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Original Article

Tetraspanin 8 promotes radioresistance of human nasopharyngeal carcinoma via the activation of PI3K/AKT pathway

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Abstract: Aims: The present study aimed to detect the expression of tetraspanin 8 (TSPAN8) in human nasopharyngeal carcinoma (NPC) tissues, and to investigate the role of TSPAN8 in NPC radioresistance and related molecular mechanism. Methods: A total of 35 fresh human NPC tissues and the adjacent normal tissues were collected, and a radioresistant cell line CNE-2R was established. TSPAN8-pcDNA3.1 was constructed into the CNE-2 cells to overexpress TSPAN8, and LY294002 was used to suppress phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) signaling pathway in CNE-2 cells. Expression of TSPAN8 and phosphorylated AKT was detected using RT-qPCR and/or western blot analysis. MTT, colony formation and flow cytometry analyses were used to determine cell growth, survival fraction (SF) and cell apoptosis. Results: TSPAN8 was upregulated in human NPC tissues and the radioresistant cells CNE-2R. Overexpression of TSAPN8 led to enhanced radioresistance of CNE-2 cells, by promoting cell growth and SF, as well as inhibiting cell apoptosis. Further, we found TSPAN8 positively regulated the phosphorylation of AKT so as to activate the AKT signaling. However, TSPAN8 induced-radioresistance in NPC was reversed by PI3K/AKT signaling suppression. Conclusion: This study firstly demonstrated TSPAN8 expression was elevated in human NPC tissues and radioresistant NPC cell line CNE-2R, and TSPAN8 could promote NPC radioresistance at least partially via the activation of PI3K/AKT signaling pathway. These findings may help in sensitization of NPC to radiotherapy.

Keywords: Tetraspanin 8, nasopharyngeal carcinoma, radioresistance, PI3K/AKT pathway

Introduction

Nasopharyngeal carcinoma (NPC) is a non-lymphomatous and squamous-cell carcinoma whose etiology is associated with Epstein-Barr virus infection, genetic susceptibility, and environmental exposures [1-3]. NPC is rare in most populations of the world; however, it is highly prevalent in Southern China and Southeast Asia, with an annual incidence of 15-50 cases per 100,000 [4]. Radiotherapy is the preferred treatment for nonmetastatic NPC [5]. Although the overall 5-year survival rate of NPC has improved with the technological advances in the fields of radiotherapy and comprehensive treatment, resistance of radiotherapy is still a major obstacle in the treatment of NPC [6, 7].

Tetraspanins are a family of four transmembrane proteins which interact and form complexes with a wide variety of proteins. They play important roles in regulating cellular processes, such as cell proliferation, differentiation, migration, and invasion [8-10]. Tetraspanin 8 (TSPAN8), also known as C0-029 in humans and D6.1 in rats, is a member of the tetraspanins family. TSPAN8 was firstly identified as a tumor-associated antigen present in human tumor cell lines of various histologic origins [11]. In recent years, increasing studies suggest that TSPAN8 overexpression correlates with the progression and metastasis of several cancers, such as gastric cancer, colorectal cancer, esophageal carcinoma, and melanoma [12-15]. However, the expression and function of TSPAN8 in NPC has not been reported until now.

Some multifunctional signal transduction pathways have been demonstrated to play essential

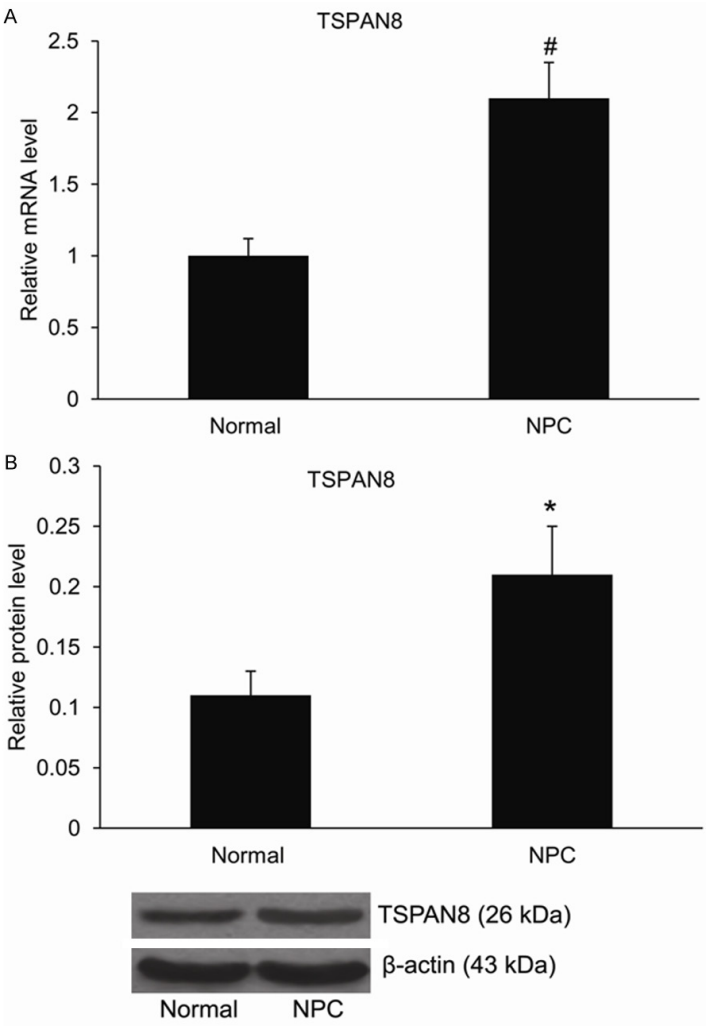

Figure 1. Expression of TSPAN8 in human NPC tissues. A. The relative mRNA level of TSPAN8. B. The relative protein level of TSPAN8. NPC, nasopharyngeal carcinoma; TSPAN8, tetraspanin 8. #P < 0.01, *P < 0.05, compared with the control.

roles in modulating tumor radiation response [16-18]. Phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) is a classical signal transduction pathway that can be activated by ionizing radiation or the receptor tyrosine kinase (RTK) of epidermal growth factor receptor (EGFR) and insulin-like growth factor 1 receptor (IGFR), and participates in the regulation of tumor radiation response [19, 20].

In the present study, we firstly detected the expression of TSPAN8 in human NPC tissues, and investigated the role of TSPAN8 in NPC radioresistance. Furthermore, whether PI3K/AKT pathway, a signaling pathway which was demonstrated to be related to radioresistance by numerous studies, mediates the effects of TSPAN8 on NPC radioresistance was explored.

Materials and methods

Clinical specimens

This study was permitted by the Ethical Committee of Huai'an First People's Hospital, Nanjing Medical University and written informed consents were obtained from all the participants. A total of 35 fresh NPC tissues and the adjacent normal tissues were collected from 35 NPC patients who underwent surgery in the Department of Otolaryngology and Head & Neck Surgery, Huai'an First People's Hospital, Nanjing Medical University. None of these patients had a prior history of radiotherapy or chemotherapy. Mean patient age was 45 years-old (range, 35-63 years-old).

Cell culture and cell transfection

The CNE-2 cells were purchased from the Cell Bank of Wuhan University (Wuhan, Hubei, China). The cells were cultured in RPMI-1640 medium (HyClone Laboratories, Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), and maintained in a humidified 95% air 5% CO_2 atmosphere at 37°C. LY294002 were obtained from Invitrogen (Carlsbad, CA, USA), and dissolved in DMSO (Sigma, St Louis, MO, USA) and stored at 50 mM. Cells were treated with 20 μM LY294002. For cell transfection, the cells were seeded at 70% confluence on 6-well plates. Next day, the cells were transfected with 1 μg plasmid DNA using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions.

Irradiation

To establish a radioresistant NPC cell line, the CNE-2 cells were expose to a range of doses of irradiation (2, 4, 6 Gy) delivered by a RS 2000 biological irradiator (Rad Source Technologies, Inc., Suwanee, GA, USA). Briefly, the CNE-2 cells

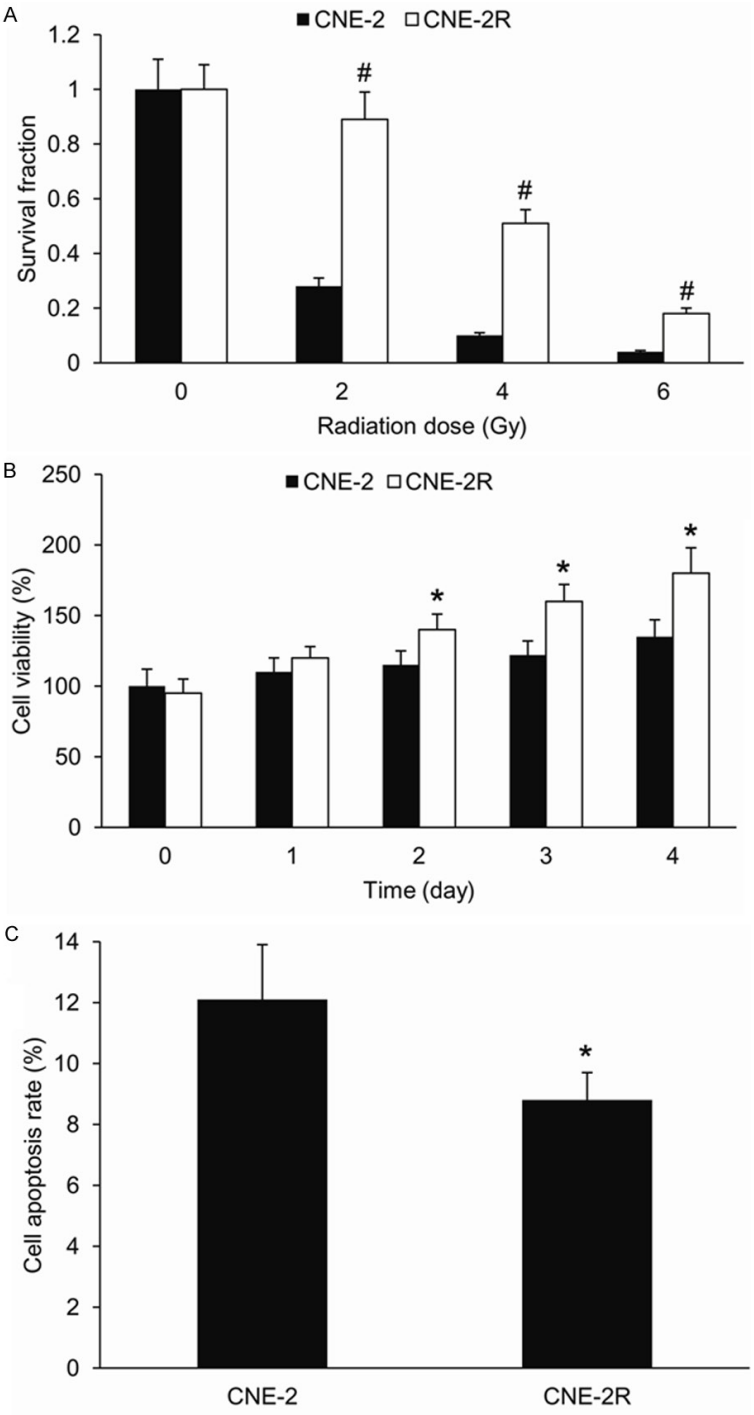

Figure 2. The radiosensitivity of CNE-2 and CNE-2R cells. A. Survival fraction determined by colony formation assay. B. Cell viability determined by MTT assay. C. Cell apoptosis rate determined by flow cytometry assay. #P < 0.01, *P < 0.05, compared with the CNE-2 cells.

in exponential growth phase were exposed to irradiation at a dose of 2 Gy. When the irradiated cells reached an exponential growth phase, the next 4 Gy and 6 Gy was delivered repeatedly in the same way.

Colony formation assay

Cells were plated into 6-well plates at the density of 0.3×10^3 cells/well, and routinely cultured overnight. Then, the cells were exposed to specified dose (0, 2, 4 and 6 Gy) of radiation for 24 h, and cultured for 14 days to allow colony growth. The colonies were fixed with 4% paraformaldehyde and stained with crystal violet. Colonies containing more than 50 cells were counted under an inverted microscope.

MTT assay

The cells were seeded at a density of 1×10^4 cells/100 μl in 96-well plates, and allowed to grow for the appropriate times. Cells were incubated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (10 μl/well of 0.5 mg/ml solution in PBS; Sigma) at 37°C for 4 h. After that, 150 μl/well DMSO was added to dissolve the formazan crystals, and the absorbance was measured at 570 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Flow cytometry (FCM) analysis

The cells were plated at 50-60% confluence, and irradiated with the dose of 10 Gy for 48 h. The apoptosis cells were detected using the FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, San Jose, CA, USA) according to the manufacturer's instructions, and quantified using a FACSCalibur flow cytometer (Becton Dickinson, Sparks, MD, *USA*).

Western blot

The tissues and cultured cells were washed with PBS, and lysed in cell lysis buffer, containing 150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1%

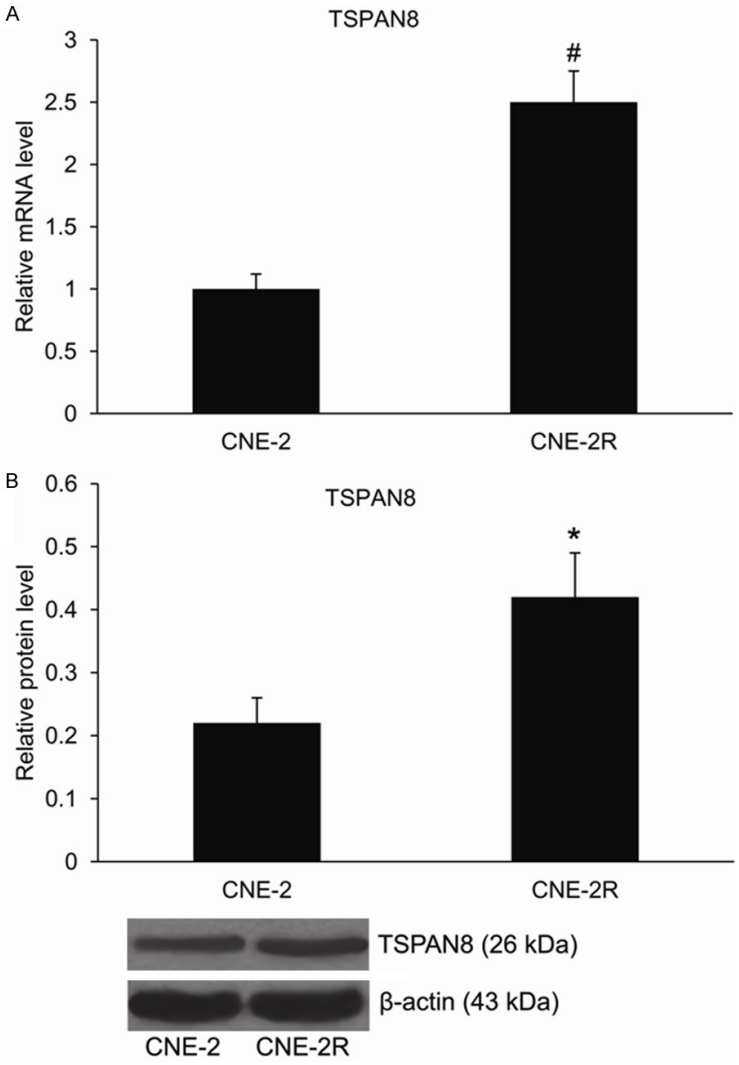

Figure 3. Expression of TSPAN8 in CNE-2 and CNE-2R cell lines. A. The relative mRNA level of TSPAN8. B. The relative protein level of TSPAN8. TSPAN8, tetraspanin 8. #P < 0.01, *P < 0.05, compared with the CNE-2 cells.

Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 2 mM DTT. After centrifugation at 15,000 for 15 min, the supernatants were collected and the protein concentration was assayed using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins (50 μg/lane) were loaded on 12% SDS-polyacrylamide gel for separation. The proteins were then electrotransferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk at 4°C overnight, the membranes were then incubated at 37°C for 1 h with different primary antibodies, including TSPAN8 rabbit polyclonal antibody (1:800; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), phospho-Akt (Ser473) rabbit monoclonal antibody (1:1000; Cell Signaling Technology, Inc., Beverly, MA, USA), Akt mouse monoclonal antibody (1:1000; Cell Signaling Technology, Inc.), and β-actin mouse monoclonal antibody (1:2000; Santa Cruz Biotechnology, Inc.). Incubations with HRP conjugated secondary antibodies were performed for 1 h at 37°C, and the signals were visualized by chemiluminescence (Western Blotting kit, Pierce Biotechnology, Inc., Rockford, IL, USA). The relative protein level of phospho-AKT was normalized to total AKT, and the TSPAN8 protein level was normalized to β-actin.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the tissues and cultured cells using the Trizol reagent (Invitrogen). 2 μg of total RNA were reverse transcribed to cDNA with a RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania). Quantitative PCR was performed on cDNA using the SYBR Green master mix (Applied Biosystems, Foster City, CA, USA). The primers used were: TSPAN8 primers, 5'-ttcacggcatctggattcct-3' (forward), 5'-atgtccacagcaacgtagga-3' (reverse); β-actin primers, 5'-ccctgga gaagagcta cgag-3' (forward), 5'-cgtacaggtctttgcggatg-3' (reverse). All the primers were obtained from GenScript (Nanjing, Jiangsu, China). Reactions were performed on the ABI Prism 7000 Sequence Detection system (Applied Biosystems).

Statistical analysis

Statistical analysis was carried out using SPSS software (IBM SPSS, Armonk, NY, USA). All the data are expressed as the means ± SD, and the differences between 2 groups were analyzed using the *Student's t test*. P < 0.05 was considered to indicate statistical significance.

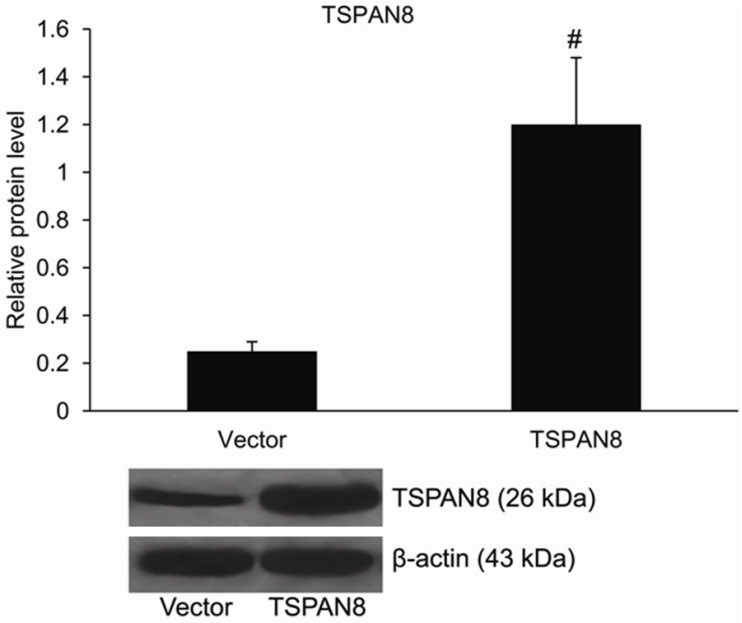

Figure 4. Expression of TSPAN8 protein in CNE-2 cells following transfection with the TSPAN8-pcDNA3.1. #P < 0.01 compared with the cells transfected with the vector control.

Results

Expression of TSPAN8 in human NPC tissues

Using RT-qPCR and western blot analysis, we detected the expression of TSPAN8 in human NPC tissues and the adjacent normal nasopharyngeal tissues. As shown in **Figure 1**, the relative mRNA and protein levels of TSPAN8 were significantly upregulated in human NPC tissues compared with that in the adjacent normal tissues ($P < 0.01$ for mRNA and $P < 0.05$ for protein).

Establishment of a radioresistant NPC cell line CNE-2R

To establish a radioresistant NPC cell line, the CNE-2 cells were exposed to a range of doses of irradiation (2, 4 and 6 Gy) for about one year, and the surviving cell line was referred as CNE-2R. The radioresistance of CNE-2R cell line was then verified. The colony formation assay revealed that CNE-2R cells showed higher survival fraction than CNE-2 cells when exposed to irradiation (2, 4 and 6 Gy) (**Figure 2A**). MTT assay was performed to examine the effect of irradiation on cell growth. As shown in **Figure 2B**, after irradiation with 2 Gy, CNE-2R cells had higher cell viability compared with the CNE-2 cells. At 48 h after irradiation with 10 Gy, FCM analysis demonstrated that CNE-2R cells exhibited lower cell apoptosis rate than CNE-2 cells (**Figure 2C**). These results indicated that CNE-2R is more radioresistant than its parent CNE-2 cells.

Expression of TSPAN8 in CNE-2 and CNE-2R cell lines

Next, we detected the expression of TSPAN8 in CNE-2 and CNE-2R cells. We found that TSPAN8 mRNA level was significantly increased in CNE-2R cells compared with CNE-2 cells (**Figure 3A**). Similar results were then confirmed by western blot analysis (**Figure 3B**).

Effect of TSPAN8 on radiosensitivity of CNE-2 cells

TSPAN8-pcDNA3.1 was constructed and transfected into CNE-2 cells to overexpress TSPAN8. As expected, TSPAN8 protein expression was significantly increased in TSPAN8-pcDNA3.1-transfected cells compared with the vector control (**Figure 4**).

To determine the role of TSPAN8 in NPC radiosensitivity, the CNE-2 cells transfected with TSPAN8-pcDNA3.1 were subjected to irradiation, and the colony formation assay, MTT, and FCM analyses were performed. As shown in **Figure 5A** and **5B**, TSPAN8 overexpression resulted in increased survival fraction and cell growth of CNE-2 cells. In addition, cell apoptosis rate was significantly decreased when TSPAN8 was overexpressed in CNE-2 cells, as shown in **Figure 5C**. These results indicated that TSPAN8 renders the radioresistance of CNE-2 cells.

Effect of TSPAN8 on PI3K/AKT pathway in CNE-2 cells

As clearly shown by western blot analysis, following trasnfection of TSPAN8-pcDNA3.1, we found that the relative protein level of phosphorylated AKT (S473) was significantly increased in CNE-2 cells compared with the vector control-transfected cells (**Figure 6**).

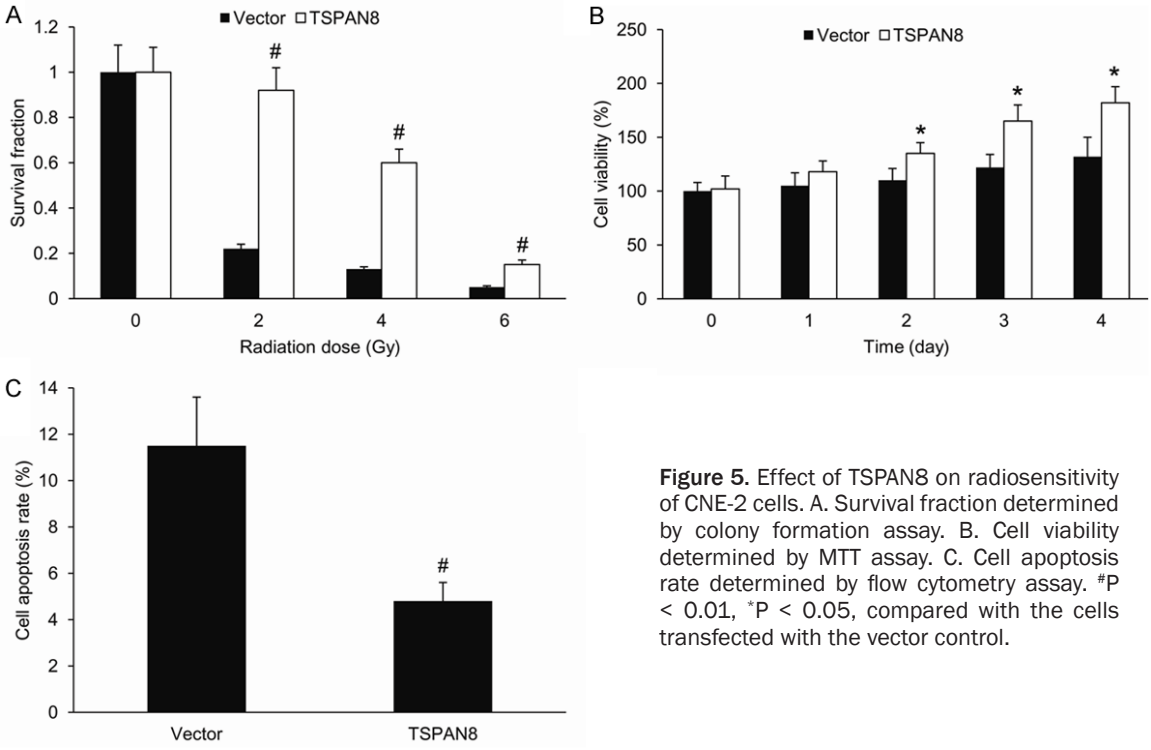

Figure 5. Effect of TSPAN8 on radiosensitivity of CNE-2 cells. A. Survival fraction determined by colony formation assay. B. Cell viability determined by MTT assay. C. Cell apoptosis rate determined by flow cytometry assay. #P < 0.01, *P < 0.05, compared with the cells transfected with the vector control.

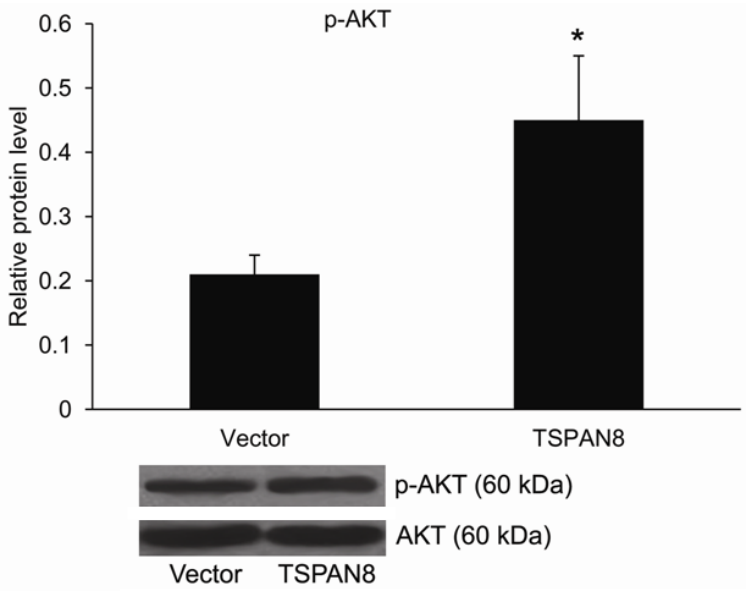

Figure 6. Effect of TSPAN8 on PI3K/AKT pathway in CNE-2 cells. p-AKT, phosphorylated AKT. *P < 0.05, compared with the cells transfected with the vector control.

PI3K/AKT pathway mediates the effects of TSPAN8 on radiosensitivity of CNE-2 cells

To investigate whether PI3K/AKT pathway mediates the effects of TSPAN8 on radiosensitivity of CNE-2R cells, TSPAN8 was overexpressed in CNE-2 cells by transfection of TSPAN8-pcDNA3.1, and the cells were treated with LY-294002 to inhibit PI3K/AKT pathway. TSPAN8 overexpression led to increased survival fraction and cell growth, as well as decreased cell apoptosis rate of CNE-2 cells exposed to irradiation; however, these effects were reversed by LY294002 treatment. As shown in **Figure 7**, the survival fraction and cell viability was significantly decreased, while the cell apoptosis rate was significantly increased in the TSPAN8+LY294002 group compared with that in the TSPAN8 group.

Discussion

In the present study, we firstly revealed that TSPAN8 was significantly upregulated in human NPC tissues, indicating that TSPAN8 may play important roles in NPC. Radioresistance is a major cause of treatment failure in NPC. Subsequently, we performed the in vitro experiments to investigate the effects of TSPAN8 on NPC radioresistance.

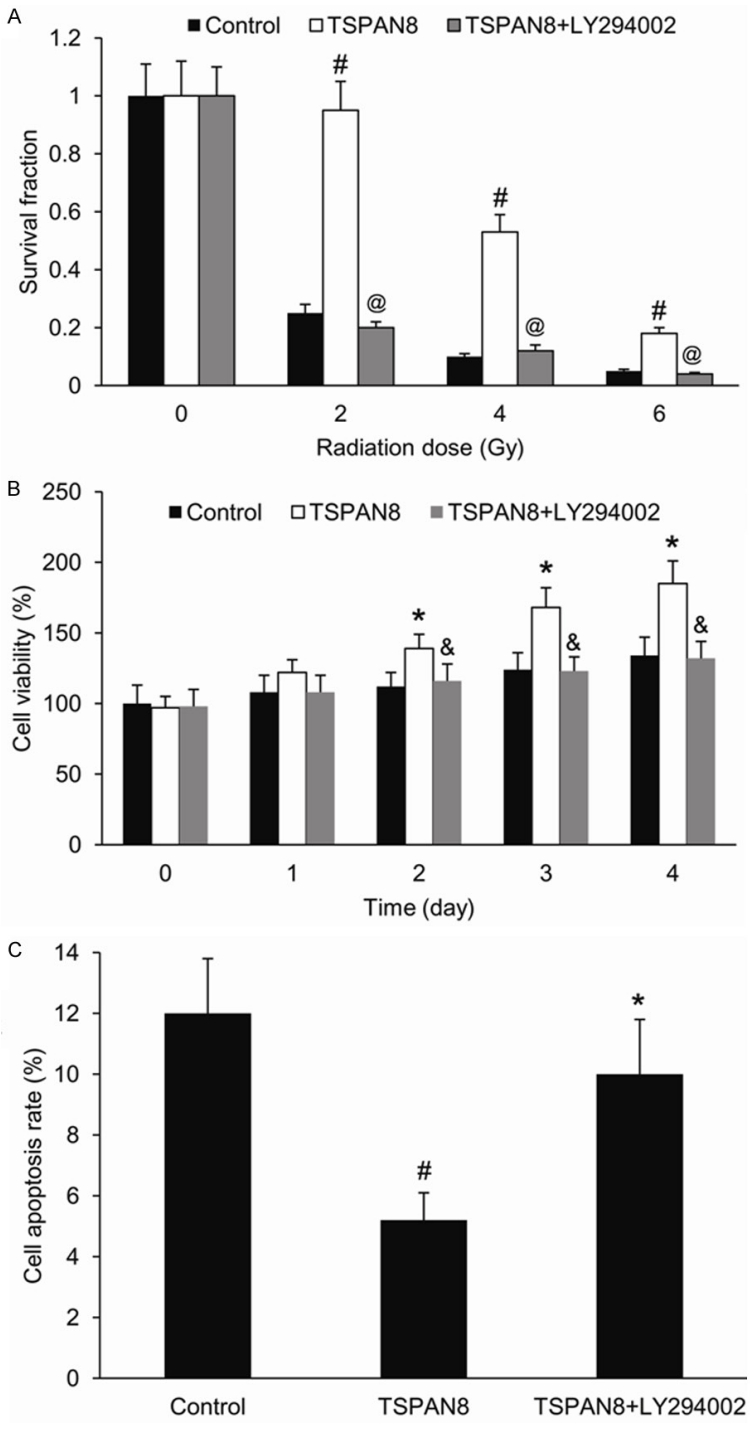

Figure 7. PI3K/AKT pathway mediates the effects of TSPAN8 on radiosensitivity of CNE-2 cells. CNE-2 cells were transfected with TSPAN8-pcDNA3.1, and treated with LY294002, a PI3K/AKT pathway inhibitor. A. Survival fraction determined by colony formation assay. B. Cell viability determined by MTT assay. C. Cell apoptosis rate determined by flow cytometry assay. $^{\#}P < 0.01$, $^{*}P < 0.05$, compared with the control cells; $^{@}P < 0.01$, $^{\&}P < 0.05$, compared with the cells transfected with TSPAN8-pcDNA3.1.

Tetraspanins act as "molecular facilitators" connecting membrane proteins involved in different signaling pathways, which influence cell motility [21-23]. TSPAN8 belongs to tetraspanins family, and it has been intensively studied in the field of cancer. Several studies have suggested that TSPAN8 is strongly expressed in cancer cells, and acts as a metastasis associated gene in many types of tumors [12-15]. Tspan8 could interact with several integrins, and signaling pathways, [24-27], thus contributing to cancer cell growth and motility. In the present study, we established a radioresistant cell line CNE-2R, and we firstly revealed that TSPAN8 was upregulated in radioresistant NPC cells compared with its parent cells. Subsequently, through gain-of-function experiments, we found that overexpression of TSPAN8 in CNE-2 cells led to increased cell growth and survival fraction, as well as decreased cell apoptosis after irradiation, indicating that TSPAN8 promotes radioresistance in NPC.

PI3K/AKT signaling pathway is an intracellular signaling pathway important in regulating various biological processes, including cell cycle, growth, invasion and metabolism [28]. PI3K generates phosphatidylinositol 3,4,5-trisphosphate (PIP3), which is an important lipid second messenger, and then PIP3 activates the serine/threonine kinase AKT. AKT translocates to the inner cell membrane and phosphorylated. It has been well-documented that the PI3K/AKT pathway is commonly activated in human cancers [29, 30], and alteration of components in this pathway may contribute to the radiotherapy resistance [31, 32]. In the present study, the results demonstrated that TSPAN8 is a crucial positive reg-

ulator of PI3K/AKT signaling pathway. TSPAN8 overexpression led to the phosphorylation of AKT so as to activate the AKT signaling. Suppression of the PI3K/AKT signaling pathway provided a novel approach for radiosensitization, and it was suggested to increase tumor radiosensitivity [33-36]. LY294002 is the first generation PI3K inhibitor that has been found to radiosensitize both in vitro and in vivo [35-37]. In this study, we used LY294002 to inhibit PI3K/AKT signaling pathway in TSPAN8-overexpressed CNE-2 cells and found that TSPAN8 induced-radioresistance in NPC was reversed by PI3K/AKT signaling suppression. These results suggested that PI3K/AKT signaling pathway is involved in mediating the effects of TSPAN8 on NPC radioresistance.

In conclusion, this study firstly demonstrated that TSPAN8 was related to radioresistance of NPC. TSPAN8 expression was elevated in human NPC tissues and radioresistant NPC cell line CNE-2R. TSPAN8 could promote NPC radioresistance at least partially via the activation of PI3K/AKT signaling pathway. Our findings suggested a novel marker in NPC radioresistance, and this study may help in sensitization of NPC to radiotherapy.

Acknowledgements

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Disclosure of conflict of interest

None.

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