

ZIP7 Maintains Colon Cancer Radioresistance by Regulating Zinc-dependent Epithelial-mesenchymal Transition

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Research Article

Keywords: ZIP7, colon cancer, radioresistance, zinc, EMT

Posted Date: May 5th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-452460/v1>

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Abstract

Background: Irradiation-induced radioresistance often leads to the therapeutic failure of colon cancer. By regulating the redistribution of intracellular zinc, ZIP7 plays a dominating role in the activation of many critical signaling pathways in the progression of tumors. However, the relationship between ZIP7 and radioresistance is still unclear.

Methods: ZIP7 expression of colon cancer was evaluated by analyzing public data from the GEPIA and CPTAC databases and validated based on immunohistochemistry (IHC) staining. Clonogenic survival assay was employed to examine the influence of intracellular zinc interference and ZIP7 knockdown on radioresistance of colon cancer, based on radioresistant colon cancer cells. At last, Western blot was performed to preliminarily explore the potential mechanisms.

Results: ZIP7 was significantly upregulated in human colon cancers compared with adjacent normal tissues. ZIP7 knockdown could significantly reduce the radioresistance of colon cancer cells, while transmembrane ionophore of zinc could partially reverse this effect. In terms of mechanisms, ZIP7 knockdown significantly reversed the radiation-induced expression of elevated ZEB1 and down-regulated E-cadherin through regulating zinc.

Conclusion: ZIP7 is crucial to maintain radioresistance of colon cancer cells through regulation of zinc distribution, and at least partially by maintenance of epithelial-mesenchymal transition (EMT).

1. Introduction

Colon cancer is one of the most commonly diagnosed malignancies, which severely threatens public health.[1] According to the latest data of the United States, the annual age-standardized colon cancer incidence rate was 37.6 per 100,000 persons, and about one third of which died from this disease.[2] Radiotherapy is an irreplaceable part of systemic therapy for solid tumors, including colon cancer.[3, 4] However, the intrinsic and irradiation-induced radioresistance often lead to the failure of treatment, while the mechanisms and coping strategies need to be further studied.[3, 5]

Zinc is the second most abundant indispensable trace nutrient for life, and is an important signal mediator of cells, including cancer cells.[6] ZIP (ZRT, IRT-like protein) family, the channels facilitating the influx of zinc into the cytosol, are the critical proteins to initiate the role of zinc to act as the second messenger.[6] Zinc dysregulation and ZIPs abnormal expressions are related to the malignant biological behaviors of various tumors, and are considered as potential therapeutic targets.[7] For esophageal cancer, high expression of ZIP5 or ZIP6 were highly responsible for poor prognosis, and down-regulation of these gene expressions significantly inhibited the progression of the disease.[8, 9] ZIP4 was significantly overexpressed in pancreatic and nasopharyngeal cancer tissue, which was implicated in chemoresistance and radioresistance.[10, 11]

ZIP7, regulating the redistribution of intracellular zinc, is a special member of the ZIPs family and just localized inside the cells.[6] Analysis of the information of the Oncomine database showed that, ZIP7 is one of the 10% of genes overexpressed in breast cancer patients with poor prognosis.[12] Taylor, *et al.* found that ZIP7 was essential for the activation of growth factor receptors, and removal of ZIP7 may be an effective strategy to prevented anti-hormone resistance in breast cancer.[13] In cervical and gastric cancer, downregulated the expression of ZIP7 significantly inhibited the proliferation, migration of tumor cells, but promoted apoptosis.[14, 15] However, the relationship between ZIP7 and colon cancer, especially in regard to radiotherapy, is still unclear.

In this study, the role of ZIP7 in colon cancer radioresistance and the preliminarily relevant mechanisms were explored. We found that ZIP7 was significant to maintain radioresistance of colon cancer cells through regulation of zinc distribution, and at least partially by maintenance of epithelial-mesenchymal transition (EMT).

2. Materials And Methods

2.1 Bioinformatics analysis.

GEPIA (<http://gepia.cancer-pku.cn/>) is an interactive website based on the Cancer Genome Atlas (TCGA) database. [16] UALCAN (<http://ualcan.path.uab.edu/>) is an integrated platform based on multi-omics data and clinic-pathological profiles. [17] In the present study, the GEPIA website and the CPTAC database, a sub-database of UALCAN, were respectively employed to explore the mRNA and proteomic levels of ZIP7 in colon cancer and adjacent normal tissues.

2.2 Tissue samples

Fifty pairs of colon cancer tissues and the paired para-tumor normal colon tissues were obtained from surgical procedures from the Wuxi People's Hospital Affiliated to Nanjing Medical University (Wuxi, China) between 2017 and 2018 with the consent of all patients. This study was approved by the Ethics Committee of Wuxi People's Hospital Affiliated to Nanjing Medical University.

2.3 Immunohistochemistry

For immunohistochemistry (IHC), the antigen repair method was employed. Briefly, the formalin-fixed and paraffin-embedded tissues were cut into 4 μ m slices. Then, deparaffinage, rehydration, antigen repair, inactivation of endogenous peroxidase, block, anti-ZIP7 (1:800 dilution, Cat. ab117560, Abcam) antibody incubation, DAB and hematoxylin counterstain were performed step by step. At last, the sections were observed using a microscope (Olympus Corporation). The percentage of positively stained cells was scored on a scale of 0 to 4, namely, 0: <1%, 1: 1–25%, 2: 25–50%, 3: 50–75% and 4: >75%. The staining intensity was scored from 0 to 3, namely, 0: negative, 1: low, 2: moderate, and 3: high. Semi-quantitative analysis was performed based on the percentage of positively stained cells and staining density by two pathologists independently.

2.4 Radioresistant HCT116 cells establishment

HCT116 cells were plated in 10-cm culture dishes with medium supplemented with 10% FBS (HyClone, UT, USA). After 24h culture, the cells were exposed to a signal dose of 6 Gy X-rays at a dose rate of 500 cGy/min. After 2 weeks of incubation, the colonies were digested and replated, and then the above radiotherapy process was repeated. The cells undergone two cycles of radiotherapy were named P2 HCT116.

2.5 Clonogenic survival assay

The cells were plated into six-well plates at a density of 300-3,000 cells/well depending on the dose of radiation. The application concentration of TPEN and pyriothione (Py) were 3 $\mu\text{mol/L}$ and 500 nmol/L, respectively. And then, the cells were irradiated with 0, 1, 2, or 4 Gy X-ray. After the irradiation, the cells were incubated for 10–14 days. And then crystal violet staining was carried out. Colonies consisting of 50 or more cells were counted as a clone. Survival fraction was calculated as: (number of colonies / number of cells plated)_{irradiated} / (number of colonies/number of cells plated)_{control}.

2.6 Lentivirus construction and transfection

Lentiviruses carrying shRNA gene sequence targeting ZIP7 and green fluorescent protein gene sequence were designed and constructed by Hanbio Biotechnology Co., Ltd. (Shanghai, China). The targeting sequence were 5'-CCACAATGACTGTCCTGCTACATGA-3' for the No. 1 lentivirus (Sh-ZIP7 #1), 5'-GCCTTTCTTGTCGTGGAGAAA-3' for the No. 2 lentivirus (Sh-ZIP7 #2), and 5'-TTCTCCGAACGTGTCACGT-3' for the control lentivirus (Sh-Con). HCT116 cells were plated into six-well plates at a density of 10^4 cells/well, and were transfected with the above lentiviruses at a multiplicity of infection (MOI) value of 20. The polybrene at the concentration of 6 $\mu\text{g/ml}$ was used during lentivirus transfection. At last, Western blot was carried out to verify the effectiveness of gene knockdown.

2.7 Western blot analysis

According to the instructions of the BCA kit (Beyotime Biotechnology, Shanghai, China), the total cell proteins were collected. Proteins were separated by SDS-PAGE and blotted onto a nitrocellulose membrane, following incubation with the specific primary antibodies, and then peroxidase-conjugating corresponding secondary antibodies. At last, the bands were visualized by chemiluminescence and were analyzed by Image J. Anti-ZIP7 (1:1000 dilution, Cat. ab117560, Abcam), ZEB1 (1:500 dilution, Cat. ab203829, Abcam), E-cadherin (1: 1000 dilution, Cat. 3195S, CST), GAPDH (1:1000 dilution, Cat. 2118S, CST) were applied as primary antibodies. For Py intervention experiment, the cells were treated with Py or control medium for 48h before protein collection.

2.8 Statistical analysis

The data were exhibited as mean \pm standard deviation (SD) and analyzed by SPSS19.0 software (V19.0 for Windows; SPSS, Inc., Chicago, IL, USA). Statistical significance was determined via a one-way analysis of the variance (ANOVA), and the Student's t-test was used when there were two groups. The

sensitizer enhancement ratios (SER) were measured according to the multi-target single hit model. $P < 0.05$ was considered statistically significant, and $P < 0.01$ and $P < 0.001$ were considered highly significant.

3. Results

3.1 ZIP7 was upregulated in human colon cancers

To explore the correlation between ZIP7 and colon cancer, the expression levels of ZIP7 in colon cancer and control normal tissues were explored using public databases and IHC staining. The mRNA expression analysis of the GEPIA database showed that ZIP7 was overexpressed in colon cancer compared with adjacent normal colon tissues ($P < 0.001$, Fig. 1A). Moreover, the analysis of the CPTAC database suggested that ZIP7 protein expression was also upregulated in colon cancer tissues ($P < 0.001$, Fig. 1B). The result of IHC staining was consistent with the GEPIA database, and the immunoreactivity score (IRS) of ZIP7 in colon cancer tissues was 5.57 ± 2.75 , significantly higher than 2.31 ± 1.40 in paired normal tissues ($P < 0.001$, Fig. 1C-D).

3.2 Colon cancer radioresistance was related to intracellular zinc

In order to identify the influencing factors of colon cancer radioresistance, the radioresistant HCT116 cells were constructed based on the method of desensitization by repeated radiotherapy (Fig. 2A). The results of clonogenic survival assay showed that, the survival fractions of all cells decreased in a dose-dependent manner, and the radioresistance of P2 HCT116 was significantly higher than that of parental cells (Fig. 2B). The mean lethal doses (D_0) for the parent cell and P2 HCT116 were 1.34 and 1.88 Gy, and the quasithreshold doses (D_q) were 0.39 and 0.10 Gy, respectively. The SER was 0.71 for desensitization radiotherapy.

Then, the relationship between radioresistance of colon cancer and zinc was studied by application of TPEN, a transmembrane zinc chelator. As shown in Fig. 2C, TPEN significantly reversed the radioresistance of P2 HCT116. The D_0 decreased from 1.91 Gy in P2 HCT116 group to 1.16 Gy in TPEN intervention group, while the D_q decreased from 0.93 Gy to 0.56 Gy. The SER was 1.64 for TPEN intervention.

3.3 ZIP7 maintains colon cancer radioresistance through regulation of zinc

To investigate the impacts of ZIP7 on colon cancer radioresistance, ZIP7 knockdown colon cancer cells were established by lentivirus mediated RNA interference technology based on P2 HCT116. As shown in Fig. 3A, the infection efficiencies were more than 90% in both targeting and control groups. The following results of western blot showed that, lentivirus with ZIP7 shRNA (sh-ZIP7) sequence could significantly

reduce the expression of ZIP7 ($P < 0.01$, Fig. 3B-C). Further results of clonogenic survival assay showed that, ZIP7 knockdown significantly reduced the radioresistance of P2 HCT116, which effect could be partially, but significantly reversed by Py, a transmembrane ionophore of zinc (Fig. 3D). Meanwhile, we found that the Py, at the corresponding concentration, had no effect on the radioresistance of the control P2 HCT116 cells (Fig. 3B). The D_0 of sh-Con, sh-Con + Py, sh-ZIP7 and sh-ZIP7 + Py group were 1.86, 1.78, 1.23 and 1.49 Gy, while the D_q of each group were 1.04, 1.23, 0.41, and 0.57, respectively. The D_0 and D_q of sh-ZIP7 group were the lowest. The SER was 1.51 for ZIP7 knockdown P2 HCT116 to sh-Con cells, and was 0.83 for Py intervention in ZIP7 knockdown P2 HCT116 cells.

3.4 ZIP7 maintains colon cancer EMT through regulation of zinc

The occurrence of EMT plays an important role in tumor radioresistance. In order to preliminarily explore the mechanisms of colon cancer acquired radioresistance regulated by ZIP7, the expressions of relating proteins were detected by Western blot. As Fig. 4A showed that, desensitization radiotherapy significantly upregulated the expression of ZIP7 and ZEB1, and the gray values of ZIP7 and ZEB1 in P2 HCT116 were about 1.85 and 2.08 times of that in parental cells, respectively ($P < 0.01$). Contrarily, the expression of E-cadherin was significantly downregulated in P2 HCT116, and the relative gray value was just about 71% of that in parental cells ($P < 0.01$). Further study showed that, ZIP7 knockdown significantly downregulated the expression of ZEB1 by 86% in P2 HCT116, while upregulated the expression of E-cadherin 1.57 times ($P < 0.01$, Fig. 4B). More meaningfully, Py treatment partially reversed the expression changes of the above protein causing by ZIP7 knockdown ($P < 0.01$, Fig. 3B). The relative gray value of ZEB1 for ZIP7 knockdown alone group was just about 20% of that for ZIP7 knockdown plus Py treatment group, while E-cadherin expression was 1.73 times higher than that for ZIP7 knockdown plus Py treatment group.

4. Discussion

Radioresistance is still a huge obstacle of colon cancer treatment, although the effectiveness of some new radiotherapy technologies, such as selective internal radiotherapy and stereotactic body radiotherapy, have been confirmed.[4, 18, 19] The exploration of radioresistance mechanisms could provide strategies for improving the efficacy of radiotherapy.[5] The results of information analyses of databases and IHC staining showed that ZIP7 was highly expressed in colon cancer tissues, indicating it may be a therapeutic target.

Zinc is an important signal mediator in the progression of various tumors, and plays key roles in many malignant biological behaviors. Cheng *et al.* confirmed that zinc supplementation could significantly increase the metastasis and invasion abilities of esophageal cancer cells, which effects were effectively reversed by zinc chelator TPEN.[20] And these phenomena may attribute to zinc activated PI3K/AKT and MAPK/ERK signaling pathway.[20] In addition, it has been reported that TPEN could cause cell death in a

dose-dependent manner by inducing oxidative stress and inhibiting autophagy, and zinc application completely reversed this kind of death.[21] For tumor radiotherapy, autophagy plays a cytoprotective role. In glioma cells, the chelation of intracellular zinc blocked the lysosomal degradation of autophagic vesicles, and further reduced the survival fraction of cells in radiotherapy. However, the equivalent dose of TPEN has no effect on glioma cells without radiotherapy.[22] In this study, we found that low concentration TPEN significantly reversed the radioresistance of progeny colon cancer cells, which provides a theoretical basis for the application of TPEN in colon cancer radiotherapy.

The impact of ZIP7 on some malignant biological behaviors of cancer has been reported, including breast cancer, cervical cancer and gastric cancer.[13–15] Sheng *et al.* found that downregulation of ZIP7 significantly inhibited cell growth and induced apoptosis in colon cancer cells.[23] However, little is known about the role of this molecule in cancer radioresistance. In this study, knockdown ZIP7 was found could significantly reduce the survival ability of radioresistant colon cancer cells after irradiation, while transmembrane ionophore of zinc partially reversed this effect. This result indicated that ZIP7 kept colon cancer radioresistance through regulation of zinc.

ZIP7 has been considered to be “a hub for tyrosine kinase activation”.[12] In the progression of cancer, some major pathways, such as MAPK, PI3K-AKT and mTOR, could be activated by ZIP7-mediated zinc release from intracellular stores.[24] Other members of ZIPs family have also been confirmed to promote tumor development through the above signaling pathways.[7, 20] In addition, ZIP7 plays an important role in maintenance the function state of endoplasmic reticulum (ER). Knockdown or knockout the expression of ZIP7 could induce ER stress and impact protein transport.[25–27] Therefore, the influence of ZIP7 on colon cancer radioresistance, as verified in this study, may be completed through a complex network.

For basic mechanisms, EMT acting as an essential process involved in acquired radioresistance has been widely accepted.[28] Irradiation can induce the occurrence of EMT through many signaling pathways. [28] During EMT, not only the interactions of cell-cell and cell-extracellular matrix are remodeled, but also the cancer stem cell properties, enhanced DNA damage response and antioxidant activity abilities are obtained.[29, 30] In this study, we found that irradiation induction promoted ZIP7 and ZEB1 expression, and the occurrence of EMT. Knockdown of ZIP7 could downregulate ZEB1 expression, but upregulate E-cadherin expression, and both of which could be partially reversed by Py treatment. These results indicated that ZIP7, through regulation of zinc, kept colon cancer radioresistance at least partially by maintenance of EMT. Therefore, ZIP7 may be a novel druggable node in radiosensitization treatment of colon cancer, and this could be possible because a small molecule inhibitor of ZIP7 has been developed. [31]

5. Conclusions

To sum up, ZIP7 was upregulated in human colon cancers compared with adjacent normal tissues. Irradiation induction increased the radioresistance of colon cancer, which could be reversed by chelation

of intracellular zinc and ZIP7 knockdown. In terms of mechanisms, the keeping role of ZIP7 on colon cancer radioresistance at least partially attributing to maintenance of EMT. Consequently, ZIP7 may be a novel target in radiosensitization treatment of colon cancer.

Declarations

Authors contribution

QZ designed the experiments. QLZ, HN, JS and JDP contributed to the experiments. All authors contributed to the analysis and discussion of the results, and preparation of the manuscript. All authors read and approved the final manuscript.

Data availability

Data are available upon request

Funding

This study was supported by the Natural Science Foundation of China (No. 81773227 and 81800490), the Top Talent Support Program for young and middle-aged people of Wuxi Health Committee (HB2020009), the Natural Science Foundation of Jiangsu Province (No. BK20170211), and High-end talents and High-end medical expert team of the 2020 Taihu Talent Program.

Compliance with ethical standards

Conflicts of Interest

The authors declare no conflicts of interest in this work.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of Wuxi People's Hospital Affiliated to Nanjing Medical University and adhered to the principles of the Declaration of Helsinki.

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Figures

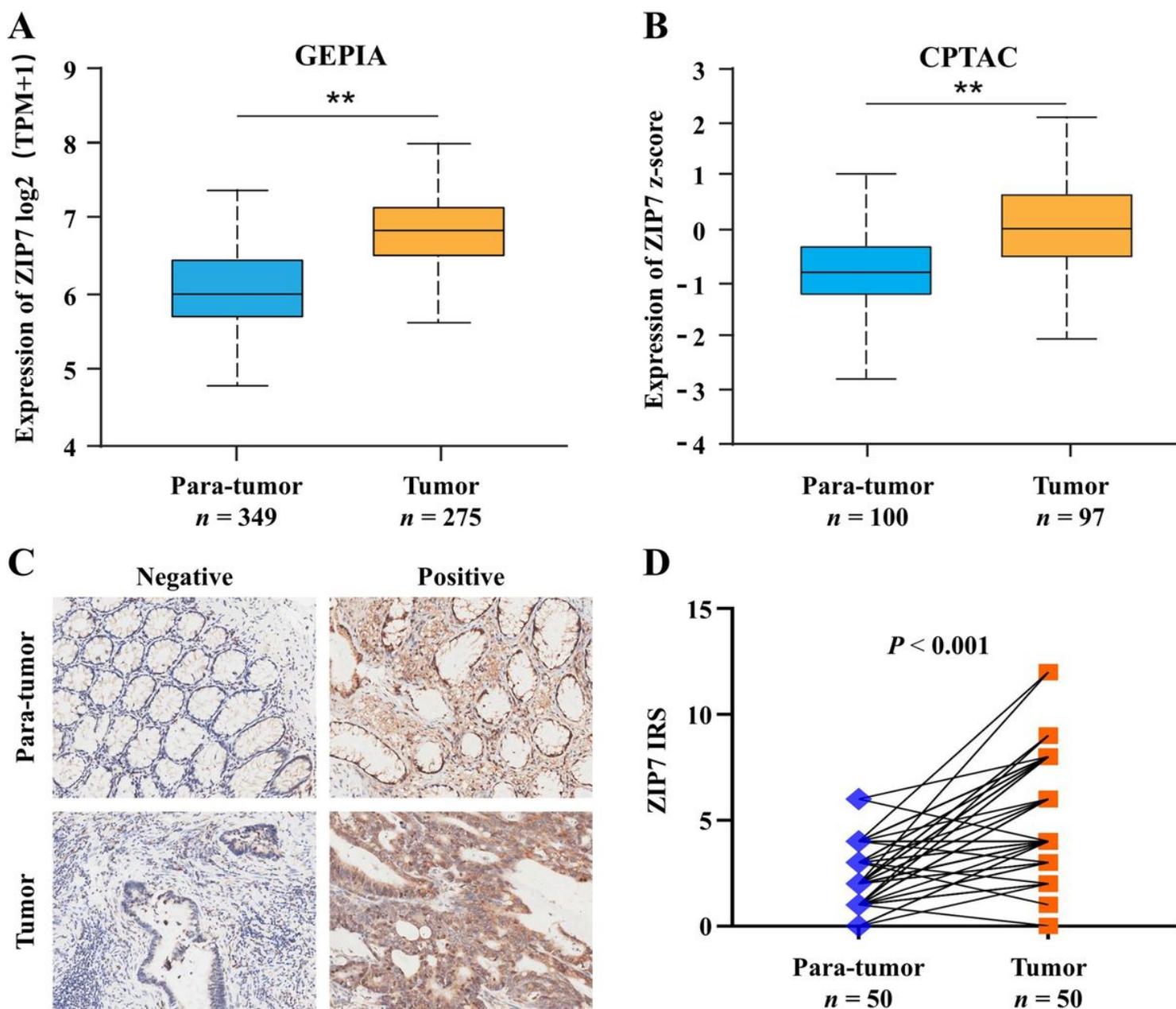


Figure 1

ZIP7 expression levels in colon cancer and normal colon tissues. (A) Transcriptional expression of the ZIP7 in cancer and adjacent normal tissues based on the GEPIA database. (B) Proteomic levels of the ZIP7 in cancer and adjacent normal tissues based on the CPTAC database. (C) Representative images from IHC staining represented negative and positive staining in colon cancer and normal tissues. Magnification: 40 X. (D) The expression levels of ZIP7 protein based IHC staining. **P < 0.001.

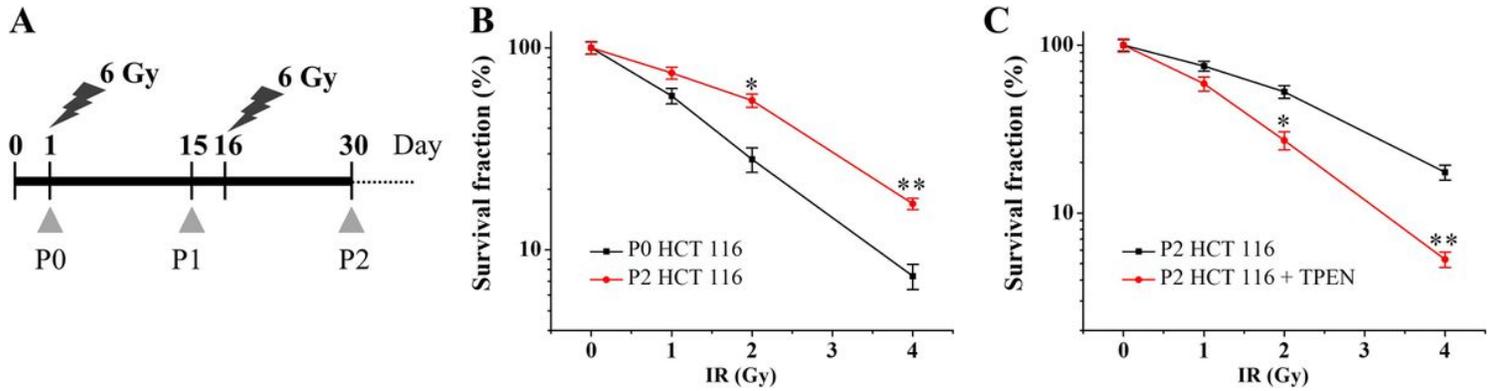


Figure 2

Zinc chelator reduced the radioresistance of colorectal cancer cells. (A) Schematic representation of radioresistant HCT116 cells establishment. (B) Survival curve of P0 and P2 HCT116 cells after different doses of radiation. (C) Survival curve of radioresistant HCT116 cells with or without TPEN treatment. n = 3 wells per group. *P < 0.01, **P < 0.001.

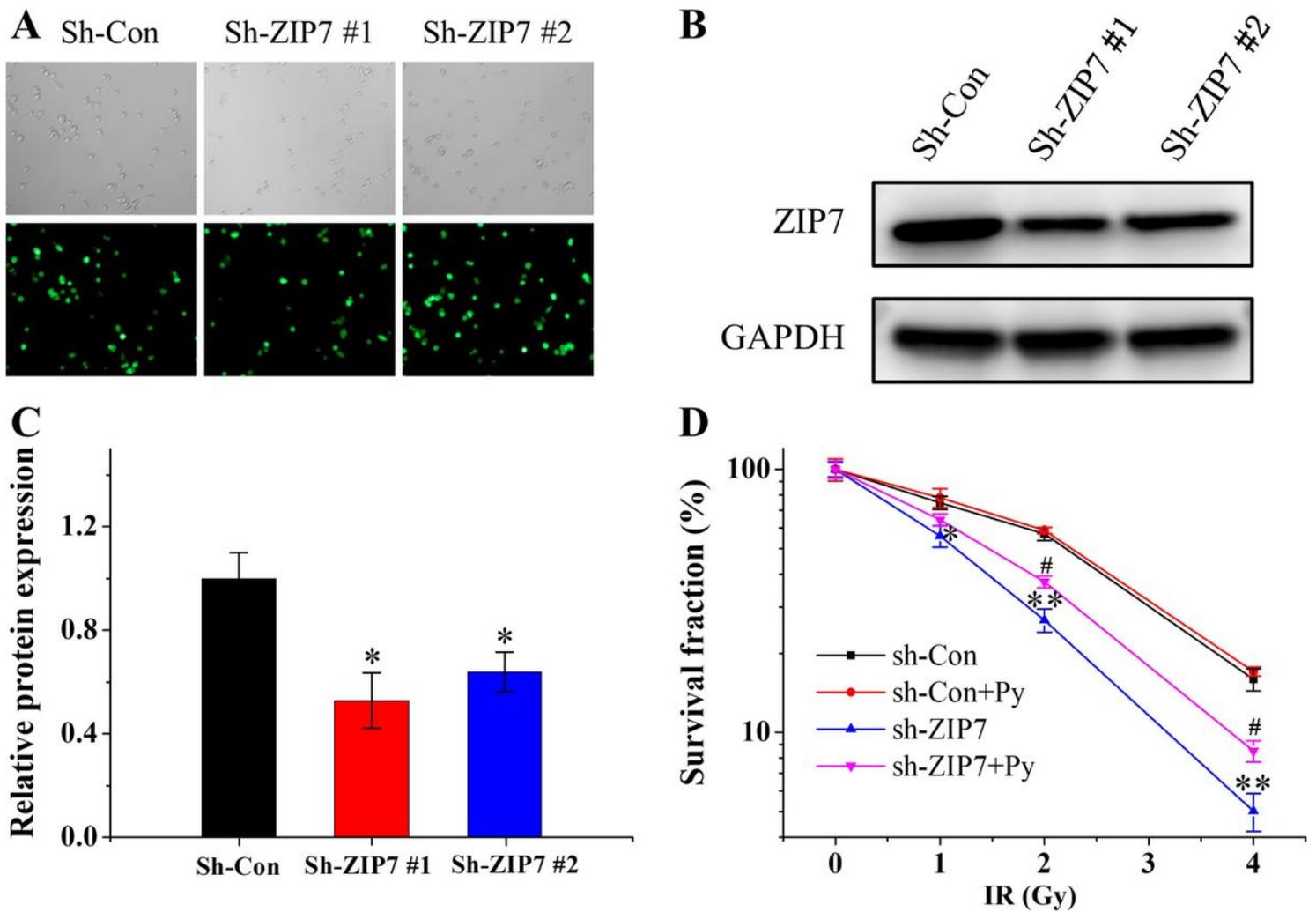


Figure 3

ZIP7 keeping colon cancer radioresistance through regulation of zinc. (A) Representative white light and fluorescence images of lentivirus infected cells from sh-ZIP7 or control group. Magnification: 200 X. (B-C) Western blot analyses of ZIP7 protein levels in P2 HTC116 cells following sh-ZIP7 or control lentivirus transfection. * $P < 0.01$. (D) Survival curves of sh-ZIP7 and sh-Con P2 HCT116 cells after different doses of radiation with or without Py treatment. $n = 3$ wells per group. sh-ZIP7 vs sh-Con * $P < 0.01$, ** $P < 0.001$, sh-ZIP7 vs sh-ZIP7+Py # $P < 0.01$.

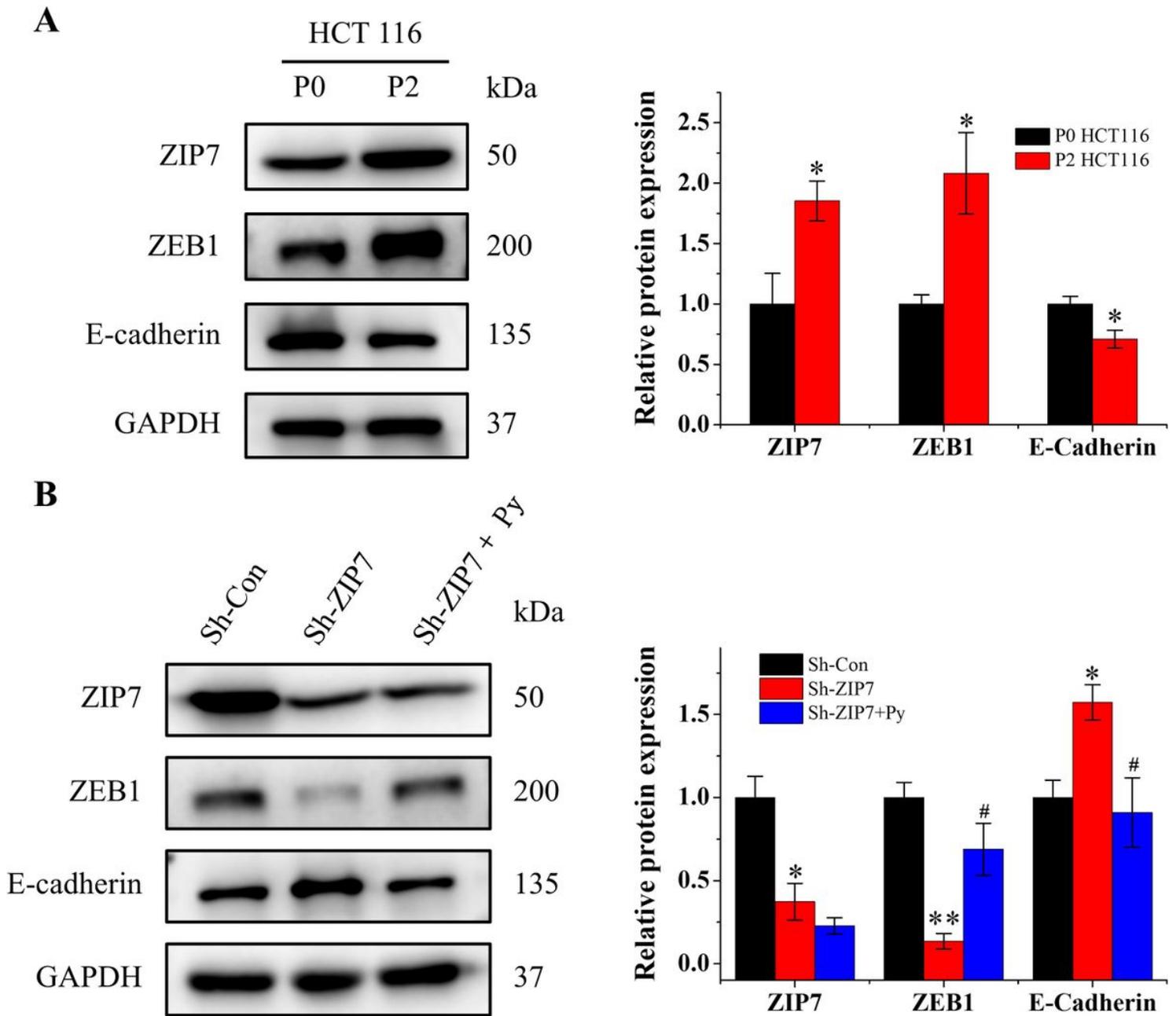


Figure 4

ZIP7 keeping colon cancer ZEB1 expression and EMT through regulation of zinc. (A) Western blot analyses of ZIP7, ZEB1 and E-cadherin protein levels in P0 and P2 HCT116 cells. $n = 3$, $*P < 0.01$. (B) Western blot analyses of ZIP7, ZEB1 and E-cadherin protein levels in sh-ZIP7 and sh-Con P2 HCT116 cells. $n = 3$, sh-ZIP7 vs sh-Con $*P < 0.01$, $**P < 0.001$, sh-ZIP7 vs sh-ZIP7+Py $\#P < 0.01$.