Silencing VEGF enhances the radiosensitivity of the radioresistant human nasopharyngeal carcinoma cell line CNE-2R by inhibiting autophagy through the activation of mTOR pathway

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Abstract

Background: Vascular endothelial growth factor (VEGF) is an important pro-angiogenic factor. VEGF was reported to promote the occurrence of autophagy, which enhanced to the radioresistance of tumors. The purpose of our study was to investigate the influence of VEGF silencing on the radiosensitivity of nasopharyngeal carcinoma radioresistant cell line CNE-2R and the underlying mechanisms.

Methods: The radiosensitivity of CNE-2R cells after silencing VEGF was detected by cell counting kit 8 (CCK-8) and clonogenic assay, cell cycle and apoptosis was subjected to flow cytometry. DNA damage and autophagy were observed by immunofluorescence and western blotting. The interaction between VEGF and mTOR was confirmed by western blotting and co-immunoprecipitation analysis. In vivo, the effect of VEGF on radiosensitivity of NPC cells was investigated through xenograft model, furthermore, immunohistochemistry and TUNEL assay were used to further verify the relationship between autophagy and radiosensitivity in NPC after VEGF depletion.

Results: Downregulation of VEGF significantly inhibited cell proliferation and induced apoptosis of CNE-2R cells after radiotherapy in vitro and in vivo. In addition, VEGF knockdown not only decreased autophagy level, but also delayed the DNA damage repair in CNE-2R cells after irradiation. Mechanistically, silencing VEGF suppressed autophagy through the activation of mTOR pathway.

Conclusion: VEGF depletion increased radiosensitivity of NPC radioresistant cell CNE-2R by suppressing autophagy via the activation of mTOR pathway.

Introduction

Nasopharyngeal carcinoma (NPC) is a common tumor dominant in Southeast Asia especially in southern China, with an annual incidence of about 20 cases per 100,000 people [1]. NPC is characterized by poor tumor differentiation, complex anatomical location, rapid invasion of adjacent organs. However, about 20% of patients with NPC develop local recurrence and distant metastasis after standard therapy due to radioresistance [2]. Therefore, exploring the factors that cause radioresistance of NPC and the underlying molecular mechanisms is important for improving the treatment efficacy and prognosis of NPC patients.

Autophagy is a conserved and essential process in organisms [3], in which unwanted cellular contents are cleared by lysosomal degradation to maintain cell survival during stress responses [4]. Its function of scavenging intracellular waste is also one of the important mechanisms by which it inhibits tumor growth [5]. Autophagy is thought to contribute to tumor radioresistance [6,7]. Our previous studies demonstrated inhibition of autophagy enhances radiosensitivity of NPC cells [8], which was also corroborated by Chu's report [9].

Vascular endothelial growth factor (VEGF) is an important pro-angiogenic factor that promotes tumor angiogenesis. VEGF causes tumor cell reoxygenation, which leads to

excessive DNA replication, increased synthesis of radioresistance genes, and radioresistance [10]. Hu [11] showed that radioresistance in esophageal cancer was associated with radiation-induced abnormal secretion of VEGF, which protected tumor vessels from radiation-related damage and subsequently increased the radioresistance of tumors. In addition, VEGF inhibited autophagy process, as evidenced by the finding that the autophagic flux and cell activity decreased significantly in VEGF-silenced tumor cells [12].

Mammalian target of rapamycin (mTOR) is a receptor for amino acids, ATP and hormones [13]. Increasing evidence have demonstrated that mTOR inhibition activate the occurrence of autophagy [14]. Furthermore, studies have indicated VEGF is involved in the regulation of the mTOR pathway[15-16]. However, whether VEGF regulates autophagy and affects radiosensitivity of NPC through mTOR signaling pathway has not yet been clarified.

We have constructed the NPC radioresistant cell line CNE-2R in vitro by fractional induction of radiation on CNE-2 cells [17], and we found that inhibition of autophagy enhanced radiosensitivity of CNE-2R cells [18]. In our study, we assessed the biological function of VEGF on radiosensitivity of CNE-2R cells. In vitro and in vivo assays indicated that VEGF knockdown inhibited cell proliferation, facilitated cell apoptosis and enhanced the radiosensitivity of NPC cells. We also found that Downregulation of VEGF repressed autophagy through the activation of mTOR pathway. Our study reveal that the molecular regulatory mechanism of VEGF, which could also be a prognostic biomarker in NPC.

Materials And Methods

Cell lines and transfection

In our previous studies, we had established radiation-tolerant CNE-2R cells. Cells were maintained in RPMI-1640 (Gibco, Grand Island, NY, USA) medium containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) at 37°C in 5% CO₂ incubator. The GV248-Puromycin-EGFP-shRNA-VEGF lentiviral vectors and GV358-3FLAG- Puromycin-EGFP-VEGF lentiviral vectors (Genechem, Shanghai, China) were transfected into CNE-2R cells. The transfection effect was verified by qPCR and western blotting. The molecular sequence of VEGF was obtained in GenBank (No.NM_001171626).

RT-qPCR analysis

Total RNA was isolated from cells with TRIzol reagent (Takara, Dalian, Japan), and then reverse-transcribed into cDNA. The SYBR[®] Premix Ex Taq[™] II (Takara, Dalian, Japan), dH₂O, cDNA, and primers were mixed to prepare a qPCR reaction system, and a RT-qPCR reaction was performed. The relative quantitative analysis of mRNA was conducted by the 2^{-ΔΔct} method. The primers were as follows: (5'→3'): VEGF-F: TCACAGGTACAGGGATGAGGACAC, VEGF-R: CAAAGCACAGCAATGTCCTGAAG; GAPDH-F: CAGGAGGCATTGCTGATGAT, GAPDH-R: GAAGGCTGGGGCTCATTT.

Western blot analysis

Total protein was isolated from cells by RIPA (Beyotime, Shanghai, China) protein lysate. Quantitative detection of protein was conducted by the BCA (Beyotime, Shanghai, China) method. Thereafter, 20 µg of protein sample was added to appropriate amount of 4× loading buffer and denatured by heating. After electrophoresis by SDS-PAGE (6%-12%), the proteins were then transferred to PVDF membranes and blocked with 5% skimmed milk. The corresponding primary and secondary antibodies were incubated with the membranes. The bands were exposed with ECL chemiluminescence and analyzed with gel imaging system.

Clonogenic Assay

Cells at densities of 200, 200, 400, 600, 1000 cells/well were added to a six-well plate according to the 0, 2, 4, 6 and 8 Gy irradiation doses. After 24 hours of culture, cells were subjected to different irradiation with 6 MV X-rays, and then cultured for 2 weeks. The medium was discarded, and the cells were fixed with 4% paraformaldehyde (Solarbio, Beijing, China) and stained with Giemsa stain (Solarbio, Beijing, China). More than 50 cells were considered to be an effective colony, and the relevant parameters were calculated, clonal formation rate (PE) = number of effective colonies / number of cells inoculated, survival fraction (SF) = PE of irradiated cells / PE of non-irradiated cells. Based on the multi-target model, we fitted the cell survival curve by using the GraphPad Prism 5.0 software, and D_0 ($=1/k$), D_q ($= \ln N * D_0$) and SF_2 were calculated.

Cell Counting Kit 8

Cell suspensions at 4×10^3 cells/well were added to 96-well plates. After incubating for 24 hours, the cells were subjected to different irradiation with 6 MV X-rays and then cultured for another 48 hours. After that, adding 10 µL of CCK-8 (Dojindo, Shanghai, China) for every 100 µL medium and incubating for 1 h. The microplate reader (Thermo Fisher, USA) was used to measure the absorbance value of each group at 450 nm and the SF of cell was calculated.

Cell Cycle Analysis

After all groups of cells were irradiated with 0 Gy or 8 Gy with 6 MV X-rays for 48 hours, 5×10^5 cells were collected from each group and washed with PBS. Each group was fixed with 1mL of 75% pre-cooled ethanol at -20°C overnight, and washed with PBS. Then, 0.5 mL PI/RNA staining buffer (BD, New Jersey, USA) was added for 20 min. All cells were filtered with a nylon filter and detected by flow cytometer.

Apoptosis Analysis

After all groups of cells were irradiated with 0 Gy or 8 Gy with 6 MV X-rays for 48 hours, 5×10^5 cells were collected from each group and washed with PBS. After that, cell suspension were added 7 µL Annexin V-APC /7-AAD (BD, New Jersey, USA) for 20 min. Thereafter, the cells were filtered with a nylon filter and detected by flow cytometer (BD, New Jersey, USA).

Immunofluorescence

The two groups of cell suspensions were inoculated on coverslips, and they were irradiated with 8 Gy irradiation after 24 hours of culture. At indicated time points(1, 3, 6, 12 and 24 h), 4% paraformaldehyde was used to fix the cells, which then treated with Immunol staining blocking buffer for blocking. Briefly, the NPC cells were incubated with primary and secondary antibodies. Finally, nuclei were counter-staining with DAPI solution, and coverslips were mounted with Antifade polyvinylpyrrolidone mounting medium. The EVOS FL Auto microscope (Life technologies, USA) was used to take images, and the number of cells which expressed the target proteins in the cytoplasm and nucleus were counted.

Co-immunoprecipitation(Co-IP)

The proteins of the vector group and the VEGF group were extracted separately, and the specific steps are as described above. 40 µL of Anti-Flag[®] M2 Affinity Gel was thoroughly mixed with 1 mL PBS, centrifuged and discarded the supernatant. 500 µL of total proteins were mixed with the above precipitate, and then incubated them overnight at 4°C. Subsequently, the mixture was centrifuged again and discarded the supernatant. 4×SDS-PAGE binding buffer were put into the precipitate and denatured by heating at 100°C. Then, the next steps of gel electrophoresis were the same as that of western blot analysis.

In vivo nude mouse models

Male athymic 4-week-old BALB/C nude mice were housed under specific pathogen free (SPF) conditions in Experimental Animal Center of Guangxi Medical University. Two group cells (4×10^6) were implanted into the right groin of nude mice. There are 6 mice in each group, and each mouse is marked with an ear tag. When the tumor diameter was about 1 cm, 3 mice from each group were randomly selected for 8 Gy irradiation. All mice were sacrificed after observation for about 12 days after irradiation. During the periodical measurement of tumor size, calculate tumor volume according to the following formula: $V = \text{width}^2 \times \text{length} \times 0.5$. Tumor tissues were submerged in formalin for hematoxylin and eosin (H&E) staining.

Immunohistochemistry (IHC)

The paraffin-embedded tissue slides were baked in 60°C incubator for 2 h, and then dewaxed, hydrated, antigen recovered and the endogenous peroxidase was eliminated. Subsequently, the primary and secondary antibodies were dropped on the slides and incubated. The staining intensity was recorded: 0 (no staining), 1 (light yellow), 2 (yellow), 3 (brownish yellow). The percentage of stained cells was recorded: 1 (0-25%), 2 (26%-50%), 3 (51%-75%) and 4 (76%-100%). Then multiply the score of staining intensity and percentage stained cells to get the immunoreactivity score of each tissue, and it was classified into two grades: low (0-3) or high (4-7).

TUNEL

TUNEL assay was detected with the commercial Tunel kit (Roche, Basel, Switzerland) according to the instruction of manufacture. The tunel-positive cells were regarded as apoptotic cells by observing the

sections on the fluorescence microscope (EVOS FL Auto) and analyzing five randomly selected regions of each slide. The apoptotic index was calculated as a percentage of apoptotic nuclei to total nuclei.

Statistical Analysis

All statistical results were analyzed using SPSS 17.0 and GraphPad Prism 5.0 softwares. All data are expressed as means $\pm$ standard deviation. Statistical p values were analyzed by Students' t tests or one-way ANOVAs. $P<0.05$ was supposed to indicate statistical significance. Each experiment was repeated three times.

Results

VEGF expression is associated with radiosensitivity in radioresistant nasopharyngeal cancer cell

Our team has established radioresistant NPC cell CNE-2R derived from the CNE-2 cell. In this experiments, we found that VEGF expression was significantly higher in CNE-2R cells than that in CNE-2 cells (Fig.1a and b). Therefore, we suspected that VEGF is necessary for radioresistance in NPC cells. To test this conjecture, we silenced VEGF with two different shRNAs, which sequences was 5'-GCGCAGCTACTGCCATCCAAT-3' and 5'-CACAACAAATGTGAATGCAGA-3', the negative control scramble sequence was TTCTCCGAACGTGTCACGT. As shown in Fig.1c and d, the PCR and western blot results showed we successfully silenced VEGF in CNE-2R cells.

To detect the effect of VEGF on radioresistance, we performed clonogenic assay and CCK-8 assays. The clonogenic assay (Fig.1e and f) revealed VEGF knockdown inhibited cell survival fractions and proliferation under the irradiation, and the radiobiological parameters are shown in Table I. The same results were analyzed using CCK-8 assay (Fig.1g). As expected, these results indicated that VEGF significantly heightened the radioresistance on NPC cells.

VEGF inhibits radiation-induced G2/M cell cycle arrest, apoptosis and DNA breaks.

Firstly, to further investigate how VEGF inhibits cell growth, we used flow cytometry to assess whether VEGF affects cell cycle progression and apoptosis. Annexin V-APC/7-AAD kit was served to detect the induction of apoptosis. We found that whether 0 Gy or 8 Gy was irradiated, VEGF silencing increased the rate of cell apoptosis (Fig.2a and b), and the apoptotic cells were remarkably higher in shRNA groups compared with the NC group ($P<0.05$). We also found that compared with NC group, given 0 Gy irradiation in shRNA groups caused a slight accumulation of cells in G2/M phase ($P<0.05$), and after given 8 Gy irradiation in shRNA groups the proportion of G2/M phase was remarkably increased ($P<0.05$) (Fig.2c-e). This result demonstrated VEGF silencing can induce cell cycle arrest.

To detect the influence of VEGF on DNA repair and damage capacity, we quantified γ -H2AX foci formation in NC and shRNA groups at different time points (0, 2, 4, 6, 12, 24 h) after 8 Gy irradiation by immunofluorescence staining. As expected, the peak foci value appeared in NC and shRNA groups at 4 h after 8 Gy irradiation, which was gradually decreased to the basic level at 24 h after 8 Gy irradiation. In

addition, inhibition of VEGF induced a remarkable increase in residual DNA damage and DNA damage existed to some extent at 24 h (Fig.3a and b). The results indicated that the repair of DNA damage was repressed by VEGF-silenced NPC cells, which was manifested by the persistence of γ -H2AX foci from 4 h to 24 h after irradiation.

Silencing VEGF can inhibit autophagy induced by irradiation

Firstly, we employed western blot to investigate the effect of VEGF on autophagy-related proteins without irradiation. The expression of p62 was decreased and LC3 II was increased in shRNA cells compared with NC cells (Fig.4a). The results showed that VEGF silencing inhibited the autophagy-related proteins in NPC cells. To further explore whether VEGF influenced the activity of autophagy induced by irradiation, western blot and immunofluorescence staining were utilized to observe autophagic activity in NC and shRNA groups at different time points (0, 2, 4, 6, 12, 24 h) after 8 Gy irradiation. As shown in Fig.4b, the western blot result indicated that LC3 II increased gradually with time and reached peak at 24 h after 8 Gy irradiation in NC group, p62 declined gradually over the time, however, in shRNA group, the expression level of LC3 II reached peak at 6 h and p62 was relatively low at 6 h after 8Gy irradiation. Meanwhile, we employed immunofluorescence assay to detect the formation of LC3 II puncta in NPC cells. After 8 Gy irradiation, the number of LC3 II puncta in NC cells increased significantly at 24 h, while that in shRNA cells increased significantly at 6 h (Fig.4c and d). Overall, these results showed that VEGF knockdown inhibited radiation-induced autophagy in NPC cells.

VEGF regulates autophagy through the mTOR pathway

It is well known that mTOR is related to autophagy, so we further explored whether VEGF regulates autophagy through mTOR pathway in NPC cell. First of all, we used western blotting to confirm that phosphorylated mTOR declined significantly after silencing VEGF, whereas total mTOR protein remained almost unchanged (Fig.5a). In order to further verify the correlation between VEGF and mTOR, we overexpressed VEGF in CNE-2R cells (Fig.5c and d). We then used Co-IP assay to confirm that VEGF precipitate down together with mTOR (Fig. 5e), which indicated that VEGF interacted with mTOR. Fig.5b showed that autophagy were suppressed by given the mTOR activator (MHY1485) to CNE-2R cells, and autophagy were increased after given the mTOR inhibitor (Rapamycin). The above findings demonstrated that VEGF knockdown inhibited the autophagy by activating the mTOR pathway in NPC cells.

VEGF can regulate autophagy to enhance radioresistance in vivo

Our in vitro studies verified that VEGF can affect radiosensitivity of nasopharyngeal carcinoma by regulating autophagy. To further investigate these effects in vivo, VEGF-silenced cells were injected into nude mice. Half of the nude mice in shRNA group and NC group were randomly selected for 8 Gy irradiation on the 15th day. We found that inhibition of VEGF significantly repressed tumor growth with or without irradiation (Fig.6a and b). No matter what given 0 Gy or 8 Gy irradiation, the IHC assays confirmed that LC3 II and VEGF expression were much higher in NC group than that in shRNA group, and p62 expression was exactly the opposite (Fig.6c). The percentages of apoptotic cells were tested by

TUNEL staining in tumor sections (Fig.6d and e). Compared with the NC cells, the apoptosis level of the shRNA cells increased slightly at 0 Gy irradiation ($P<0.05$), by comparison, the apoptosis level was significantly increased in shRNA cells after 8 Gy irradiation ($P<0.05$). These results showed that silencing VEGF enhanced the irradiation sensitivity of NPC cells in vivo, which is in keeping with its functions in vitro.

Discussion

At present, radiotherapy is one of the main treatments for NPC. However, during the treatment course, treatment failure often occurs due to radiation resistance. Therefore, it is particularly important to explore the mechanism of radioresistance in NPC patients. Autophagy is the most common intracellular degradation process in organisms, and it can maintain normal homeostasis and influence tumor development. The anti-stress role of autophagy is important for cancer cell survival during therapy [19, 20]. The radioresistant NPC cell line CNE-2R were highly expressed VEGF as compared to CNE-2 cells. Therefore, this study mainly explored the possible relationships and mechanisms of VEGF and radioresistance in NPC.

Human VEGF gene is located on 6p21.3 chromosome, VEGF has certain bisulfidic bridges in its protein structure [21, 22]. Normal angiogenesis is in a dynamically balanced homeostasis, but becomes abnormally active in tumor tissue, and the most important driver of tumor angiogenesis is VEGF [23]. In 1993, Ferrara [24] found that inhibition of VEGF blocked tumor growth. Moreover, knocking down VEGF could significantly inhibit the growth of tumor cells [25, 26]. Radioresistance is a major obstacle to cancer treatment, and is thought to be involved in multiple signaling pathways in cancer [27]. In 1999, Gorski. found that irradiation increased the secretion of VEGF in tumor cells, and VEGF could also enhance the radioresistance of endothelial cells [28]. Similarly, Chen [29] and Kil [30] found that radiation therapy of tumors induces increased secretion of VEGF. Moreover, the relationship between high VEGF expression in tumor tissues and radiation therapy has been demonstrated in many cancers [31-33]. Consistent with previous reports, our results showed that inhibition of VEGF significantly repressed the tumor growth especially with irradiation, which indicating VEGF could promote the radioresistance in NPC cells.

Ionizing radiation-induced cell killing is mainly associated with DNA double-strand breaks (DSB) and cell cycle redistribution. Upon exposure to ionizing radiation, cells produce reactive oxygen species (ROS) and free radicals that induce DNA damage, the most severe being DSB [34]. The cancer cells died of necrosis and apoptosis after irradiation, so radiosensitivity can be measured by the degree of apoptosis cells [35]. Therefore, we demonstrated that CNE-2R cells had a higher apoptotic rate after silencing VEGF especially with irradiation by flow cytometry and TUNEL assays. Since DSBs are the cause of cellular death after irradiation, tumor cells can induce radioresistance by activating DNA repair and cell cycle checkpoints [36]. The G2 phase of the cell cycle is a late stage of DNA synthesis and numerous proteins are required for mitosis phase [37]. The cell cycle is an important factor of electro-radiation sensitivity and the G2/M phase cells have the highest radiosensitivity. Hence, partial simultaneous radiotherapy of cells during the radiosensitivity phase of the cell cycle is more effective [38]. Cui [39] found that arsenic trioxide enhances

radiosensitivity by promoting arrest G2/M phase in esophageal cancer cells, reducing the repair of sublethal damage and inducing apoptosis. In this study, it is confirmed that the G2/M phase of NPC cells was arrested by silencing VEGF. There was a linear correlation between γ -H2AX foci and DSBs, the formation and disappearance kinetics of γ -H2AX are consistent with the successful repair of DSBs [40]. By IF assay, we observed that γ -H2AX expression increased to the maximum about 4 hours after irradiation, and the residual γ -H2AX in VEGF-silenced cells lasted for 24 hours. Therefore, these evidence suggested that VEGF silenced could weaken the ability of double-strand DNA repair, which makes tumor cells sensitive to radiation.

In the process of tumor development, autophagy can protect tumor cells from cancer therapy by opposing pressure, providing energy, and regulating homeostasis [41-44]. Furthermore, more and more evidences have confirmed the existence of autophagy is the basic mechanism of cell survival in stressful environments caused by radiotherapy and chemotherapy. The increase of autophagy will enhance the radioresistance of tumor, and tumor with autophagy defect are more sensitive to radiotherapy [45].

The expression of autophagy-related genes light chain 3 (LC3), Beclin-1 and p62 could be used as indicators of tumor recurrence and poor prognosis [46]. The transition from LC3 I to LC3 II is considered to be a sign of autophagy activity. P62 is another marker of autophagy, which known as polypeptide binding protein chelator 1. When autophagy was inhibited, the expression level of p62 was increased, while when autophagy was induced, the expression level of p62 was decreased [47]. In this experiment, we found that VEGF silencing could inhibit autophagy, and since autophagy is a dynamic process, we measured autophagy-related proteins at different time points after irradiation by western blotting and immunofluorescence. Interestingly, we found that knockdown of VEGF advanced the occurrence of autophagy in NPC cells after irradiation. These results indicated that silencing VEGF can inhibit the autophagy in NPC cells and make them sensitive to radiation.

As we all know, autophagy is regulated by an intricate network, and mTOR pathway has been well characterized. MTOR can be activated to negatively regulate autophagy [48]. Recent studies also show that mTOR pathway is connected with tumor development and treatment resistance [49-51]. In this study, silencing VEGF increased mTOR phosphorylation. Similarly, co-ip assay also confirmed the interconnection between VEGF and mTOR. In addition, we used mTOR activator (MHY1485) and inhibitor (Rapamycin) respectively to evaluate their effects on VEGF-induced autophagy in radioresistant NPC cells. As expected, MHY1485 activated the expression of p-mTOR and p62, and inhibited the expression of LC3 II in CNE-2R cells, while the effect of rapamycin was the opposite. These findings indicated that silencing VEGF inhibited the autophagy-related proteins by the the activation of mTOR pathway.

Conclusions

Our research shows that inhibition of VEGF exerts anti-tumor effects and increases the radiosensitivity of NPC cells. In addition, it is evident that the role of VEGF in radioresistance is closely related with the induction of autophagy. We also found that silencing VEGF inhibits autophagy via activating the mTOR

pathway. Our findings demonstrate that inhibition of VEGF-mediated autophagy may provide a better therapeutic strategy for addressing radioresistance in NPC.

Declarations

Ethics approval and informed consent

The animals studies was approved by the Ethics Committee of Guangxi Medical University Cancer Hospital (No. LW2020001).

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Competing interests

The authors have no conflict of interest to declare.

Authors' contributions

XZ conceived and designed the experiments, LC, GL, KC, FW, YS, RL and XC performed the experiments, LC and GL contributed to the data analysis and wrote the manuscript.

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Table

Table I. Radiobiological parameters of the three groups (Mean±SD)

Cell lines	D ₀	D _q	SF ₂
NC	5.091±0.178	2.362±0.309	0.787±0.008
ShRNA1	4.31±0.391	1.667±0.026	0.724±0.008
ShRNA2	4.193±0.189	1.465±0.083	0.735±0.008
<i>p</i>	0.082	<0.05	<0.05

D₀ is the average lethal dose, which is the dose required to hit each cell once. D_q is a quasi-threshold dose, which refers to the ability to repair sublethal injury. SF₂ is the survival fraction at 2 Gy radiation dose.

Figures

Figure 1

Effect of VEGF on radiosensitivity of nasopharyngeal carcinoma cells. (a, b) The expression of VEGF protein and mRNA in CNE-2R and CNE-2 cells. *** p < 0.001, CNE-2 versus CNE-2R. (c, d). The expression of VEGF protein and mRNA in CNE-2R cells treated with control or VEGF short hairpin RNAs (shRNAs). (e, f) The number of colonies and the size of the colonies were analyzed in NC and shRNAs cells after

irradiation. (g) Cell proliferation in NC and shRNAs cells were detected by CCK-8 assay after irradiation. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, shRNAs versus NC.

Figure 2

Silencing VEGF increased radiation-induced apoptosis and G2/M cell cycle arrest. (a,b) Flow cytometry was used to detect cell apoptosis in NC and shRNAs cells with or without 8 Gy irradiation. (c-e) The cell cycle in NC and shRNAs cells were subjected to flow cytometry with or without 8 Gy irradiation. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, shRNAs versus NC.

Figure 3

Silencing VEGF decreased DNA damage repair in CNE-2R cells. (a) Immunofluorescence for γ -H2AX at different time point after 8 Gy irradiation in NC and shRNA cells. (b) The statistical result of mean γ -H2AX foci per nucleus in NC and shRNA cells. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, shRNA versus NC, size bars=100 μ m.

Figure 4

Silencing VEGF inhibited radiation-induced autophagy. (a) The expression of p62 and LC3 II proteins in NC and shRNAs cells. (b) The expression of p62 and LC3 II proteins in NC and shRNA cells at different time point after 8 Gy irradiation. (c) Quantitative analysis of LC3 II protein in immunofluorescence assay. (d) Effect of VEGF on autophagy detected by using immunofluorescence of LC3 II at different time point after 8 Gy irradiation. Size bars=100 μ m.

Figure 5

VEGF regulated autophagy through mTOR pathway. (a) The expression of mTOR and p-mTOR proteins in NC and shRNAs cells. (b) The quantify levels of mTOR, p-mTOR, LC3II, p62 and VEGF was detected by western blotting when mTOR activators (MHY1485) and inhibitors (Rapamycin) were given to CNE-2R cells. (c, d) VEGF overexpression vector was used to transfect into CNE-2R cells . Transfection effociencies were tested by western blotting and qPCR assays. (e) The formation of the VEGF/mTOR complex was detected by Co-IP.

Figure 6

Silencing VEGF promoted the radiosensitivity of nude mouse models. (a) Tumor size of NC and shRNA cells inoculated subcutaneously in nude mice at 27 days. (b) Growth curve of tumor nodules with or without 8 Gy irradiation in each group (black arrow indicates the time point of radiation). (c) Expression of LC3 II, p62 and VEGF was analyzed by IHC assay. (d) Tumor nodules were subjected by TUNEL assay. (e) Quantitative analysis of TUNEL assay results. ** $p < 0.01$, shRNA versus NC, size bars=100 μm .