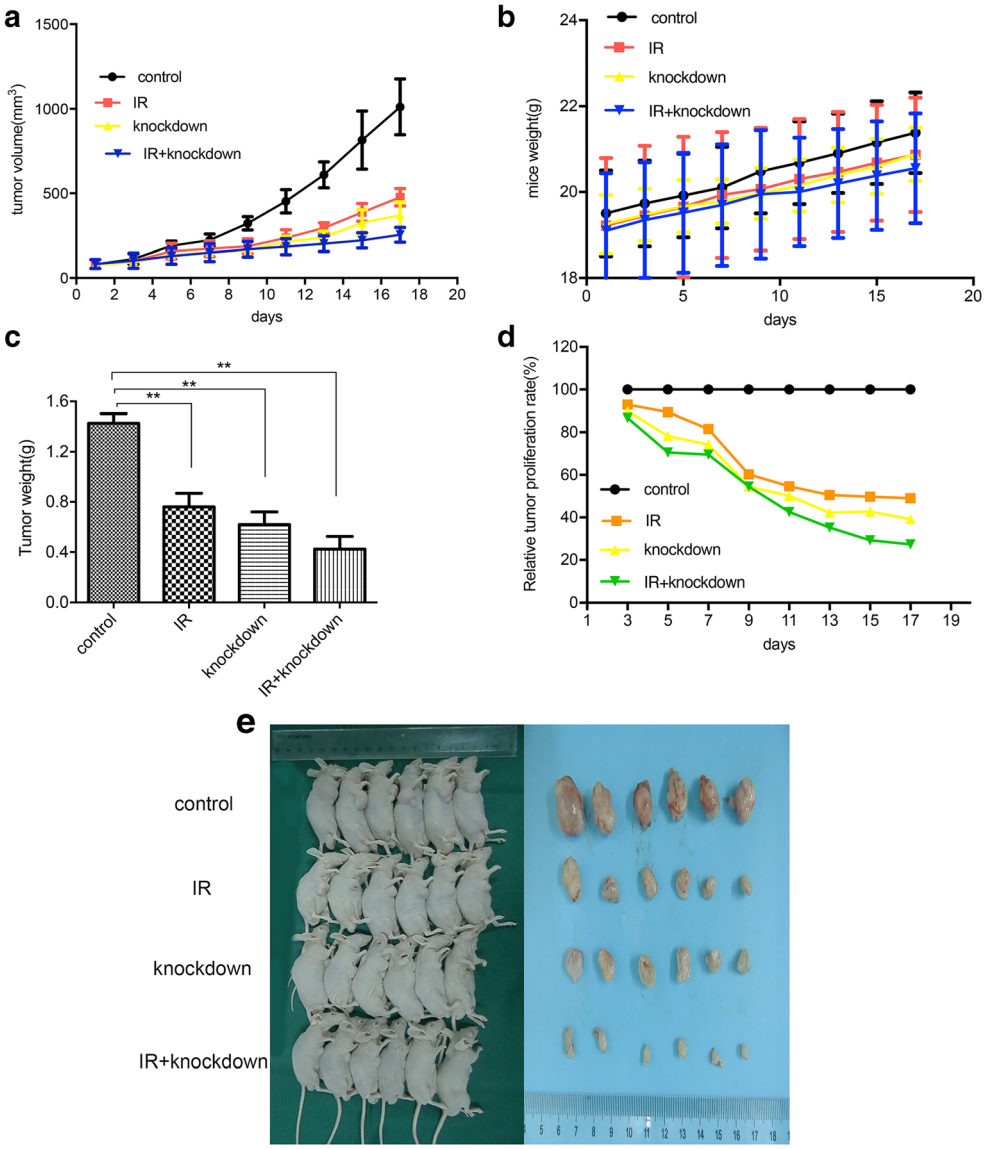

Fig. 3 PBX1 silencing increases radiosensitivity in KYSE150 OSCC cells in vivo. (**a**) Measurement of tumour volume in nude mice every two days. The data represent the average tumour volume; error bar, SD. (**b**) Measurement of mouse weight every other day. (**c**) Measurement of tumour weight in nude mice after sacrifice. ** $p < 0.01$. (**d**) Effect of radiotherapy and/or downregulation of PBX1 on the relative tumour proliferation rate of xenograft tumours in nude mice. (**e**) Observation of mice and tumours in each group

Table 3 The radiosensitization of PBX1 in KYSE150 xenografted tumor

Group	Inhibition[a]	Doubling time (days)	Absolute growth delay (days)[b]	Normalized growth delay (days)[c]	EF
control		4.4 ± 0.4			
IR	0.48 ± 0.18	6.2 ± 0.8	1.8		
knockdown	0.60 ± 0.14	7.3 ± 1.6	2.9		
IR + knockdown	0.72 ± 0.15	9.8 ± 1.8	5.4	2.5	1.39

[a] Tumour growth inhibition rate was evaluated based on the tumour volume on the final day

[b] The doubling tumour time in the treatment group minus that in the control group

[c] The time of absolute growth delay of the tumour in the IR combined with knockdown group minus that in the knockdown group

functional similarities in normal and tumour cell metabolism due to mediation by protein motifs in conserved regions [31]. Qiu Y [32] and Liu D B [23] et al. used an immunohistochemical method to detect the expression of the PBX1 and PBX2 proteins and suggested that PBX1 and PBX2 show significantly high expression in OSCC patient tissues. Moreover, PBX1 positively regulates STAT3 transcription and plays a key role in tumour proliferation via the JAK2/STAT3 pathway in ovarian carcinomas and renal clear cell carcinoma [22, 29]. The activation of STAT3 is based on the phosphorylation of a conserved tyrosine residue by JAK2 and contributes to tumourigenesis by regulating proliferation, migration, angiogenesis and resistance to apoptosis [33]. In this research, we investigated whether PBX1 silencing promotes sensitivity to IR in OSCC cells in vivo and in vitro and probed the underlying mechanism.

To demonstrate the effect of the downregulation of PBX1 on OSCC cells, we conducted cell proliferation assays, clonogenic survival assays and flow cytometry analysis. We observed that the PBX1 silencing inhibited cell proliferation and colony formation. Additionally, we demonstrated that compared with the IR or PBX1 knockdown alone group, the combination of PBX1 knockdown and IR had a pronounced synergistic pro-apoptotic effect on the KYSE450 and KYSE150 cells. The downregulation of PBX1 improved IR-induced cell apoptosis, increasing the sensitivity of OSCC cells to radiation. In addition, we assessed the expression of the STAT3 and p-STAT3 proteins. Our results indicate that the PBX1 knockdown was sufficient to suppress the activation of STAT3 and p-STAT3 and reduced the expression of STAT3 and p-STAT3 in response to IR. We demonstrated that the PBX1 knockdown increased the sensitivity of the KYSE450 and KYSE150 cells to radiation in vivo by building a xenograft nude mouse model. Compared with the other groups, the combination of PBX1 knockdown and IR clearly inhibited the tumour weight. Overall, we found that the PBX1 knockdown increased radiosensitivity in OSCC cells in vitro and in vivo. Our research study is the first to demonstrate that the downregulation of PBX1 can be used to increase the sensitivity of OSCC cells to IR by targeting STAT3 and p-STAT3. Consistent with the findings in vivo, we also found that the downregulation of PBX1 inhibits the tumour weight, tumour volume and relative tumour proliferation rate in a xenograft nude mouse model of OSCC cells. Our prior work verified that the STAT3/HIF-1α/VEGF signalling pathway participates in the resistance to radiation in OSCC. To detect whether PBX1 is sensitized by the STAT3/HIF-1α/VEGF signalling pathway, the results of our study must be further expanded to determine the expression of the HIF-1α and VEGF proteins.

Overall, this study indicated that PBX1 knockdown substantially enhances sensitivity to radiation in OSCC cells by inhibiting STAT3 expression. Our results imply that PBX1 may be a potential target for radiotherapy in clinical therapies for OSCC.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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