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Original Article

RNA binding motif protein 3 (RBM3) drives radioresistance in nasopharyngeal carcinoma by reducing apoptosis via the PI3K/AKT/Bcl-2 signaling pathway

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Abstract: Radioresistance is an important obstacle to nasopharyngeal carcinoma (NPC) therapy. In this study, we explored the role of RNA-binding motif protein 3 (RBM3) in the radioresistance of NPC and its underlying mechanism. We measured the expression of RBM3 in 20 clinical NPC tissues and in NPC cell lines. We found that RBM3 was upregulated in radioresistant NPC tissues and cells. Radioresistant NPC cells (CNE1/IR) and parental NPC cells (CNE1) were subjected to RBM3-shRNA knockdown and RBM3 overexpression, respectively. RBM3 depletion in CNE1/IR cells sensitized cells to radiotherapy, increased DNA damage, and accelerated the rate of apoptosis. In contrast, RBM3 overexpression in CNE1 cells significantly enhanced radioresistance and reduced the rate of apoptosis. Additionally, radioresistance conferred by RBM3 was attributed to the activation of the AKT/Bcl-2 signaling pathway and reduction of caspase 3. Inhibition of AKT signaling attenuated RBM3-mediated radioresistance. Furthermore, RBM3 directly interacted with PI3K subunit p85 in NPC cell lines. Altogether, our data demonstrate that RBM3 enhances radioresistance by inhibiting the apoptotic response to radiotherapy through the PI3K/AKT/Bcl-2 signaling pathway. RBM3 may serve as a novel factor for predicting radioresistance and as a molecular target in the treatment of NPC.

Keywords: RBM3, nasopharyngeal carcinoma, radioresistance, apoptosis, Bcl-2, PI3K

Introduction

Nasopharyngeal carcinoma (NPC) is a common malignant tumor that has the highest incidence and mortality rate among malignant head and neck tumors. NPC is unsuitable for surgical resection due to its anatomical structure and local infiltration characteristics. Moreover, conventional chemotherapy drugs are ineffective. Thus, radiotherapy is the primary treatment modality for NPC [1]. The most frequently observed NPCs are sensitive to radiotherapy and are characterized by poorly differentiated squamous cells and undifferentiated carcinoma. However, a large number of studies have shown that ionizing radiation (IR) induces changes at the protein and gene expression level that lead to reduced sensitivity to IR and contribute to the development of radiation resistance, or radioresistance [2], These factors often lead to recurrence of the tumor. Radioresistance is the leading cause of unsuccessful therapy in NPC cases. Therefore, exploring the molecular mechanisms that regulate radioresistance in NPC is critical to improve the disease prognosis.

Numerous factors affect radioresistance, and accumulating evidence indicates that apoptosis may play a critical role in modulating the response to radiotherapy. However, the molecular mechanism of radioresistance remains unknown.

RNA-binding motif protein 3 (RBM3) has been demonstrated to bind to both DNA and RNA [3]. Previous studies have documented the role of RBM3 in various malignancies and suggest that high expression of RBM3 is a predictor for improved survival in prostate [4], colorectal cancer [5], and breast cancer [6]. Overexpression of exogenous RBM3 inhibits polyurethane induced apoptosis [7]. Although accumulating evidence has emphasized the importance of RBM3 in carcinogenesis, there is currently no systematic study investigating the impact of RBM3 on the radiation response of NPC.

In our study, we found that RBM3 was markedly overexpressed in radioresistant human NPC samples and radioresistant NPC cells. RBM3 overexpression increased resistance to IR. In contrast, knockdown of RBM3 promoted radiosensitivity of NPC cells. Furthermore, we explored the molecular mechanism of RBM3 participation in radioresistance. Our results provide insight into the mechanism of radioresistance in NPC and support a role for RBM3 in the promotion of radioresistance.

Materials and methods

Clinical samples

NPC paraffin-embedded tissue specimens (n = 20) from patients who had undergone radiation therapy (70-75 Gy) were collected from the Pathology Department of Xijing Hospital between January 2010 and January 2014. Specimens were classified as radiation sensitive (RS, 10 cases), with no residual tumor following radiation therapy, or as radiation resistant (RR, 10 cases), with viable residual tumors. All paraffin sections were subjected to immunohistochemistry and RT-qPCR analysis. This study was approved by the ethics committee of Xijing Hospital.

Immunohistochemistry (IHC)

All paraffin-embedded patient specimens were baked at 56°C for 1 h before deparaffinization in xylene followed by rehydration in graded alcohol (100%-75%). To perform antigen retrieval, tissue samples were boiled for 15 minutes in a microwave in 0.1 mM sodium citrate buffer (pH = 6.0). Endogenous peroxidases were inactivated in 3% H_2O_2 followed by blocking in goat serum for 20 minutes. All specimens were incubated with RBM3 polyclonal antibody (1:50, Cell Signaling Technology, Danvers, MA, USA) and Bcl-2 polyclonal antibody (1:200, Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. A DAB kit (Beyotime, China) was used for immunohistochemical staining.

Cell line culture and transfection

The NPC cell line, CNE1, was obtained from the Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences, Shanghai, China). CNE1 cells were irradiated with X-ray doses of 2 Gy every other day for 40 days, resulting in 20 total irradiation treatments. Surviving cells were considered radioresistant (CNE1/IR) and were cultured in RPMI-1640 (Gibco; Invitrogen, Carlsbad, California, USA) supplemented with 10% FBS (HyClone, Thermo, USA) and 1% penicillin-streptomycin in a humidified incubator at 37°C and 5% CO_2. We designed the shRNA-RBM3 (5'-CCGGCCCAATGTACCTATAAGAAATCTCGAGATTTCTTATAGGTACATTGGGTTTTT-3') and negative control (5'-TTCTCCGAACGTGTCACGTTTC-3') constructs, and the viral vectors were synthesized by Gene Pharma (Gene Pharma, Shanghai, China) and infected in CNE1/IR cells. RBM3 (pCDNA3.1-RBM3) and negative control (pCDNA3.1-NC) plasmids were obtained from Gene Pharma and transfected in CNE1 cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, California, USA), according to the manufacturer's instructions.

Radiation treatment

Cells were exposed to radiation in a Gamma Cell Radiator (RS-2000 XE Biological Irradiator, RAD. SOURCE) using X-rays as a radioactive source. A radiation dose of 1.47 Gy/min was applied to all groups. The culture medium was changed immediately following IR.

Real-time quantitative PCR (RT-qPCR)

DNA was isolated from paraffin-embedded NPC specimens using Takara MiniBEST FFPE DNA Extraction kit (Takara, Dalian, China). Total RNA was extracted from NPC cells with Trizol reagent (Takara, Dalian, China). cDNA was synthesized from RNA using the Prescript Reagent Kit (Takara, Dalian, China), according to the manufacturer's instructions. Reverse transcription was performed using SYBR Premix Ex Taq II (Tli

RNaseH Plus, Takara), and all qPCR reactions were performed using SYBR Green qPCR Master Mix (Clontech, USA). The primer sequences used in this study have been previously described [8] and are as follows: RBM3 5'-TGGG AGGGCTCAACTTTAAC-3'/5'-ATGCTCTGGGTTGGTGAAG-3' and GADPH 5'-CAGCCTCAAGATCATCAGCA-3'/5'-GTCTTCTGGGTGGCAGTGAT-3'. GAPDH was used as an internal control. The RT-PCR consisted of 40 cycles with an annealing temperature of 60°C.

Western blotting assay

Western blotting was performed as previously described [8]. The primary Rabbit antibodies for RBM3, Bcl-2, phosphorylation-AKT, AKT, and p-PI3K (p85) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) and were applied at a 1:500 dilution. A 1:1,000 dilution of mouse antibodies against human caspase 3 (Cell Signaling Technology, Danvers, MA, USA) and β-actin (Cell Signaling Technology, Danvers, MA, USA) were also used. β-actin was used as a loading control.

Clonogenic survival assay

Cells were seeded in 6-well plates at increasing cell densities that corresponded to the IR dose (400 cells for 0 Gy and 2 Gy; 800 cells for 4 Gy; 2,000 cells for 6 Gy; 4,000 cells for 8 Gy; and 10,000 cells for 10 Gy). IR was performed as described above. After 2 weeks, the cells were stained with 0.5% crystal violet in anhydrous ethanol, and colonies were regarded as more than 50 cells. Survival fraction was calculated as follows: [number of colonies counted/(number of cells seeded × plating efficiency/100)] (mean ± SD, n = 3). The survival fractions after the indicated IR doses were used to determine radiosensitivity. GraphPad Prism 5.0 Software (GraphPad Software Inc., La Jolla, CA, USA) was used to calculate and fit the dose survival curve using the linear-quadratic model.

Immunofluorescence (IF)

CNE1/IR cells transfected with shRNA-RBM3 or negative control were plated into confocal culture dishes at a density of 1×10^5 cells per dish. All cells were exposed to 6 Gy IR. One hour after exposure to IR, the cells were prepared for IF with phospho-H2AX (Ser139) antibody. IF staining was detected with a fluorescence microscope (Olympus, Shinjuku-ku, Tokyo, Japan). Five sections from each group were counted and averaged. Each experiment was repeated 3 times.

Apoptosis detection

CNE1/IR cells were transfected with shRNA-RBM3 or negative control and then exposed to 6 Gy IR. Twenty-four hours after transfection, the cells were harvested and fixed in 75% alcohol followed by staining for annexin V and propidium iodide (PI). After incubation for 30 minutes, cell apoptosis was analyzed using a BD FACS Calibur Flow Cytometer (Becton-Dickinson, USA).

Co-immunoprecipitation (Co-IP)

For immunoprecipitation, cells were lysed using RIPA buffer. Pierce protein A/G beads were incubated with antibodies (RBM3 and p85), and immunoprecipitation was performed using a Co-IP kit (Pierce, USA), according to the manufacturer's instructions. The purified target protein was detected by western blotting.

In vivo tumor radio response assay

Male nude mice aged 4 weeks were obtained from the Laboratory Animal Center of the Fourth Military Medical University (Xi'an, China) and were maintained under specific pathogen-free conditions. For the in vivo tumor radio response assay, mice (n = 5 each group) were subcutaneously injected with 1×10^6 cells/mouse into the right flank. When the tumor volumes reached approximately 500 mm^3, the tumors were exposed to 6 Gy IR. After 3 weeks, the mice were sacrificed by cervical dislocation, and the tumors were excised, cut into half, and embedded in polyoxymethylene for immunohistochemistry. Tumor volume (in mm^3) was measured using caliper measurements every 3 days and calculated using the following formula: (volume = length × $width^2/2$).

Statistical analysis

SPSS13.0 software was used for all statistical analyses. Quantitative data are presented as mean ± standard deviation of at least three independent replicates. In addition, the clonogenic survival assay was analyzed using one-way analysis of variance for factorial design

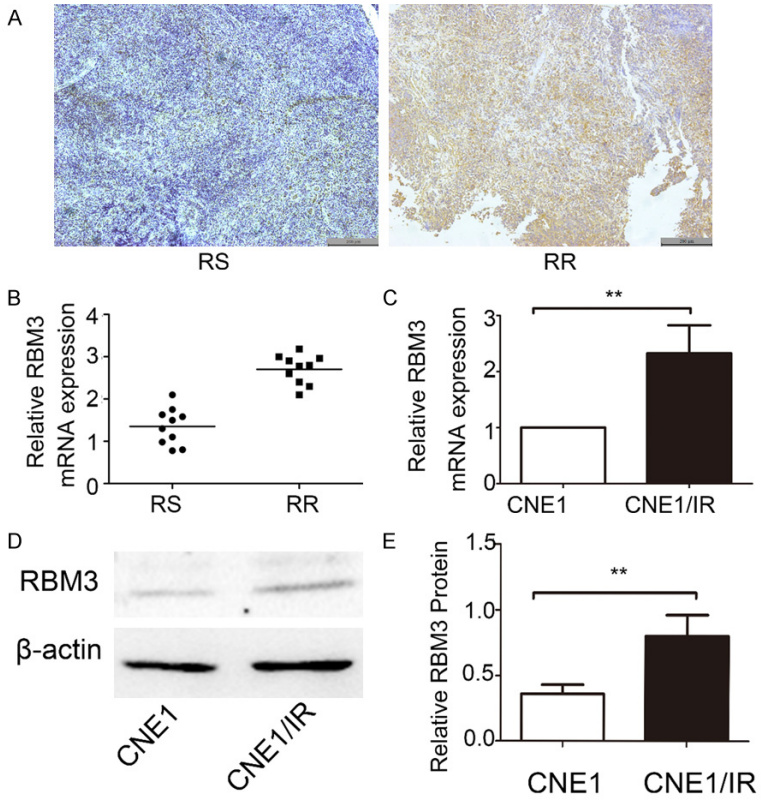

Figure 1. RBM3 expression is elevated in radioresistant NPC tissues and cell lines (A) RBM3 expression was examined by immunohistochemistry in clinical NPC tissue samples from radiation resistant (RR) and radiation sensitive (RS) individuals (magnifications: ×100). RBM3 expression was elevated in RR clinical specimens at the protein level. (B) The RBM3 mRNA level was higher in RR tissues than RS tissues when evaluated by RT-qPCR. (C) The RBM3 mRNA level in NPC RR cell lines (CNE1/IR) was higher than NPC parental cell lines (CNE1) when evaluated by RT-qPCR. (D) RBM3 was upregulated in NPC RR cell lines (CNE1/IR) at the protein level when evaluated by western blotting, and (E) densitometry was performed using Image J software **P<0.01.

Two groups were analyzed by two-tailed paired Student's t-test. Significance was defined as P<0.05.

Results

RBM3 is overexpressed in NPC radioresistant patient specimens and cell lines

To analyze whether RBM3 is involved in NPC radioresistance, we measured the expression of RBM3 in 10 pairs of clinical RS and RR patient specimens using immunohistochemistry and RT-qPCR. The results suggested that RBM3 is overexpressed in RR specimens in comparison to RS clinical tissues (**Figure 1A** and **1B**). Expression of RBM3 was also detected by RT-qPCR and western blot in CNE1/IR cells and CNE1 cells. As shown in **Figure 1C**, RBM3 mRNA levels were significantly increased in radioresistant CNE1/IR cells compared with the parent CNE1 cells (P<0.01). RBM3 protein level was similarly elevated in CNE1/IR cells compared to CNE1 cells (**Figure 1D** from the original Figure S1 and **Figure 1E**). Together, these results demonstrate that RBM3 is overexpressed in radioresistant NPC clinical specimens and cells.

RBM3 enhances radioresistance of NPC cells

Based on the high endogenous expression of RBM3 in CNE1/IR cells and low RBM3 expression in CNE1 cells, we performed respective RBM3 plasmid transfection and shRNA knockdown. To confirm successful incorporation of these constructs, RT-qPCR and western blot assays were employed to detect RBM3 mRNA and protein levels, respectively. As shown in **Figure 2A**, the transcription level of RBM3 mRNA was significantly elevated in pCDNA3.1-RBM3 CNE1 cells compared with negative control CNE1 cells. In addition, shRNA knockdown of RBM3 was confirmed in CNE1/IR cells relative to negative control CNE1/IR cells (**Figure 2B**). Western blot results further confirmed that RBM3 protein expression increased in pCDNA3.1-RBM3 CNE1 cells compared with negative control CNE1 cells (**Figure 2C** from the original Figure S1), and that RBM3 expression was substantially reduced in CNE1/IR cells infected with LV3-shRBM3 compared to negative control CNE1/IR cells (**Figure 2D** from the original Figure S1).

To evaluate the effect of RBM3 on the radiation response of NPC cells, the cell survival fraction of each group was measured by colony formation assays in cells exposed to increasing doses (0-10 Gy) of IR. As shown in **Figure 2E**, cell survival was clearly enhanced in pCDNA3.1-RBM3 CNE1 cells. In contrast, we found that the survival rate after IR treatment was substantially

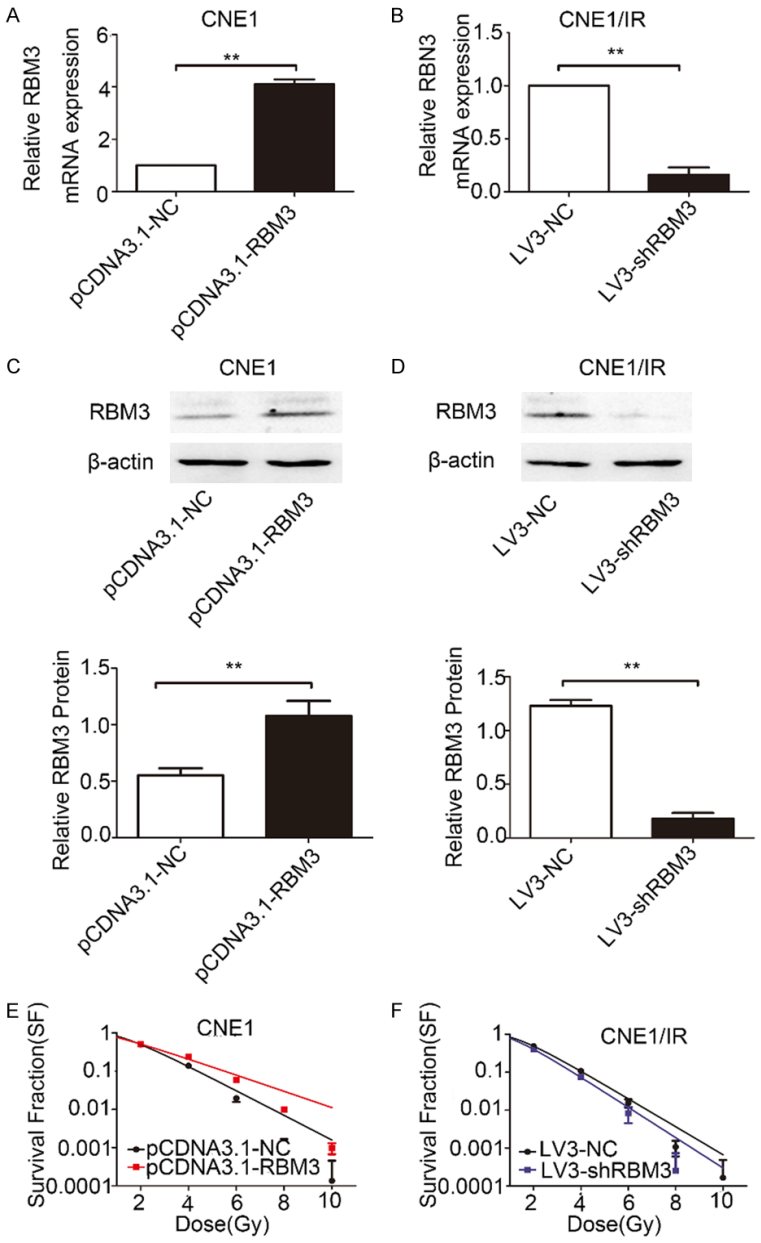

Figure 2. RBM3 enhances radioresistance in response to IR in NPC cells CNE1 cells were transfected with pCDNA3.1-RBM3, pCDNA3.1-NC and were infected with LV3-shRNA, LV3-NC. A. RT-qPCR showed that CNE1 cell lines transfected with pCDNA3.1-RBM3 had high RBM3 expression compared with negative control. B. LV3-shRNA significantly downregulated RBM3 mRNA in CNE1/IR cells when evaluated by RT-qPCR. C. Top panel: Western blot analysis was used to measure the RBM3 expression in CNE1 cells transfected with pCDNA3.1-RBM3 and pCDNA3.1-NC plasmid, Bottom panel: Quantitative results for Top panel. The data confirmed that the pCDNA3.1-RBM3 plasmid significantly upregulated RBM3 protein in CNE1 cells. D. Top panel: Western blot analysis was used to measure the RBM3 expression in CNE1/IR cells infected with LV3-shRBM3 and LV3-NC, Bottom panel: Quantitative results for Top panel. RBM3 protein was significantly reduced in CNE1/IR cells infected with LV3-shRBM3. E. CNE1 cells overexpressing RBM3 had significantly higher surviving fractions compared to CNE1 cells after irradiation at 2, 4, 6 8 and 10 Gy after 2 weeks. F. CNE1/IR cells were also treated with increasing doses of IR and evaluated for survival after 2 weeks. The survival fraction was substantially reduced in RBM3-shRNA cells compared to LV3-NC cells. *P<0.05.

reduced in CNE1/IR cells after RBM3 knockdown (**Figure 2F**). These results indicate that RBM3 enhances the radioresistance of NPC cells.

RBM3 decreased DNA damage in NPC cells

To analyze whether DNA damage was affected by RBM3 expression in NPC cells, IF was used to detect γH2AX foci, a marker of DNA damage. Quantification of γH2AX foci revealed that the number of foci per cell was reduced in pCDNA3.1-RBM3 CNE1 cells compared to negative control CNE1 cells (**Figure 3A** and **3B**). In contrast, the number of γH2AX foci increased in LV3-shRBM3 CNE1 cells compared to the negative control group, indicating that knockdown of RBM3 expression enhanced radiation-induced DNA damage and/or delayed DNA repair in CNE1/IR cells (**Figure 3C** and **3D**). These results suggest that RBM3 reduces DNA damage.

Radiation-induced apoptosis of NPC cells is inhibited by RBM3

The percentage of apoptotic NPC cells was detected by annexin V-FITC staining, followed by flow cytometry. CNE1 cells transfected with the negative control plasmid or pCDNA3.1-RBM3 were irradiated with 6 Gy. After 24 h, the percentage of apoptotic cells were 50.70% ± 0.69 and 33.19% ± 4.03 ($P<0.01$) for negative control and pCDNA3.1-RBM3 CNE1 cells, respectively (**Figure 3E**). In contrast, LV3-shRBM3 CNE1/IR cells had a significantly higher percentage of apoptotic cells compared to negative control cells (**Figure 3F**). Thus, these results show

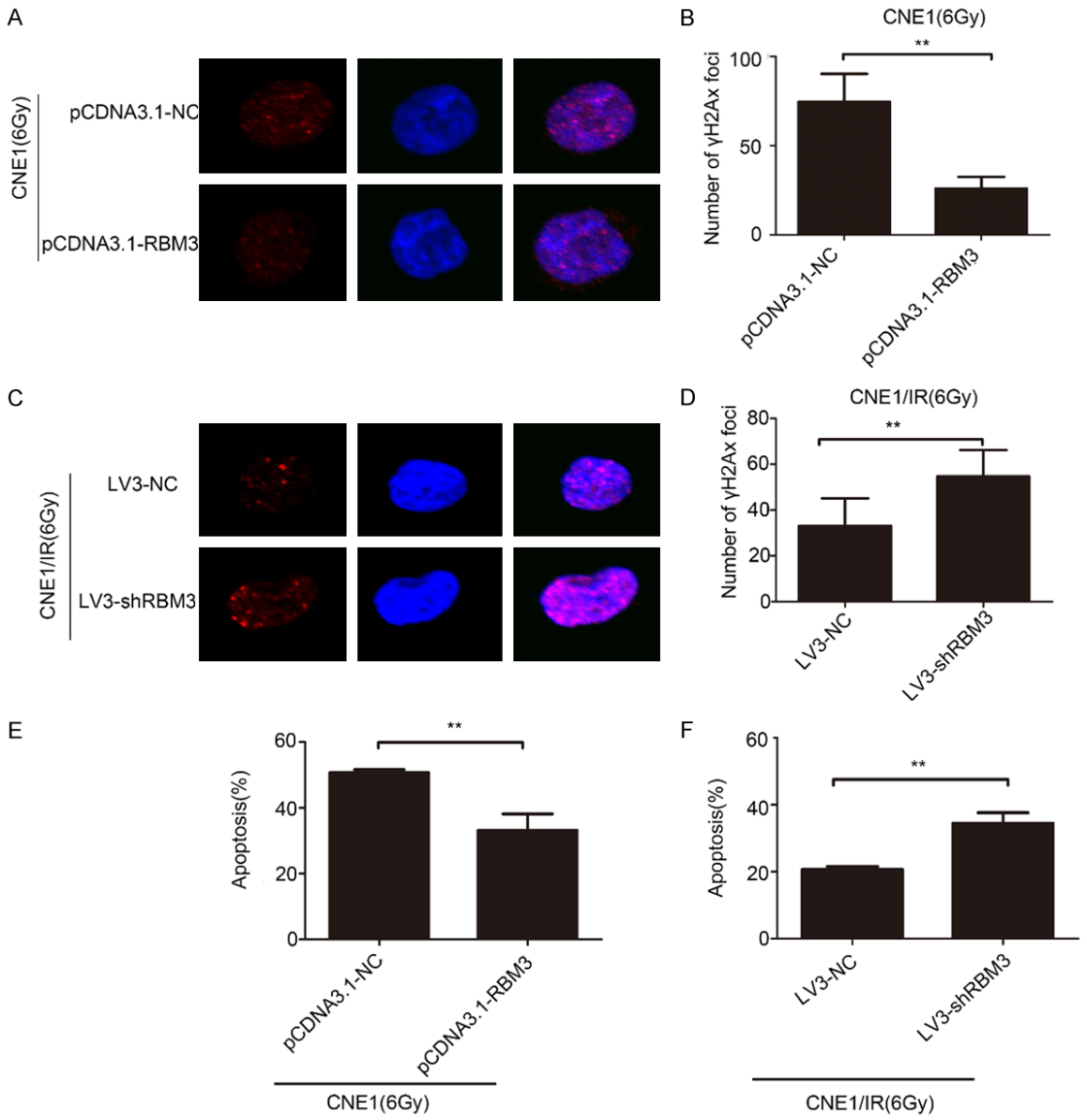

Figure 3. RBM3 reduces DNA damage and apoptosis in NPC. A. One hour after 6 Gy IR treatment, γH2AX foci were visualized by immunofluorescence in CNE1 cells. B. The number of γH2Ax foci was quantified and was significantly decreased in RBM3 overexpressing CNE1 cells. C, D. Similar evaluation of RBM3 knockdown cells showed an increased number of γH2AX foci in CNE1/IR cells. E. Annexin V/PI staining followed by flow cytometry revealed that RBM3 overexpression in CNE1 cells reduced the proportion of apoptotic cells in response to IR. F. In CNE1/IR cells, RBM3 depletion increased the proportion of apoptotic cells. **P<0.01.

that RBM3 inhibits radiation-induced apoptosis in NPC cells.

RBM3 induces caspase 3 and activates the AKT/Bcl-2 signaling pathway in CNE1/IR cells

To explore the molecular mechanism of radioresistance in NPC, we measured the changes in apoptosis related protein signaling in response to IR and modulation of RBM3 expression. RBM3 overexpression in CNE1 cells reduced the level of caspase 3, whereas RBM3 depletion induced a significant increase in caspase 3 in CNE1/IR cells exposed to 6 Gy IR. The apoptosis suppressor protein, Bcl-2, and its upstream AKT signaling pathway is pivotal for NPC radioresistance. Western blotting revealed that proteins in this signaling pathway were upregulated in CNE1 cells transfected with pCDNA3.1-RBM3. In contrast, RBM3 knock-

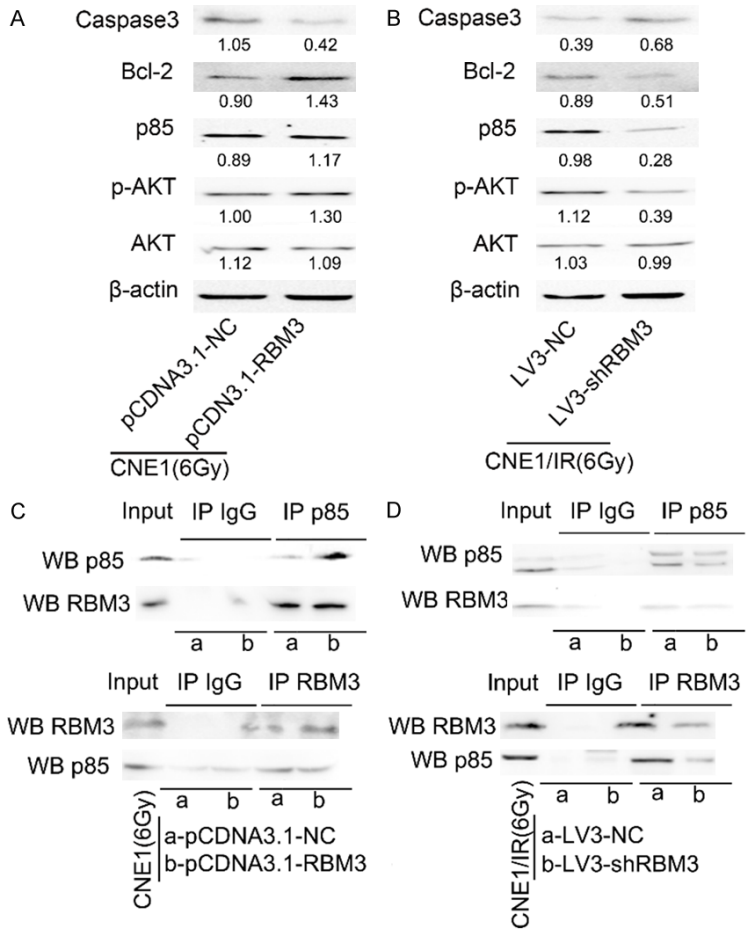

Figure 4. RBM3 reduces apoptosis and activates the PI3K/AKT/Bcl-2 signaling pathway in NPC cells. A. In CNE1/IR cells overexpressing RBM3, downregulation of caspase 3 and upregulation of Bcl-2, p-AKT, and p-PI3K p85 were observed by western blot. B. RBM3 depletion induced significant upregulation of caspase 3 and downregulation of Bcl-2, p-AKT, and p-PI3K p85 by western blot in CNE1/IR cells transfected with RBM3-shRNA. C, D. Co-immunoprecipitation revealed that RBM3 interacts with the p85 subunit of PI3K in NPC cell lines.

down in CNE1/IR cells suppressed Bcl-2 and AKT phosphorylation. Moreover, RBM3 knockdown downregulated p-PI3K (p85), whereas overexpression of RBM3 resulted in the opposite effect (**Figure 4A** and **4B** from the original Figure S2). Subsequently, we investigated the mechanism of RBM3-induced AKT activation by co-immunoprecipitation. RBM3 was associated with the p85 subunit of PI3K in both CNE1 and CNE1/IR cell lines. Upregulation of RBM3 strengthened the interaction, whereas knockdown of RBM3 attenuated it (**Figure 4C** and **4D**). Our results suggest that RBM3 reduced apoptotic response to IR through PI3K/AKT/Bcl-2 signaling.

Inhibition of the AKT pathway enhances IR-induced apoptosis regardless of RBM3

To confirm the involvement of the AKT signaling pathway in RBM3-induced radioresistance, we treated CNE1 and CNE1/IR cells with 6 Gy IR and the AKT inhibitor, LY294002 (5 μmol/L). Flow cytometry analysis with annexin V revealed that LY294002 treatment dramatically enhanced cell apoptosis. In CNE1 and CNE1/IR cells treated with LY294002, the effect of RBM3 was not significant compared with that of the untreated group (**Figure 5A** and **5B**). LY294002 was confirmed to effectively inhibit AKT phosphorylation in both cell lines. Furthermore, we investigated the impact of LY294002 on caspase 3 and Bcl-2 levels in CNE1 and CNE1/IR cells. Western blotting revealed that AKT inhibition increased caspase 3 and decreased Bcl-2. AKT inhibition also eliminated the effect of RBM3 and RBM3-shRNA on Bcl-2 and caspase 3 in CNE1 and CNE1/IR cells (**Figure 5C** and **5D** from the original Figure S3). Our results indicate that RBM3 causes resistance to IR-induced apoptosis by activating the AKT/Bcl-2 signaling cascade.

RBM3 increases the radioresistance of NPC in vivo

To further verify the effect of RBM3 on radioresistance of NPC, we subcutaneously injected mice (n = 5 per group) with approximately 1.0×10^6 cells (CNE1 cells transfected with pCDNA3.1-NC and pCDNA3.1-RBM3 CNE1; CNE1/IR cells infected with LV3-NC and LV3-shRBM3). When tumors grew to an approximate size of 500 mm^3, mice were exposed to 6 Gy IR. After 3 weeks, the tumors were excised and photographed. As shown in **Figure 6A**, tumor volume was dramatically enlarged in the RBM3 upregulated group when compared to the negative control group. Conversely, knock-

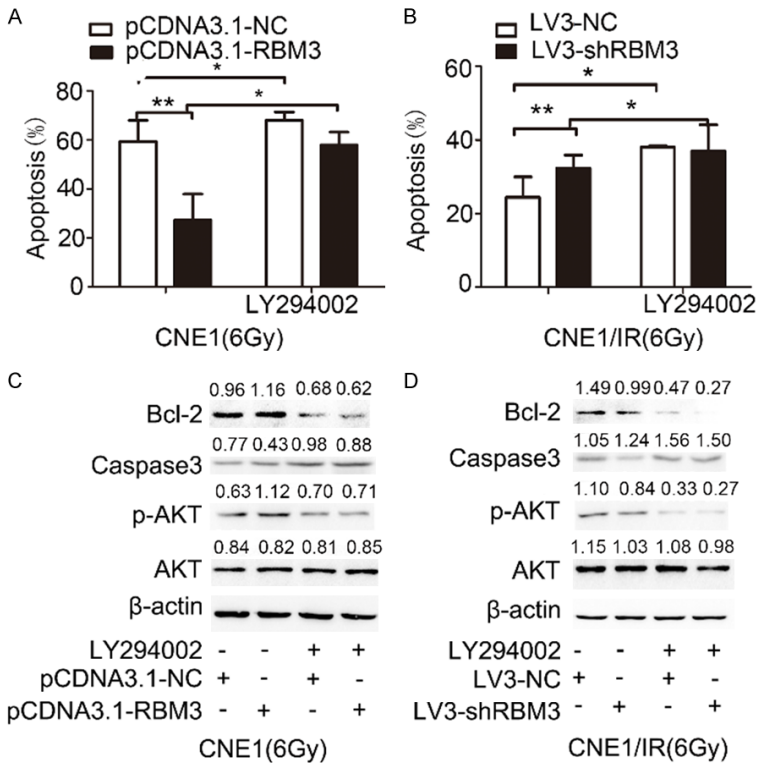

Figure 5. Inhibition of the AKT pathway eliminates the role of RBM3 in IR-induced apoptosis. A, B. Annexin V/PI staining and flow cytometry showed that LY294002 treatment upregulated the percentage of apoptosis. In LY294002 treated CNE1 cells transfected with RBM3 overexpression, the effect of RBM3 was not significant compared with the untreated group. Similar results were observed in the CNE1/IR cell line with RBM3 downregulation. C, D. AKT inhibition with LY294002 induced caspase 3 and Bcl-2 downregulation. LY294002 treatment also abolished the effect of RBM3 and its shRNA on Bcl-2 and caspase 3 in CNE1/IR cells. Contrasting results were observed in CNE1 cells with RBM3 overexpression. *P<0.05.

down of RBM3 significantly decreased the tumor volume (**Figure 6C**). Tumor size was measured every 2 days, and the results showed that the tumor growth rate of the RBM3 overexpression group was significantly higher than that of the control group (**Figure 6B**), whereas RBM3 knockdown in CNE1/IR cells exhibited contrasting results (**Figure 6D**). Subsequently, immunohistochemical staining for Bcl-2 was found to be substantially lower with RBM3 overexpression compared to the control group (**Figure 6E**), whereas knockdown of RBM3 had the opposite effect (**Figure 6G**). The apoptosis percentage in solid tumors was detected by flow cytometry. We found that overexpression of RBM3 in the CNE1 group showed lower apoptosis than negative control (**Figure 6F**) and knockdown of RBM3 revealed elevated apoptosis (**Figure 6H**). Together these results indicate that RBM3 increases radioresistance in NPC.

Discussion

The involvement of RBM3 in tumorigenesis and in the prediction of clinical outcomes has been widely studied with varying conclusions in many cancers, including breast, urothelial bladder, prostate, colorectal, and gastric cancer [4-6, 9, 10]. However, the role of RBM3 in the radiation response of NPC has not been reported. Accordingly, we evaluated RBM3 expression in 10 pairs of clinical NPC tissue samples as well as in NPC cell lines. Our data demonstrate that RBM3 is highly expressed in radioresistant clinical NPC. Consistent with our clinical results, we also found that RBM3 is upregulated in radioresistant NPC cells (CNE1/IR), further implicating RBM3 in the radiation response of NPC.

As radioresistance is a driving factor of tumor cell survival and recurrence, the survival fraction of cells is an important method used to determine the radiosensitivity of tumors [11]. Therefore, to investigate the role of RBM3 in radioresistance of NPC, we detected survival fraction following IR treatment using a clonogenic assay. We found that inhibition of RBM3 enhanced radiosensitivity, suggesting that RBM3 is indeed involved in NPC radioresistance. Consistent with cell experiments, *in vivo* xenografts demonstrated that RBM3 downregulation inhibits NPC radioresistance and tumor growth.

Exposure to IR results in single strand breaks, double strand breaks (DSBs), base damage, and DNA-protein crosslinking in genomic DNA. DSBs are particularly critical as they result in genomic instability and unrepaired or falsely repaired cells [12, 13]. Protein that sense DNA damage are recruited to the site of DSBs within minutes or hours of exposure to IR. This rapid recruitment results in radiation-induced foci, such as γH2AX, and provides an indirect mea-

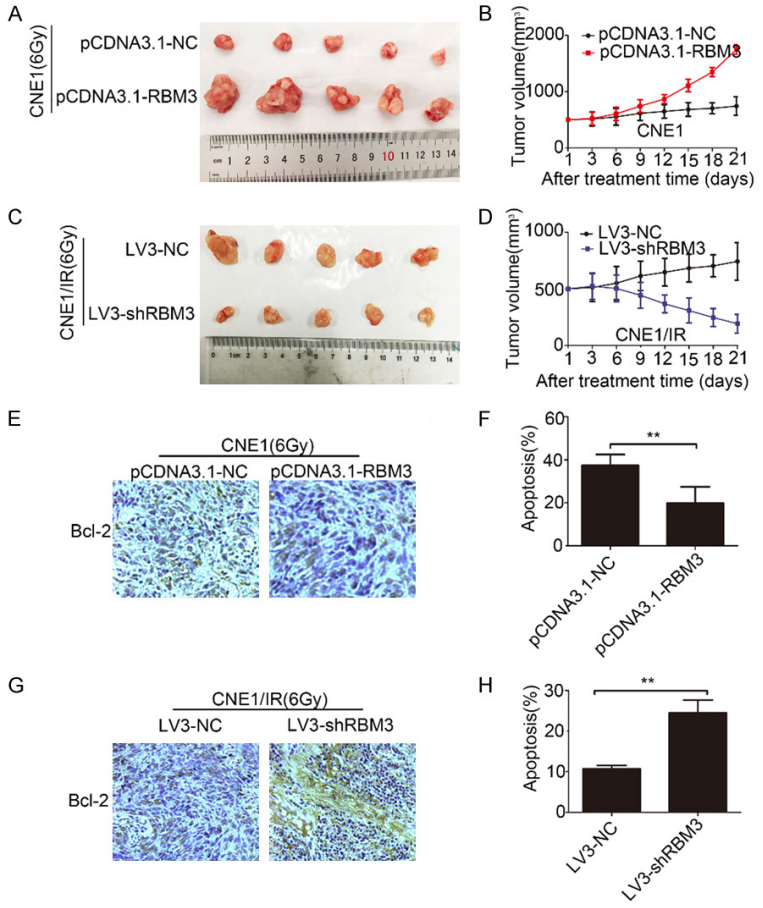

Figure 6. RBM3 enhances radioresistance of xenograft NPC tumors via Bcl-2. Mice (n = 5 each group) were subcutaneously injected with 1×10^6 cells into the right flank and monitored for tumor growth. Tumors were exposed to 6 Gy ionizing radiation and, after 3 weeks, tumors were excised, halved, and embedded for immunohistochemistry. A, C. Representative xenograft tumors depicting the increase in tumor size with RBM3 overexpression in CNE1 tumors. In contrast, RBM3 downregulation inhibited tumor size in CNE1/IR tumors. B, D. Growth curves of tumor volumes were calculated and suggest that the tumor growth rate was higher in RBM3 overexpressing tumors compared to negative control, whereas RBM3 shRNA tumors exhibited contrasting growth curves. E, G. Bcl-2 expression in tumors was analyzed by immune-histochemistry (magnifications: ×100). Bcl-2 was low in CNE1 cells transfected with pCDNA3.1-RBM3. In contrast, high expression of Bcl-2 was detected in RBM3-shRNA CNE1/IR cells. F, H. Flow cytometry was used to detect apoptosis of xenograft NPC tumor cells. Results showed that overexpression of RBM3 in CNE1 cells reduced apoptosis compared to the negative control. Conversely, CNE1/IR cells infected with LV3-shRBM3 demonstrated opposite effects. **P<0.01.

sure of the DNA damage response [14]. Thus, we evaluated radiation-induced DSBs by visualization of discrete γH2AX foci in response to IR treatment of NPC cell lines with modulated RBM3 expression. We found that overexpression of RBM3 resulted in reduced recruitment of γH2AX. In contrast, knockdown of RBM3 expression resulted in enhanced recruitment of γH2AX. As expected, our study suggests that DNA damage after IR is decreased when RBM3 expression is upregulated.

Apoptosis is widely recognized as the desired response to radiation of various tumor cells [15-19], including NPC. The loss of apoptotic response to IR has been associated with the radioresistance phenotype. Therefore, it is believed that inhibition of apoptotic protein antagonists may enhance radiosensitivity of human cancers by inducing apoptosis [20, 21]. Moreover, overexpression of genes that inhibit apoptosis or downregulation of apoptotic genes may lead to radioresistance in tumor cells [22]. Our experimental findings suggest that RBM3 reduces the apoptotic response to IR in NPC cells, resulting in radioresistant cells.

It has been previously suggested that multiple proteins participate in the development of radioresistance, including Bcl-2, caspase 3, and Bax [23, 24]. Further, downregulation of Bcl-2 is involved in sensitizing NPC to IR [25-28]. In this study, we measured the expression of Bcl-2 and caspase 3 by western blotting and demonstrated that the expression of Bcl-2 and caspase 3 could be inhibited by downregulation of RBM3 expression. Conversely, RBM3 overexpression enhanced the expression of Bcl-2 and caspase 3. This positive correlation between RBM3 and Bcl-2 has also been reported in breast cancer [6, 29]. Together these observations support a model where RBM3 reduces apoptotic response to IR through upregulation of Bcl-2 in NPC cells.

We therefore further explored the molecular mechanism of Bcl-2 regulation in NPC cells. Many studies have reported that AKT activation

participates in the induction of IR resistance through Bcl-2 [30-33]. Our study suggested that shRNA-mediated knockdown of RBM3 decreases the level of AKT phosphorylation. We also detected a direct interaction between RBM3 and PI3K, as demonstrated by immunoprecipitation with RBM3 antibody. RBM3 and PI3K subunit p85 were detected in the immunoprecipitate, confirming an interaction between RBM3 and PI3K following NPC radiation. Moreover, the AKT inhibitor, LY294002, attenuated the effect of RBM3 on Bcl-2-mediated IR resistance, demonstrating that the PI3K/AKT/Bcl-2 signaling pathway is involved in RBM3-induced radioresistance. Altogether, our study suggests that RBM3 is involved in NPC radioresistance through the PI3K/AKT/Bcl-2 signaling pathway.

In conclusion, we have demonstrated a role for RBM3 in the development of radioresistance in NPC. RBM3 inhibits DNA damage and reduces apoptosis in response to IR. RBM3 interacts with p85 and activates the AKT/Bcl-2 pathway, which accounts for the reduced apoptotic response to IR in NPC cells. Thus, RBM3 is a novel candidate to predict the development of radioresistance and is a potential therapeutic target for NPC.

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Disclosure of conflict of interest

None.

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References

[1] Du C, Ying H, Zhou J, Hu C and Zhang Y. Experience with combination of docetaxel, cisplatin plus 5-fluorouracil chemotherapy, and intensity-modulated radiotherapy for locoregionally advanced nasopharyngeal carcinoma. Int J Clin Oncol 2013; 18: 464-471.

[2] Luftig M. Heavy LIFting: tumor promotion and radioresistance in NPC. J Clin Invest 2013; 123: 4999-5001.

[3] Wright CF, Oswald BW and Dellis S. Vaccinia virus late transcription is activated in vitro by cellular heterogeneous nuclear ribonucleoproteins. J Biol Chem 2001; 276: 40680-40686.

[4] Jonsson L, Gaber A, Ulmert D, Uhlen M, Bjartell A and Jirstrom K. High RBM3 expression in independently predicts a reduced risk of biochemical recurrence and disease progression. Diagn Pathol 2011; 6: 91.

[5] Yang G, Fu H, Zhang J, Lu X, Yu F, Jin L, Bai L, Huang B, Shen L, Feng Y, Yao L and Lu Z. RNA-binding protein quaking, a critical regulator of colon epithelial differentiation and a suppressor of colon cancer. Gastroenterology 2010; 138: 231-240, e231-235.

[6] Schneider J, Gomez-Esquer F, Diaz-Gil G, Torrejon R and Pollan M. mRNA expression of the putative antimetastatic gene BRMS1 and of apoptosis-related genes in breast cancer. Cancer Genomics Proteomics 2011; 8: 195-197.

[7] Kita H, Carmichael J, Swartz J, Muro S, Wyttenbach A, Matsubara K, Rubinsztein DC and Kato K. Modulation of polyglutamine-induced cell death by genes identified by expression profiling. Hum Mol Genet 2002; 11: 2279-2287.

[8] Venugopal A, Subramaniam D, Balmaceda J, Roy B, Dixon DA, Umar S, Weir SJ and Anant S. RNA binding protein RBM3 increases beta-catenin signaling to increase stem cell characteristics in colorectal cancer cells. Mol Carcinog 2016; 55: 1503-1516.

[9] Boman K, Segersten U, Ahlgren G, Eberhard J, Uhlen M, Jirstrom K and Malmstrom PU. Decreased expression of RNA-binding motif protein 3 correlates with tumour progression and poor prognosis in urothelial bladder cancer. BMC Urol 2013; 13: 17.

[10] Ye F, Jin P, Cai X, Cai P and Cai H. High RNA-binding motif protein 3 (RBM3) expression is independently associated with prolonged overall survival in intestinal-type gastric cancer. Med Sci Monit 2017; 23: 6033-6041.

[11] Bjork-Eriksson T, West C, Karlsson E and Mercke C. Tumor radiosensitivity (SF2) is a prognostic factor for local control in head and neck cancers. Int J Radiat Oncol Biol Phys 2000; 46: 13-19.

[12] Vallard A, Rancoule C, Guy JB, Espenel S, Sauvaigo S, Rodriguez-Lafrasse C and Magne N. [Biomarkers of radiation-induced DNA repair processes]. Bull Cancer 2017; 104: 981-987.

[13] Mavragani IV, Nikitaki Z, Souli MP, Aziz A, Nowsheen S, Aziz K, Rogakou E and Georga-

kilas AG. Complex DNA damage: a route to radiation-induced genomic instability and carcinogenesis. Cancers (Basel) 2017; 9.
[14] Rogakou EP, Pilch DR, Orr AH, Ivanova VS and Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem 1998; 273: 5858-5868.
[15] Pan X, Mou J, Liu S, Sun Z, Meng R, Zhou Z, Wu G and Peng G. SHP-1 overexpression increases the radioresistance of NPC cells by enhancing DSB repair, increasing S phase arrest and decreasing cell apoptosis. Oncol Rep 2015; 33: 2999-3005.
[16] Zhang J, Zhou L, Nan Z, Yuan Q, Wen J, Xu M, Li Y, Li B, Wang P, Liu C, Ma Y, Chen S and Xie S. Knockdown of cMyc activates Fas-mediated apoptosis and sensitizes A549 cells to radiation. Oncol Rep 2017; 38: 2471-2479.
[17] Silva MF, Khokhar AR, Qureshi MZ and Farooqi AA. Ionizing radiations induce apoptosis in TRAIL resistant cancer cells: in vivo and in vitro analysis. Asian Pac J Cancer Prev 2014; 15: 1905-1907.
[18] Alan Mitteer R, Wang Y, Shah J, Gordon S, Fager M, Butter PP, Jun Kim H, Guardiola-Salmeron C, Carabe-Fernandez A and Fan Y. Proton beam radiation induces DNA damage and cell apoptosis in glioma stem cells through reactive oxygen species. Sci Rep 2015; 5: 13961.
[19] Kumar C, Jayakumar S, Pandey BN, Samuel G and Venkatesh M. Cellular and molecular effects of beta radiation from I-131 on human tumor cells: a comparison with gamma radiation. Curr Radiopharm 2014; 7: 138-143.
[20] Fulda S. Targeting IAP proteins in combination with radiotherapy. Radiat Oncol 2015; 10: 105.
[21] Fulda S. Inhibitor of Apoptosis (IAP) proteins as therapeutic targets for radiosensitization of human cancers. Cancer Treat Rev 2012; 38: 760-766.
[22] Kyprianou N, King ED, Bradbury D, Rhee JG. Bcl-2 over-expression delays radiation-induced apoptosis without affecting the clonogenic survival of human prostate cancer cells. Int J Cancer 1997; 341-348.
[23] Mackey TJ, Borkowski A, Amin P, Jacobs SC and Kyprianou N. bcl-2/bax ratio as a predictive marker for therapeutic response to radiotherapy in patients with prostate cancer. Urology 1998; 52: 1085-1090.
[24] Zhuang RJ, Ma J, Shi X, Ju F, Ma SP, Wang L, Cheng BF, Ma YW, Wang M, Li T, Feng ZW and Yang HJ. Cold-inducible protein RBM3 protects UV irradiation-induced apoptosis in neuroblastoma cells by affecting p38 and JNK pathways and Bcl2 family proteins. J Mol Neurosci 2017; 63: 142-151.
[25] He JH, Liao XL, Wang W, Li DD, Chen WD, Deng R, Yang D, Han ZP, Jiang JW and Zhu XF. Apogossypolone, a small-molecule inhibitor of Bcl-2, induces radiosensitization of nasopharyngeal carcinoma cells by stimulating autophagy. Int J Oncol 2014; 45: 1099-1108.
[26] Lu ZX, Ma XQ, Yang LF, Wang ZL, Zeng L, Li ZJ, Li XN, Tang M, Yi W, Gong JP, Sun LQ and Cao Y. DNAzymes targeted to EBV-encoded latent membrane protein-1 induce apoptosis and enhance radiosensitivity in nasopharyngeal carcinoma. Cancer Lett 2008; 265: 226-238.
[27] Ou J, Pan F, Geng P, Wei X, Xie G, Deng J, Pang X and Liang H. Silencing fibronectin extra domain A enhances radiosensitivity in nasopharyngeal carcinomas involving an FAK/Akt/JNK pathway. Int J Radiat Oncol Biol Phys 2012; 82: e685-691.
[28] Sun X, Xu R, Deng Y, Cheng H, Ma J, Ji J and Zhou Y. Effects of tetrandrine on apoptosis and radiosensitivity of nasopharyngeal carcinoma cell line CNE. Acta Biochim Biophys Sin (Shanghai) 2007; 39: 869-878.
[29] Martinez-Arribas F, Agudo D, Pollan M, Gomez-Esquer F, Diaz-Gil G, Lucas R and Schneider J. Positive correlation between the expression of X-chromosome RBM genes (RBMX, RBM3, RBM10) and the proapoptotic Bax gene in human breast cancer. J Cell Biochem 2006; 97: 1275-1282.
[30] Shimura T, Noma N, Sano Y, Ochiai Y, Oikawa T, Fukumoto M and Kunugita N. AKT-mediated enhanced aerobic glycolysis causes acquired radioresistance by human tumor cells. Radiother Oncol 2014; 112: 302-307.
[31] Ramalingam M and Kim SJ. Insulin involved Akt/ERK and Bcl-2/Bax pathways against oxidative damages in C6 glial cells. J Recept Signal Transduct Res 2016; 36: 14-20.
[32] Yuan L, Yi HM, Yi H, Qu JQ, Zhu JF, Li LN, Xiao T, Zheng Z, Lu SS and Xiao ZQ. Reduced RKIP enhances nasopharyngeal carcinoma radioresistance by increasing ERK and AKT activity. Oncotarget 2016; 7: 11463-11477.
[33] Dong Q, Fu L, Zhao Y, Liu Y, Li Q, Qiu X and Wang E. Derlin-1 is a target to improve radiotherapy effect of esophageal squamous cell carcinoma. Oncotarget 2017; 8: 55135-55146.

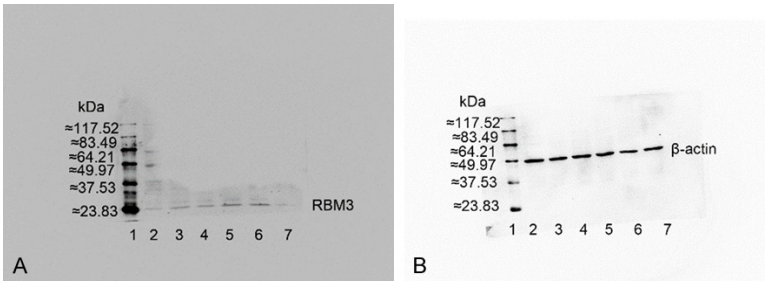

Figure S1. Original western images for RBM3 relevant western blots. A. The expression of RBM3 in CNE1, CNE1/IR transfected with related plasmid; B. The expression of β-actin in CNE1, CNE1/IR transfected with related plasmid. 1-Marker (yisemed, shanghai, china); 2-CNE1; 3-CNE1/IR; 4-CNE1 transfected with pCDNA3.1-NC; 5-CNE1 transfected with pCDNA3.1-RBM3; 6-CNE1/IR infected with LV3-NC; 7-CNE1/IR infected with LV3-shRBM3. 2 and 3 refer to **Figure 1D**. 4 and 5, 6 and 7 refer to **Figure 2C**, **2D**, respectively.

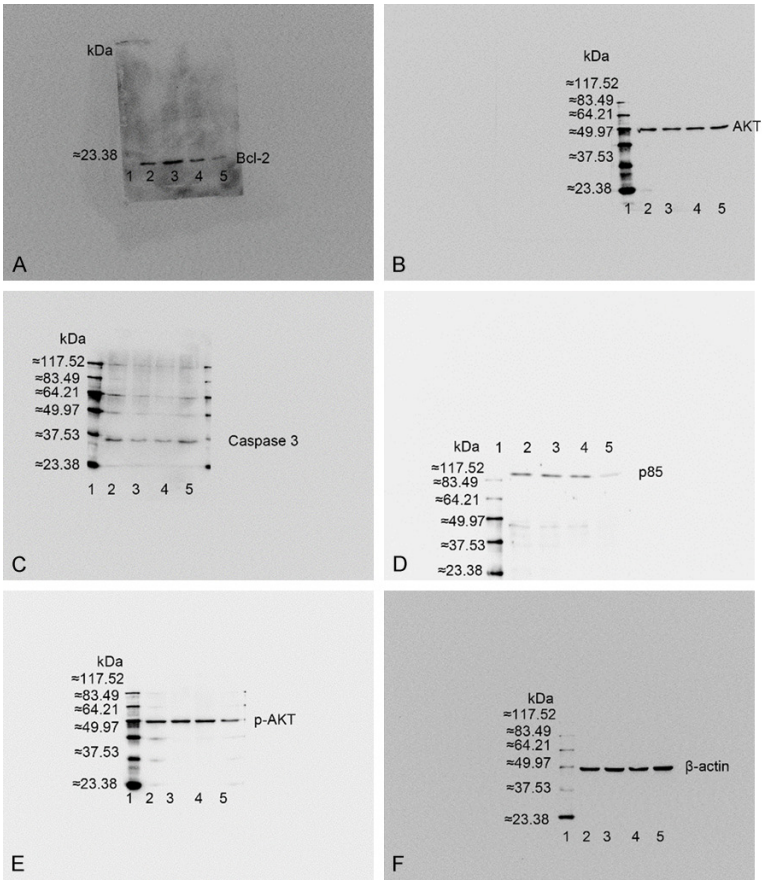

Figure S2. Original western images for **Figure 4A**, **4B** western blots. A. The expression of Bcl-2 protein; B. The expression of AKT protein; C. The expression of Caspase3 protein; D. The expression of p85 protein; E. The expression of p-AKT protein; F. The expression of β-actin protein. 1-Marker (yisemed, shanghai, china); 2-CNE1 transfected with pCDNA3.1-NC; 3-CNE1 transfected with pCDNA3.1-RBM3; 4-CNE1/IR infected with LV3-NC; 5-CNE1/IR infected with LV3-shRBM3.

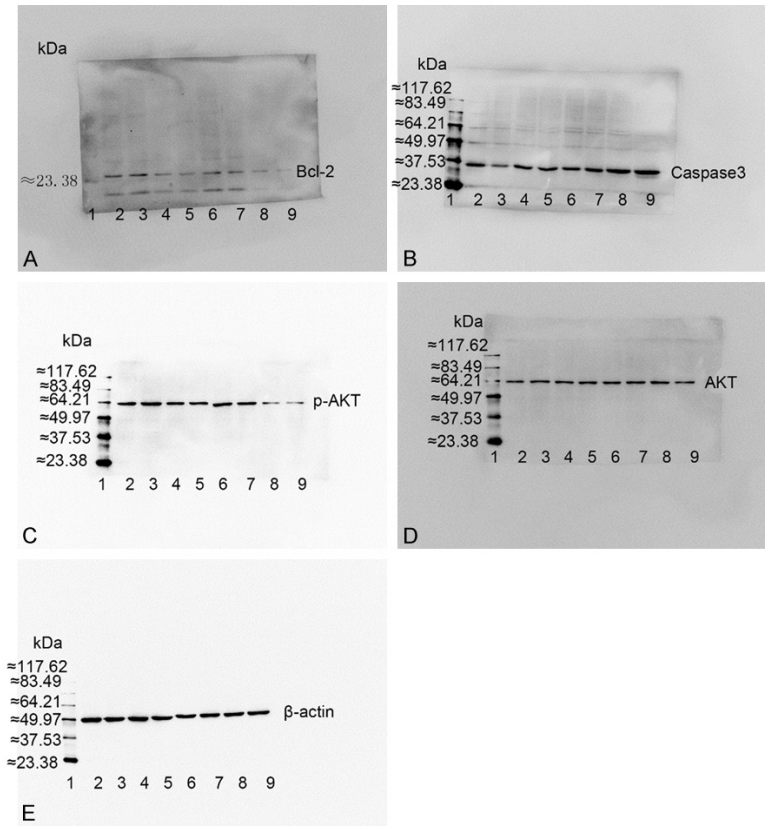

Figure S3. Original western images for **Figure 5C**, **5D** western blots. A. The expression of Bcl-2 protein; B. The expression of Caspase3 protein; C. The expression of p-AKT protein; D. The expression of AKT protein; E. The expression of β-actin protein. 1-Marker (yisemed, shanghai, china); 2-CNE1 transfected with pCDNA3.1-NC; 3-CNE1 transfected with pCDNA3.1-RBM3; 4-CNE1 transfected with pCDNA3.1-NC and treated with LY294002; 5-CNE1 transfected with pCDNA3.1-RBM3 and treated with LY294002; 6-CNE1/IR infected with LV3-NC; 5-CNE1/IR infected with LV3-shRBM3; 7-CNE1/IR infected with LV3-NC and treated with LY294002 8-CNE1/IR infected with LV3-shRBM3 and treated with LY294002.