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In vivo study of breast carcinoma radiosensitization by targeting eIF4E

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ABSTRACT

Background: Eukaryotic initiation factor eIF4E, an important regulator of translation, plays a crucial role in the malignant transformation, progression and radioresistance of many human solid tumors. The overexpression of this gene has been associated with tumor formation in a wide range of human malignancies, including breast cancer. In the present study, we attempted to explore the use of eIF4E as a therapeutic target to enhance radiosensitivity for breast carcinomas in a xenograft BALB/C mice model.
Materials and methods: Ninety female BALB/C mice transfected with EMT-6 cells were randomly divided into six groups: control, irradiation (IR), pSecX-t4EBP1, pSecX-t4EBP1 + irradiation, pSecX and pSecX + irradiation. At the end of the experiments, all mice were sacrificed, the xenografts were harvested to measure the tumor volume and mass, and the tumor inhibition rates were calculated. Apoptosis was detected with a flow cytometric assay. Immunohistochemistry was used to detect the expression of HIF-1α.
Results: The xenografts in pSecX-t4EBP1 mice showed a significantly delayed growth and smaller tumor volume, with a higher tumor inhibition rate compared with the control and pSecX groups. A similar result was obtained in the pSecX-t4EBP1 + IR group compared with IR alone and pSecX + irradiation. The expression of HIF-1α in the tumor cells was significantly decreased, while the apoptosis index was much higher.
Conclusions: pSecX-t4EBP1 can significantly inhibit tumor growth and enhance the radiosensitivity of breast carcinoma xenografts in BALB/C mice. This is possibly associated with the downregulation of HIF-1α expression, which suggests that pSecX-t4EBP1 may serve as an ideal molecular target for the radiosensitization of breast carcinoma.

1. Introduction

Breast cancer is the commonest type of primary malignancy that affects women [1], with an estimated 1,200,000 new cases worldwide each year [2], and this trend is likely to continue for some time to come. Radiotherapy, which is one of the most effective means for cancer treatment, plays a crucial role in combined modality therapies for breast cancer [3,4]. However, acquired radioresistance is a significant impediment to the effects of radiotherapy. Therefore, we attempted to elucidate the mechanisms causing radioresistance of breast cancer, and explore the possibility of eukaryotic initiation factor 4E (eIF4E) as a therapeutic target to enhance radiosensitivity and reverse radioresistance [5,6].

eIF4E plays an important role in the initiation of protein translation by binding the 5′-cap structure of the mRNA and, combined with eIF4G and eIF4A, is known as the eIF4F complex. Among the three subunits, eIF4A functions as an ATP-dependent helicase and eIF4G provides a scaffold for eIF4E and eIF4A. eIF4E interacts with eIF4G via a site through which it also binds inhibitory proteins that are termed 4E-BPs. The association between eIF4E and 4E-BP prevents the formation of productive initiation complexes with eIF4G; the 4E-BPs therefore act as inhibitors of cap-dependent translation [7–9].

eIF4E, an important regulator of translation, plays a crucial role in malignant transformation, tumor progression and the radioresistance of many human solid tumors. It has been reported that it is modified upon the induction of apoptosis [10] and could modulate programmed cell death [11,12]. The overexpression of the gene has been associated with tumor formation in a wide range of human malignancies, including head and neck squamous cancer, colon carcinoma, leukemias, lymphomas, cervical neoplasias and breast cancer [13–16], which suggests that upregulated eIF4E expression promotes cellular transformation, tumorigenesis, invasion and metastasis [17–22]. Furthermore, in transgenic models, the overexpression of eIF4E yields a high incidence in a variety of cancers [20]. Moreover, many studies have shown over the last decade that the overexpression of eIF4E is involved in the development of breast

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cancer radioresistance [23–26]. Collectively, these data suggest that eIF4E could be a promising therapeutic target for breast cancer, as well as a broad array of other cancers. Other researchers have reported that targeting eIF4E with antisense or small interfering RNAs (siRNAs) can induce tumor cell apoptosis as well as enhancing chemosensitivity to cisplatin [27,28], but these methods are inefficient and lack a high degree of tumor specificity.

As a consequence, we initially constructed a eukaryotic expression vector, pSecX-t4EBP1, which contained phosphorylation-defective 4E-BP1 and the protein transduction domain. The former downregulated the expression of eIF4E in direct binding, and the latter domain could cross the cellular membrane and improve the efficiency of the vector's spread. Consequently, we found that this plasmid could effectively inhibit tumor growth and enhance radiosensitivity through downregulating the expression of eIF4E in vitro. In the present study, we transfected pSecX-t4EBP1 into a mouse xenograft model, and demonstrated experimentally that it inhibited the expression of eIF4E, leading to the downregulation of HIF-1a expression. This resulted in the inhibition of cell proliferation, the promotion of apoptosis and the enhancement of radiosensitivity. In addition, there was no increase in toxicity or injury to normal tissues during the 29-day follow-up period. The study suggests that it is feasible to use pSecX-t4EBP1 alone or in combination with radiotherapy for the treatment of breast cancer as it possesses high levels of both tumor specificity and efficacy.

2. Materials and methods

2.1. Cell culture

The EMT-6 mouse breast cancer cell line was obtained from the Experimental Animal Center, Fourth Military Medical University (FMMU). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco, Gaithersburg, MD, USA), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/L L-glutamine. All cultures were incubated in a humidified atmosphere of 5% CO_2 and 95% room air at 37 °C.

2.2. Tumor xenograft mouse models

Ninety female BALB/C mice aged 6–8 weeks (purchased from Experimental Animal Center, FMMU, Xi'an, china) were used for the experimental tumorigenicity assays. All animals were maintained in specific pathogen-free rooms and all procedures were approved by the FMMU Animal Care and Use Committee. A total of 2×10^7 EMT-6 cells in 0.2 ml were injected subcutaneously into the abdomen of each mouse. Tumor diameters were assessed using a digital Vernier caliper every day and tumor volume was calculated according to described previously [29]: $V = \prod/6 \times L \times W^2$, where "$L$" represents the largest tumor diameter, and "W" the smallest one.

When the tumor had increased to $\geqslant 300\,mm^3$, the 90 mice were assigned randomly into six groups: controls, irradiation (IR), pSecX-t4EBP1, pSecX-t4EBP1 combined with IR (pSecX-t4EBP1 + IR), pSecX and pSecX combined with IR (pSecX + IR). In the groups where mice were subjected to irradiation, the mice were given three doses of local irradiation at a dose of 4 Gy at 2-day intervals. Mice in the pSecX-t4EBP1 and pSecX-t4EBP1 + IR groups were treated as follows: 80 μg of the pSecX-t4EBP1 plasmid was given by intravenous (IV) injection in 2 ml saline, in a series of four doses at 1-week intervals. Mice in the pSecX and pSecX + IR groups were given 80 μg of the pSecX plasmid in 2 ml saline by IV injection, in a series of four doses at 1-week intervals (Fig. 1). Mice were weighed, and the tumor sizes were measured twice a week with an electronic caliper. At the end of the experiments (on day 29), all mice were sacrificed and the tumors were extracted, weighed, measured, and stored for histological and immunohistochemical injection. The tumor growth suppression rate was calculated using the following equation: $[1 - (Wt/Wc)] \times 100$, where Wc was the tumor weight in the control mice and Wt was the tumor weight in the treated groups.

2.3. Irradiation conditions

The mice were fixed upon a mouse shelf while awake. Tumors were then irradiated with a 6-MV X-ray source at a dose rate of 400 cGy/min at room temperature. The mice were otherwise shielded with lead so that only the tumor was irradiated. Radiotherapy was performed in the Department of Radiation Oncology, Xijing Hospital, FMMU.

2.4. Flow cytometric analysis of apoptosis

Tumors were harvested from anesthetized mice and flash into homogenates. A 1-ml sample of cells was removed by pipetting, washed with ice-cold PBS twice, and resuspended in binding buffer. After this, cells were stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide following the manufacturer's protocols. After a 15-min incubation in the dark at room temperature, stained cells were immediately analyzed on an Epics XL-MCL flow cytometer. All of the samples were assayed in triplicate, and

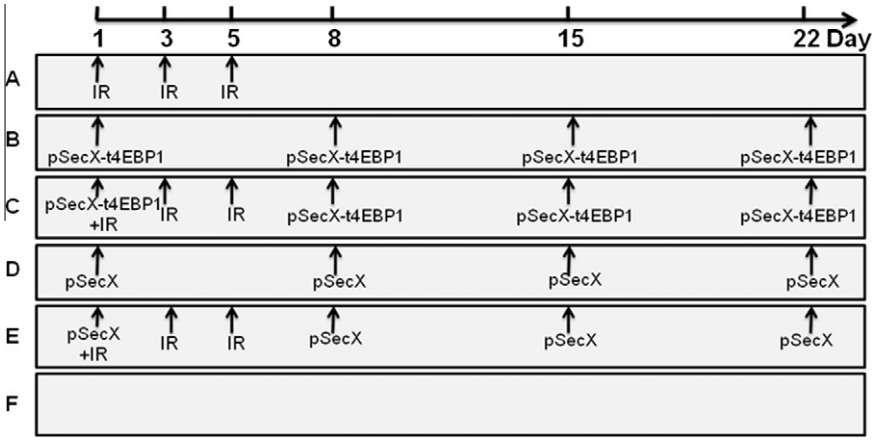

Fig. 1. Treatment modalities of the six groups. (A) Irradiation (IR); (B) pSecX-t4EBP1; (C) pSecX-t4EBP1 combined with irradiation; (D) pSecX; (E) pSecX combined with irradiation and (F) the control group.

the cell apoptosis rate was calculated using the following formula: (Napoptotic cell/Ntotal cell) × 100%.

2.5. Immunohistochemical analysis

Xenograft tumors were fixed in 4% paraformaldehyde for 12 h at room temperature, paraffin-embedded, cut into 5–7 μm sections and placed on slides. The slides were incubated with 100 μL rabbit anti-HIF-1α overnight at 4 °C. After they were rinsed with tris-buffered saline (TBS) five times for 5 min each rinse, slides were incubated in rabbit anti-mouse secondary antibodies for 30 min at 37 °C, and subsequently rinsed again with TBS five times for 5 min each. Then, 100 μL DAB solution was added to each slides and incubated for 1 min at room temperature. A negative control was run for each slide using an irrelevant, isotype antibody. Slides were scored for positivity (brown-stained nuclei) and negativity (blue nuclei) using a light microscope.

2.6. Statistical analyses

The data are reported as the means ± standard deviation (SD). The SPSS 13.0 statistical software was used to perform the statistical analyses. Comparisons of the mean values were performed by Student's *t*-tests and one-way analysis of variance (ANOVA). The statistical level of $P < 0.05$ was considered as significant.

3. Results

3.1. pSecX-t4EBP1 reduced tumor volume and enhanced radiosensitivity

Ten days before treatment, the growth in tumor volume was relatively slow, and there were no significant differences in tumor growth between the six groups. Ten days after treatment, the tumor growth curves showed major changes. The tumor growth rate in the pSecX-t4EBP1 + IR, pSecX + IR and IR group was significantly more inhibited than those in the pSecX-t4EBP1, pSecX and control groups. This finding suggested that radiotherapy could significantly inhibit tumor growth. pSecX-t4EBP1 treatment had a significant larger inhibitory effect on tumor growth than the pSecX or control groups ($P < 0.05$), while the rate of tumor growth did not differ significantly between the control and pSecX groups ($P > 0.05$). These data also indicate that pSecX-t4EBP1 significantly inhibited tumor growth compared with the control and pSecX group, which was transfected with vector only.

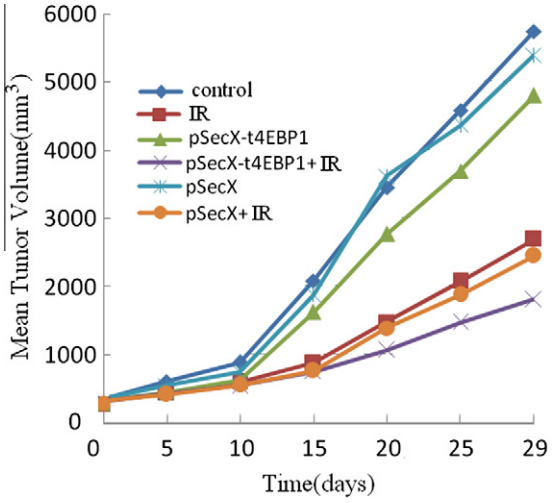

Fig. 2. Tumor growth curve. Tumor growth was monitored over 29 days. Tumor diameters were assessed using digital vernier calipers every day and tumor volume was calculated with the following formula: $V = \prod/6 \times L \times W^2$, where "*L*" represents the largest tumor diameter, and "*W*" the smallest. ANOVA testing was performed to determine whether the change in volume during the time course was significant. $P < 0.05$ was considered statistically significant.

Table 1
Tumor weight at the end of the experiments.

Group	Tumor weight (g)	Inhibition (%)	*P*-value
Control	3.51 ± 0.963	–	–
IR	1.96 ± 0.489	44.16	0.000
pSecX-t4EBP1	3.02 ± 0.849*	13.96	0.011
pSecX-t4EBP1 + IR	1.51 ± 0.872**	56.98	0.000
pSecX	3.48 ± 1.220	0.85	0.281
pSecX + IR	1.88 ± 0.931	46.44	0.000

Values were represented as means ± S.D, $P < 0.05$ was considered statistically significant.
* $P < 0.05$ compared with the control or pSecX group, paired Student's *t*-test.
** $P < 0.05$ compared with the IR or pSecX + IR group, paired Student's *t*-test.

A similar result was obtained in the three groups combined with IR, in which the mean relative tumor volume for the pSecX-t4EBP1 + IR treatment was initially significantly lower ($P < 0.05$) than the pSecX + IR or IR alone groups, and remained low until the end of the trial. There were no significant differences in tumor volumes between the pSecX + IR or IR alone groups ($P > 0.05$) (Fig. 2), which suggested that the mice in the pSecX-t4EBP1 group were markedly sensitized to radiotherapy compared with pSecX transfected with vector only.

3.2. pSecX-t4EBP1 decreased tumor weight and enhanced radiosensitivity

To evaluate the tumor growth suppression effect of pSecX-t4EBP1, tumors were dissected and tumor weight was measured at the end of the experiments. The tumor weight values in the pSecX-t4EBP1, pSecX and control groups were 3.02 ± 0.849, 3.48 ± 1.220 and 3.51 ± 0.963 g, respectively. The tumor weights in the pSecX-t4EBP1 + IR, pSecX + IR and IR groups were 1.51 ± 0.872, 1.88 ± 0.931 and 1.96 ± 0.489 g, respectively (Table 1). Treatment with pSecX-t4EBP1 significantly decreased the weights of the initial tumors ($P < 0.05$) and showed significantly better inhibition of tumor growth than in the pSecX or control groups. pSecX-t4EBP1 + IR treatment significantly decreased the weights of the initial tumors ($P < 0.05$) than pSecX + IR or IR alone, which demonstrated that it had significantly enhanced the sensitivity of tumors to radiotherapy.

3.3. The effect of pSecX-t4EBP1 on apoptosis

Cell death was detected by FCM in the present study, as shown in Fig. 3 and Table 2. The rates of cell apoptosis for the pSecX-t4EBP1 treatment group were initially significantly higher ($P < 0.05$) than in the pSecX or control groups, whereas there were no obvious differences in pSecX + IR and IR alone groups ($P > 0.05$). The rates of cell apoptosis in tumors of mice that underwent the pSecX-t4EBP1 + IR treatment were initially significantly higher ($P < 0.05$) than in the pSecX + IR or IR alone groups, whereas there were no obvious differences between the pSecX + IR and IR alone groups ($P > 0.05$).

3.4. pSecX-t4EBP1 downregulated HIF-1α expression

Substantial evidence has previously demonstrated that the expression of HIF-1a might be closely related to radiosensitivity [24,30,31]. To demonstrate whether HIF-1a was indeed suppressed

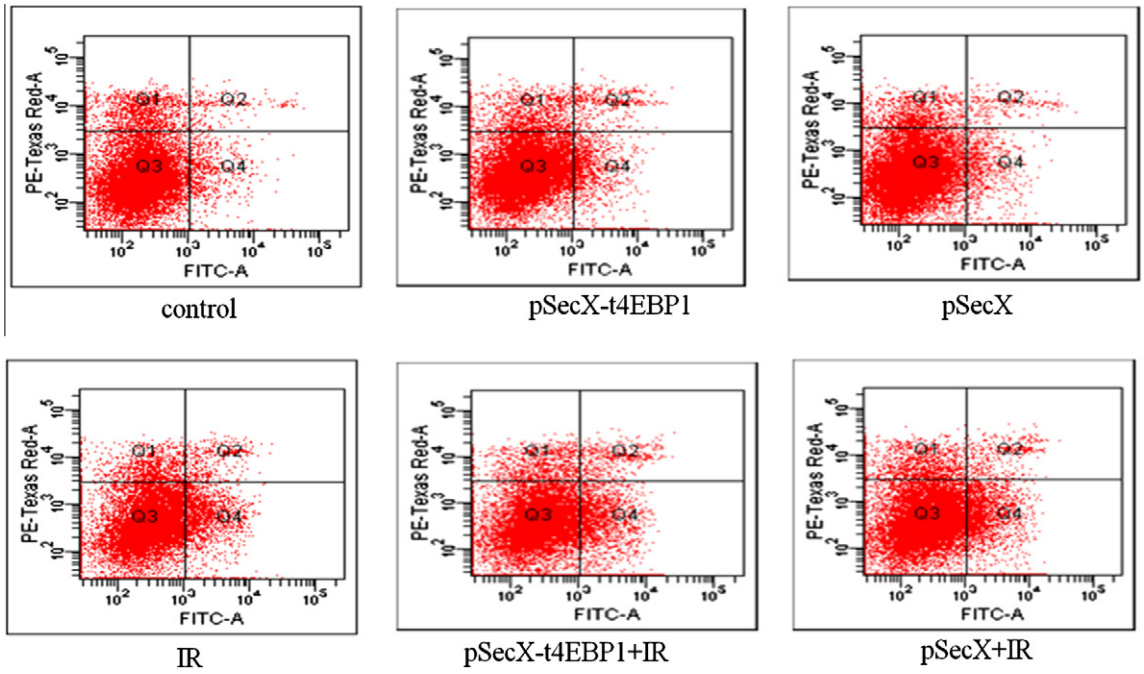

Fig. 3. Representative histograms of the flow cytometric analysis using double-staining with annexin-V (FITC-A) and PI (PE-A) in six groups. Q1: Necrotic cells; Q2: cells in late apoptosis; Q3: normal cells and Q4: cells in early apoptosis.

Table 2
Apoptotic index of six groups (%).

Group	Apoptosis (%)
Control	5.31 ± 0.97
IR	15.1 ± 2.34
pSecX-t4EBP1	8.3 ± 1.46*
pSecX-t4EBP1 + IR	18.5 ± 3.38**
pSecX	4.5 ± 0.34
pSecX + IR	14.8 ± 1.98

Values were represented as means ± S.D, $P < 0.05$ was considered statistically significant.
* $P < 0.05$ compared with the control or pSecX group, paired Student's *t*-test.
** $P < 0.05$ compared with the IR or pSecX + IR group, paired Student's *t*-test.

in the tumor tissues, immunohistochemical staining was conducted in the tumors from different treatment groups. HIF-1a expression was shown to be markedly reduced in the pSecX-t4EBP1-treated tumors in comparison with the pSecX and control groups, while HIF-1a expression was shown to be markedly reduced in the pSecX-t4EBP1 + IR-treated tumors in comparison with the pSecX + IR and IR groups (Fig. 4). Taken together, these results showed that pSecX-t4EBP1 could inhibit its target expression in tumors, which led to the significant suppression of tumor growth and radiosensitization of breast cancer. This inhibitory effect might be caused by the reduced expression of HIF-1a, which was in agreement with the in vivo data.

4. Discussion

To date, many investigators have demonstrated that the overexpression eIF4E was significantly correlated with aggressive tumor progression, resistance to radiotherapy, and the poor prognosis for human cancers [17,24,32]. This implies that the specific downregulation of eIF4E might be a potential therapeutic strategy for either genetic or conventional drug interventions against human cancers, including breast cancer. Moreover, eIF4E is the rate-limiting factor in the 5′-cap structure, and its bioavailability is strictly controlled by its association with the eIF4E-binding protein 1 (4E-BP1) [9]. Thus, in order to enhance the tumor response to radiotherapy and improve the prognosis of patients, it is essential to identify the molecular mechanisms of eIF4E overexpression.

Substantial evidence has demonstrated that the upregulation of HIF-1a expression in many human cancers might be closely related to the overexpression of eIF4E gene. HIF-1a has been reported to be associated with radiosensitivity, and the inhibition of HIF-1a expression could markedly enhance tumor response to radiotherapy. Therefore, we hypothesized that decreasing the level of eIF4E expression would downregulate HIF-1a expression and enhance the radiosensitivity of tumors. Therefore, in our previous studies, we successfully assembled a recombinant fusion protein by combining 4E-BP1 and a protein transduction domain, and constructed the eukaryotic expression vector, pSecX-t4EBP1. In vitro, it has been demonstrated to effectively inhibit tumor growth and also significantly enhance tumor response to radiotherapy by downregulating the expression of HIF-1a.

In the present report, we transfected pSecX-t4EBP1 into a mouse xenograft model of breast cancer. The results indicated that the xenografts treated with pSecX-t4EBP1 had a significantly delayed rate of growth and smaller tumor volumes, with a higher tumor inhibition rate compared with control and pSecX-treated mice. A similar result was obtained in the pSecX-t4EBP1 + IR group compared with IR alone and pSecX + IR, which suggested that this vector could significantly inhibit tumor growth and enhance tumor radiosensitivity. Furthermore, FCM assays also indicated that the targeting of eIF4E promoted apoptosis. To explore the molecular mechanisms of tumor suppression, HIF-1a expression was also examined and shown to be markedly decreased in the pSecX-t4EBP1 groups compared with the control and pSecX, and pSecX-t4EBP1 + IR groups compared with IR and pSecX + IR groups, respectively. All the experimental data indicated that targeting eIF4E with pSecX-t4EBP1 had a significant inhibitory effect on tumor growth by decreasing the expression of HIF-1a.

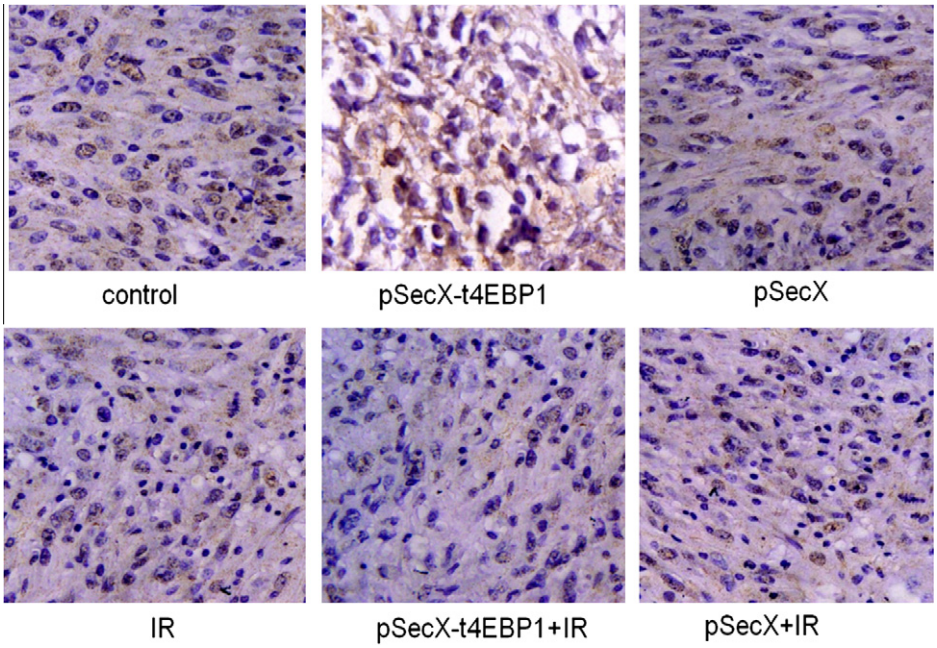

Fig. 4. The expression of HIF-1a in tumor tissues was detected by histochemical analyses (200×).

Hypoxia is a common phenomenon that occurs in the majority of human solid tumors larger than 1 mm^3 in volume. This results in the promotion of cell proliferation and disordered vasculogenesis. Hypoxia-inducible factor-1 (HIF-1) is a heterodimer that consists of the HIF-1a and HIF-1b subunits; HIF-1a is inducible by hypoxia [33]. Clinical and experimental studies have confirmed that HIF-1a expression correlates with radioresistance and poor prognosis in a variety of human cancer cells [34]. Furthermore, transfecting siRNA that is specific to HIF-1a, or creating dominant negative mutants of HIF-1a, significantly improves the radiosensitivity of tumor cells [31,35–37].

It has been reported that hypoxic cancer cells are more likely to survive radiotherapy if HIF-1a is upregulated. The subsequent enhancement of VEGF and bFGF expression could provide survival signals to both cancer cells and adjacent vascular endothelial cells by autocrine or paracrine signaling pathways [38,30,36,39]. Moreover, substantial evidence has demonstrated that the initiation of translation of HIF-1a, VEGF and bFGF mRNA are all controlled by the expression of eIF4E [40]. Thus, we hypothesized that targeting eIF4E with the pSecX-t4EBP1 vector might inhibit the transcription of HIF-1a, VEGF and bFGF, leading to an enhanced tumor response to radiotherapy. In the present study, as reported in other human malignancies by other researchers, our results showed that the pSecX-t4EBP1-induced downregulation of HIF-1a expression significantly inhibited tumor growth and enhanced the radiosensitivity of breast carcinoma xenografts in BALB/C mice. We postulate that the use of pSecX-t4EBP1 alone or in combination with radiotherapy for the treatment of breast cancer will be clinically highly effective. Thus, targeting eIF4E will be a novel strategy in targeted cancer gene therapy and may be a highly efficacious approach in the future for the radiosensitization of breast cancers with low levels of toxicity.

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