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Original Research Article

Effect of overexpression of Rac1 on radiosensitivity of nasopharyngeal carcinoma xenografts in nude mice, and the underlying mechanism

Kai Liao, Ronghui Zheng, Jianhui Feng, Hui Liu, Guofeng Xie*
Department of Radiation Oncology, Affiliated Cancer Hospital & Institute of Guangzhou Medical University, Guangzhou 510030, Guangdong Province, China

For correspondence:* ***Email:* *yangaohe71700@163.com*

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Abstract

Purpose: *To study the effect of overexpression of Rac1 on the radiosensitivity of transplanted nasopharyngeal carcinoma (NPC) in nude mice, and the mechanism involved.*
Methods: *Forty Lucky SPF-grade male thymus-free nude mice were used. Mice were divided into 4 groups: overexpression control, overexpression Rac1, Rac1 inhibition, and inhibition control groups, each with 10 mice. The prepared cell lines were treated with 6 MV x-ray. Before and after radiation, the growth of tumors in each group was monitored. Histomorphological images of nude mice tumors were obtained using hematoxylin and eosin (H&E) stains. Protein expressions of p67, P47, and Rac1 were evaluated bu Western blotting.*
Results: *Transplanted tumor growth slowed down after 20 days. Growth rate was significantly higher in Rac1 and Rac1 overexpression groups than in overexpression and inhibition control groups ($p < 0.05$). Overexpression of Rac1 resulted in more cell necrosis, incomplete cellular structure, severe nuclear fragmentation, nuclear pyknosis, and cytotoxic red staining in endoscopic tumor tissues ($p < 0.05$). There were significantly lower expression levels of p67, P47, and Rac1 in Rac1 invasion group than in invasion control and overexpression control groups, while the expression levels of p67 and P47 were significantly higher in overexpression Rac1 group and IR overexpression Rac1 group than in inhibition control ($p < 0.05$). However, concentrations of p67 and P47 were significantly higher in overexpression Rac1 and IR overexpression Rac1 groups than in inhibition control mice.*
Conclusion: *Rac1 increases the radiosensitivity of NPC xenografts in nude mice via a mechanism related to the regulation of expressions of proteins associated with Rac1/NADPH signaling pathway. Thus, Rac1 is potentially a new target for radio-sensitization of NPC.*

Keywords: *Rac1, Nude mice, Nasopharyngeal carcinoma xenograft, Radiotherapy, Radiation, Radiosensitivity*

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a malignant tumor that occurs on the top and lateral walls of the nasopharyngeal cavity, with clinical symptoms of nasal obstruction, bleeding nose, hearing loss, ear condensation, headache, and amblyopia [1]. The disease has a high and

increasing incidence among Chinese people, and it is a serious challenge to life and health [2]. It is difficult to treat NPC due to the hidden location of the lesion areas and multiple etiologies [3].

Clinically, radiotherapy and surgery are the main ways of treating NPC, but radiotherapy is more widely used [4]. Resistance to radiation often occurs during NPC radiation therapy. Therefore, it is important to improve chemotherapy efficiency and reduce or eliminate radiation resistance so as to improve treatment effectiveness in NPC patients [5]. It is known that PAC1 is a RAS-related Rho GTPase protein that is overexpressed in gastric cancer, colon cancer, breast cancer, testicular cell cancer, as well as head and neck tumors, and it is closely related to tumor occurrence, invasion, and metastasis [6]. Recent studies have suggested that PAC1 affects the radiosensitivity of NPC xenografts in nude mice [7]. The purpose of this research was to determine the effect of overexpression of Rac1 on the radiosensitivity of NPC xenografts in nude mice, and the molecular mechanism involved.

EXPERIMENTAL

Animals and cell lines

Male Lucky SPF grade and thymus-free nude mice aged 4 – 5 weeks (mean weight = 17 ± 30 g) provided by Changzhou Cavens Laboratory Animal Co. Ltd. were used in this study. The mice were housed in a well-ventilated environment with a 12-h light/12-h dark photoperiod at a mean temperature of 23 ± 1 ℃ and relative humidity of 55 ± 5 %. They were fed *ad libitum* with sterilized pellet feed and allowed free access to clean drinking water.

The study received approval (approval no. GMUAR2020034) from the Animal Ethics Authority of Affiliated Cancer Hospital and Institute of Guangzhou Medical University, and was performed in line with NIH guidelines [8].

Cell lines

Lentiviral LV5 empty vector CNE-2 cells were used in this study. The cells were provided by Hao di huatop Bio-Technology Co. Ltd., Shenzhen, China. The CNE-2 cell lines overexpressing lentiviral LV5 vector encoding Rac1 and an untreated CNE-2 cell line were used.

Preparation of cell suspension

When the density of cultured cells reached 80 – 90 %, the culture medium was discarded, and the cells were trypsinized with PBS buffer solution containing 0.25 % trypsin. The digest was taken up in a 15-mL centrifuge tube and centrifuged at 1000 rpm for 5 min. Then, the cells were rinsed with PBS, and serum-free RPMI 1640 culture solution was added to make a single-cell suspension. The cells were adjusted to a final concentration of 5×10^6 cells/0.2 mL.

Subcutaneous injection

The nude mice were fixed on a table, with the outer parts of their hind limbs exposed. Then, the skins were disinfected with 75 % alcohol, after which 200 µL of the cell suspension was injected into the skin of each mouse using a medical micro-syringe at a depth of 1 – 1.5 cm, and at an angle of 30 ° between the injection point and skin. The cell suspension was injected into the skin to form a 25-mm^2 pilus on the lateral side of the hind limb of the nude mouse.

Animal grouping

Forty mice were randomly assigned to overexpression control, overexpression Rac1, inhibition Rac1, and inhibition control groups, each with 10 mice. The cell suspension was injected into the left and right hind limbs of the four groups. The left hind limbs were the non-infection treatment group, while the right hind limbs were the infection treatment group. The non-radiation treatment group did not receive the radiation treatment. During the radiation treatment, the nude mice were fixed on the plate, with the hind limbs exposed to the radiation field, while the other parts were not exposed to radiation. The radiation treatment involved the use of 6 MV X-rays, with source skin distance (SSD) of 110 cm, dose rate of 269 mu/min, and exposure dose of 4 Gy. In the 4 groups without radiation treatment, the cell suspension was injected outside the left limb of nude mice, i.e., the overexpression control group, overexpression Rac1 group, inhibition Rac1 group, and inhibition control group. In 4 groups of combined radiation treatment, the cell suspension was injected outside the right limb of nude mice, i.e., in the IR overexpression control group, IR overexpression Rac1 group, IR invasion Rac1 group, and IR invasion control group.

Determination of parameters/indices

Tumor long diameter (*a*) and short diameter (*b*) were measured every other day after tumor formation, using a Vernier caliper. The tumor volume (V) was calculated using Eq 1.

$V\ (mm^3) = ab^2/2$ ..... (1)

After 2 weeks of xenograft growth, a local radiation experiment was carried out. The body weight and tumor volume were measured and recorded for 10 days, and the nude mice were sacrificed via cervical dislocation, with xenograft strapped. The weight of the tumor in each mouse was obtained, and the tumor was photographed. Histomorphological image analysis of nude mice tumors was done using hematoxylin and eosin (H&E) staining. The samples were sequentially fixed in 10 % neutral formalin, washed, dehydrated, cleared, embedded in wax, embedded in paraffin, sliced, and kept at 4 °C, prior to use. They were serially soaked in xylene, ethanol, and tap water for H&E staining using standard protocol. The stained sections were examined under a light microscope.

Immunoblotting was employed to assay relative protein concentrations of p67, P47, and Rac1. After the mice were sacrificed, the xenograft blocks were removed and put directly into labeled cryopreservation tubes. The xenografts were sliced into 5-μm sections using a cryo-sectioning machine. Tumor tissue total protein extraction was done with RIPA buffer in line with standard protocol. Lysed tissue suspensions were centrifuged for ½ h at 12,000 rpm at 4 °C. Thereafter, equal amounts of protein were subjected to SDS-PAGE, transfer to PVDF membranes, incubated with appropriate primary and secondary antibodies, and enhanced chemiluminescence.

Statistical analysis

The data obtained were analyzed using SPSS 20.0 software package. Measurement data are expressed as mean ± SD, and comparison between the 2 groups was made with *t*-test. Enumeration data are expressed as numbers and percentages {n, (%)}, and comparison among groups was made with χ^2-test. Statistical significance was assumed at $p < 0.05$.

RESULTS

Tumor growth before and after radiation

At various time points of radiation exposure, the volume of transplanted tumor was comparable in the negative control and the empty vector groups ($p > 0.05$). In contrast, transplanted tumor volumes of nude mice in the overexpression Rac1 groups were significantly low, relative to empty vector and negative control groups (Figure I).

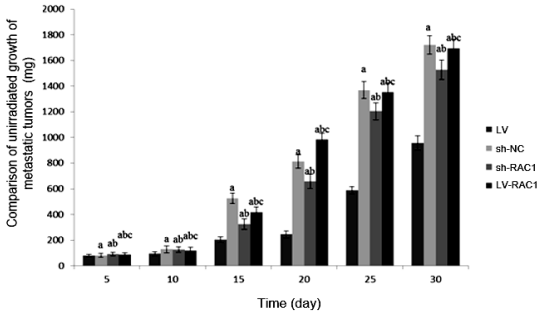

Figure 1: Comparison of the growth of metallic tumors before and after inflammation amongst the groups. [a]$P < 0.05$, vs. overexpression control group; [b]$p < 0.05$, vs. inhibition control group; [c]$p < 0.05$, vs. inhibition Rac1 group

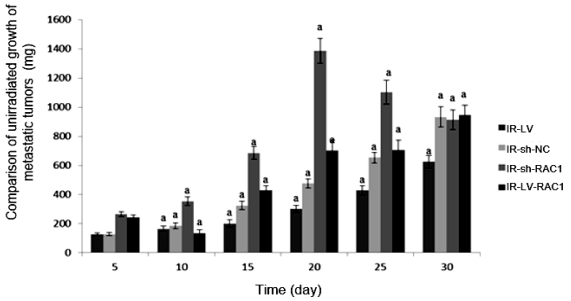

Figure 2: Comparison of the growth of metallic tumors before and after inflammation in each group. [a]$P < 0.05$, compared to another group at the same time point

Histo-morphological changes in metastatic tumor tissues

The grafted tumors from the negative control and empty vector groups contained dense and compact arrays of cells with large differences in cell size, hyperchromatic nuclei, atypia and few necrotic areas, and the cells showed proliferative state. In the group that overexpressed Rac1, there were more necrotic cells, and a vast majority of cells had incomplete structures, severe nuclear fragmentation and high degree of pyknosis. Moreover, the necrotic tumor tissues showed cytoplasmic red staining. These results are shown in Figure 3.

Expression levels of RAC1/NADPH signal pathway-related proteins

Immunoblot assay revealed higher relative concentrations of p67, P47, and Rac1 in the group that overexpressed Rac1 than those in the negative control group and empty vector group. However, relative protein concentrations of p67, P47, and Rac1 were comparable in negative control and empty vector mice, as shown in Figure 4.

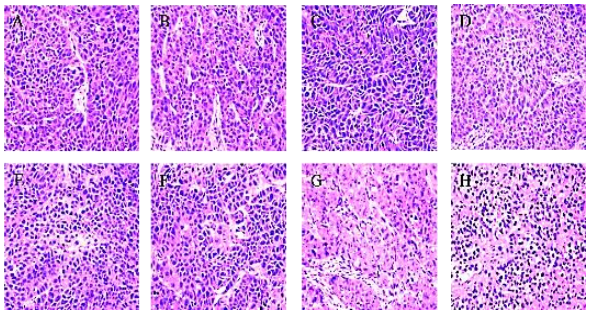

Figure 3: Comparison of histo-morphological changes in metastatic tumor tissues amongst the groups. A: H&E-stained tissue images of the negative control group; B: H&E-stained tissue images of empty vector group; C: Images of H&E-stained tissues in the group that overexpressed RAC1

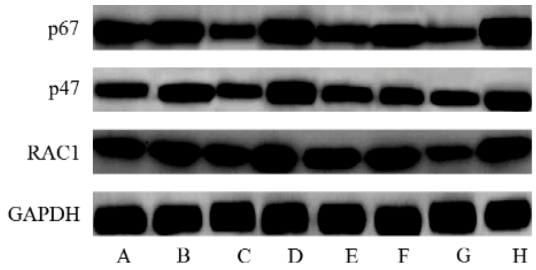

Figure 4: Expression levels of RAC1/NADPH signal pathway-associated proteins in metastatic tumor tissues of each group. A: Negative control group; B: empty vector group; C: overexpressed RAC1 group

DISCUSSION

Radiotherapy is the treatment method used for nasopharyngeal carcinoma and other malignant tumors showing moderate sensitivity to the rays. This method not only results in cell apoptosis by destroying cellular DNA, but it also damages mitochondria and produces additional oxidative stress. Long radiation therapy results in radiation resistance, thereby causing treatment failure [9]. It has become the focus of medical scholars in China and elsewhere to explore more feasible ways of maximizing radio-sensitization-induced tumor damage while reducing the negative impact of radiation on the normal surrounding tissues.

It is known that Rac1 plays an important role in cell morphology, proliferation, adhesion, lipid metabolism, membrane transport, endothelial cell connectivity, and regulation of the cell cycle. Moreover, Rac1 affects the activities of apoptotic regulatory proteins and transcription factors [10]. In this study, at various time points of radiation exposure, the volume of transplanted tumor was comparable in negative control and empty vector mice. In contrast, transplanted tumor volumes of nude mice in the overexpression Rac1 mice were significantly reduced, relative to the negative control and empty vector groups. The grafted tumors from the negative control and empty vector groups contained dense and compact arrays of cells, with large differences in cell size, hyperchromatic nuclei, atypia, and fewer necrotic areas.

Furthermore, the cells were in a proliferative state. In the group that overexpressed Rac1, there were more necrotic cells, and a vast majority of cells had incomplete structure, severe nuclear fragmentation, and aggravated pyknosis. Moreover, the necrotic tumor tissue showed cytoplasmic red staining. These data suggest that overexpression of Rac1 increased the sensitivity of tumors to radiation in NPC xenograft tumor model in nude mice. The results are consistent with the findings of Wang et al [11].

The protein Rac1 is an important control element for nicotinamide adenine dinucleotide oxidase. Recent studies have suggested that Rac1/NADPH signaling pathways are closely related to the radiosensitivity of NPC [12]. When external stimulation of growth factors, cytokines, inflammatory media, and rays puts the body in an abnormal state, phosphorylation of nicotinamide adenine dinucleotide oxygenase in cytoplasmic subunit P47 leads to conformational changes. Phosphorylated P47, P67, P40, and Rac1RAC1 are close to the cellular membrane. This increases active oxygen concentration and enhances oxidative damage to lipids, nucleic acids, and proteins, thereby causing irreversible damage and death to tumor cells [13-15].

In this research, relative protein concentrations of p67, P47, and Rac1 in the group overexpressing Rac1 were higher than the corresponding expression levels in the negative control group and empty vector group. Thus, the mechanism by which overexpressed Rac1 increased radiation sensitivity of NPC radiotherapy may be related to its regulation of the expressions of proteins related to Rac1/NADPH signaling pathway.

CONCLUSION

The results obtained in this study demonstrate that Rac1 protein significantly increases the radiosensitivity of NPC xenografts in nude mice through a mechanism involving the regulation of expressions of proteins associated with Rac1/NADPH signaling pathway. Thus, RAC1 has the potential of becoming a new target for radio-sensitization of NPC.

DECLARATIONS

Acknowledgements

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Funding

None provided.

Ethical approval

This study received approval (approval no. GMUAR2020034) from the Animal Ethics Authority of Guangzhou Medical University, and was performed in line with NIH guidelines [8].

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors, all authors read and approved the manuscript for publication. Guofeng xie conceived and designed the study, Ronghui Zheng, Jianhui Feng and Hui Liu collected and analysed the data. Guofeng Xie wrote the manuscript.

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