Radiosensitization of $[Ru(bpy)_2(phen)]Cl_2$ on nasopharyngeal carcinoma cells

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ABSTRACT

Background: To investigate effect of radiosensitization of $[Ru(bpy)_2(phen)]Cl_2$ complex on nasopharyngeal carcinoma cell line CNE1 and its mechanism. ***Materials and Methods:*** Nasopharyngeal carcinoma cell line CNE1 in vitro culture was divided into control group, light irradiation group (4 Gy, 6 MV photonic line), simple metal ruthenium complex treatment group (Ru group, 100 μmol/L $[Ru(bpy)_2(phen)]Cl_2$) and metal ruthenium complex combined with radiotherapy group (Combined radiotherapy group, cells were irradiated with 4 Gy and 6 MV photons at 2 days after 100 μmol/L $[Ru(bpy)_2(phen)]Cl_2$). ***Results:*** Transcriptional level of *P53* gene in combined radiotherapy group was higher than that in the other groups ($P<0.001$). Inhibition rate of combined radiotherapy group was higher than that of Ru group and irradiation group ($P<0.001$). Apoptotic rate was the highest ($P<0.05$) in the combined radiotherapy group, and irradiation group was higher than Ru group and control group ($P<0.05$). Survival rate of Ru group was lower than that of control group under the same radiation dose ($P<0.05$), and the radiotherapy sensitization ratio was 1.227 (Dq ratio). ***Conclusion:*** $[Ru(bpy)_2(phen)]Cl_2$ increases the sensitivity of nasopharyngeal carcinoma cell line CNE1 to X-ray, which may be related to increase of *P53* gene expression.

Keywords: *$[Ru(bpy)_2(phen)]Cl_2$, nasopharyngeal carcinoma cells, P53 gene, radiosensitization.*

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is an uncommon cancer arising from the nasopharynx epithelium with a very unique pattern of geographical distribution [(1)]. Currently recognized and effective treatment is radiation therapy, or radiotherapy-based comprehensive treatment. Because there is a certain proportion of radiation-resistant cells (such as hypoxic cells) in NPC, treatment of tumor cells is restricted by radiation sensitivity, which is the main cause of local residual, recurrence and metastasis of NPC. Therefore, improved sensitivity of NPC to radiation therapy is an effective means to improve its cure rate and

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control rate.

Studies have shown that metal ions can improve the tissue absorption rate of radiation [2]. The reinforce of DNA damage and inhibition of DNA repair can also improve radiation sensitivity [3]. Therefore, all kinds of metal complexes can be served as a candidate radiotherapy sensitization because of its targeting advantage of DNA, and platinum complex is one of the typical representatives [4-6], such as cisplatin, carboplatin, and oxaliplatin. However, the toxic and side effects of platinum are obvious with long-term use and could result in drug resistance. So scholars want to search other metal complexes. Ruthenium complexes have attracted a great interest due to their higher activity and lower toxicity than platinum complexes. Although the research was still at the laboratory level, a series of encouraging results had been reported on ruthenium complexes as radiotherapy sensitizers. Study had shown that many ruthenium complexes as radiotherapy sensitizers have strong DNA binding capacity [7]. Because the robustness of the ruthenium–arene unit hold a high potential for antitumour candidates, neutral or cationic arene ruthenium complexes provided both hydrophilic and hydrophobic properties [8]. Ruthenium has been considered to be an attractive alternative to platinum, particularly since many ruthenium complexes are low-toxic and some ruthenium complexes have been shown to be quite selective for cancer cells [9].

In this study, [Ru(bpy)$_2$(phen)]Cl$_2$ was synthesized by the combination of polypyridyl ruthenium and o-phenanthroline ligand. Compared with other ruthenium complexes, it has good water solubility, can pass through cell membrane, has high DNA insertion ability, and shows high concentration of drugs in hypoxic conditions [10]. Because of tumor growth too fast, internal vascular support is lacked and some internal hypoxic cells is existed, which also makes the tumor resistance to radiotherapy and chemotherapy [11]. The use of such ruthenium complexes on the role of tumor hypoxia to study the treatment of solid tumors is very promising [12].

Therefore, radiosensitization of [Ru(bpy)$_2$(phen)]Cl$_2$ on NPC CNE-1 cell was firstly studied to provide a good basis for clinical trials and the basis for the development of a new generation of radiotherapy sensitizer, and the important clinical significance to improve the control rate, recurrence rate and cure rate of NPC were explored.

MATERIALS AND METHODS

Cell line and culture method

CNE-1 cells of wild-type human NPC (donated by the Central Laboratory of the First Affiliated Hospital of Fujian Medical University) were cultured with RPMI 1640 (Macgene, China) containing 10% fetal bovine serum and exponential growth phase cells were taken to test.

Drugs and main reagents

[Ru(bpy)$_2$(phen)]Cl$_2$ were prepared and supplied by Institute of Material Science, Chinese Academy of Sciences, Fuzhou, with a concentration of 600 μmol/L. Fetal protein and MTT powder were purchased from Fuzhou Bioman Biotechnology Co., Ltd.; Annexin V-FITC/PI cell apoptosis detection kit was purchased from Nanjing KeyGEN BioTECH Co., Ltd.; Trizol extract was purchased from Aidlab company.

Irradiation method

The cells were irradiated by 600CD linear accelerator (Varian, USA) in Department of Radiation Oncology, First Affiliated Hospital of Fujian Medical University. Irradiation conditions: 6 MV X-ray, target skin distance was 100 cm, irradiation field area of 35 cm × 35 cm cell culture dish was placed 1cm at the bottom of the tissue compensation, the rack angle was 180°, and dose rate was 200 cGy / min.

Primer design and synthesis

According to *P19* gene sequence published in the relevant literature, the primer and oligonucleotide sequence template were designed to meet the requirements, and were synthesized by Sangon Biotech (Shanghai) Co.,

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Ltd.. Internal reference b-actin, *P19* gene polymerase chain reaction (PCR) primers:
Forward 5'- AGCGAGCATCCCCCAAAGTT -3';
Reverse5'-GGGCACGAAGGCTCATCATT-3'(285 bp);
Forward5'- AGGTTGGCTCTGACTGTACC-3',
Reverse5'-GATTCTCTTCCTCTGTGCGC-3'(195 bp).

Transcription condition of P53 gene in human NPC CNE-1 cells

CNE-1 cells were inoculated into 6-well plates, divided into control group, irradiated group, Ru group and combined radiotherapy group, and cultured for 48 hours to extract RNA, and then real-time reverse transcription polymerase chain reaction (RT-PCR) and real-time quantitative PCR detection were carried out.

3-(4,5-dimethyl-2-Thiazyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay was used to detect the cell proliferation

The cells were divided into control group, radiotherapy group, Ru group and combined radiotherapy group including 4000 cells per well, and then cultured in 96-well plate. After 48 hours of culture, MTT assay was performed and the OD value of A_{490nm} was measured. The cell growth inhibition rate was calculated according to the formula "cell growth inhibition rate (%) = A_{490} value of the control group - A_{490} value of the control group".

Cell apoptosis was detected by flow cytometry

The cells were treated and cultured for 48 hours. Apoptosis rate was detected according to Annexin V-FITC/PI cell apoptosis assay kit (BD Pharmingen).

Clone formation assay

The cells of Ru group and control group were inoculated into 6-well plate with 100 cells per well, until the cells adhered to irradiate, and irradiation doses were 0, 2, 4, 6, and 8 Gy. The treated cells were allowed to stand for 11-14 days, and culture was stopped. After crystal violet was stained, the number of clones were calculated by using enzyme-linked spot image automatic analyzer and clone formation number was calculated. The multi-target model parameter of dose survival curve was fitted by SPSS, and cell survival score was predicted at each dose. The predicted cell survival score was ordinate. The EXCEL software was used, and multi-target model was used to draw out cell survival curve.

Data processing

The experimental data were expressed as`x±s (Repeated three times). After established a database collation to organize by using EXCEL, the statistical chart was drawed; SPSS 20 statistical software package was used for analysis. The one-way analysis of variance was used for comparison of multiple groups, α= 0.05 , and P≠0.05 meaned that the difference was statistically significant.

RESULTS

Transcription of P53 gene in CNE-1 cells

Real-time quantitative PCR was used to measure the values of 2-ΔΔCt in each group and statistical analysis was performed (figure 1). The results showed that expression of *P19* gene in combined radiotherapy group, Ru group, control group and irradiation group were 1.959 ± 0.781, 1.009 ± 0.042, 1.000 ± 0 and 0.982 ± 0.054, respectively. The transcriptional level of *P19* gene of CNE-1 cells in combined radiotherapy group was higher than that in irradiation group, Ru group and control group (P<0.001). There was no significant difference in transcription level among irradiation group, Ru group and control group (P> 0.05).

MTT results

The growth inhibition rates of each group of cells were shown in figure 2. The growth inhibition rate of Ru group, irradiation group and combined radiotherapy group were (0.274 ± 0.052)%, (0.244 ± 0.043)% and (0.619 ± 0.036)%, respectively. Compared with the irradiation group and Ru group, increase of growth inhibition rate in combined radiotherapy

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group was significant (P<0.001). There was no significant difference between Ru group and combined radiotherapy group (P> 0.05). As for growth inhibition rate, there was no significant difference between the Ru group and irradiation group (P> 0.05).

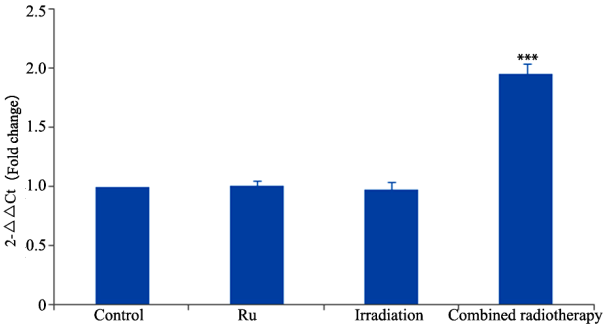

Figure 1. Statistical results of 2-△△Ct in control group, irradiation group, Ru group and combined radiotherapy group. ***,P=0.000.

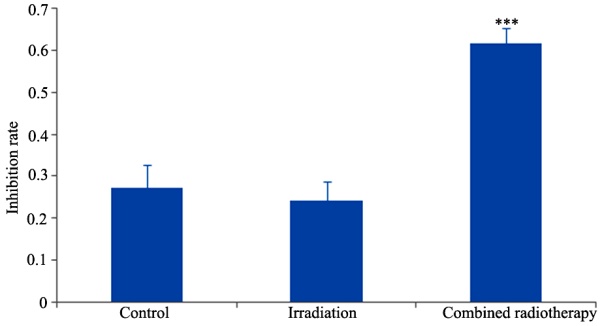

Figure 2. Inhibition rate in control group, Ru group and combined radiotherapy group.***,P=0.001.

Apoptosis results

The early apoptotic rates of combined radiotherapy group, irradiation group, Ru group and control group were (15.6 ± 4.573)%, (8.987 ± 3.567)% , (3.163 ± 1.64)% and (2.687 ± 1.75)% (P<0.05). The apoptotic rate of combined radiotherapy group was significant difference compared with other groups (P<0.05); and irradiation group was significant difference compared with Ru group and control group (P¬4.49); There was no significant difference between Ru group and control group (figure 3).

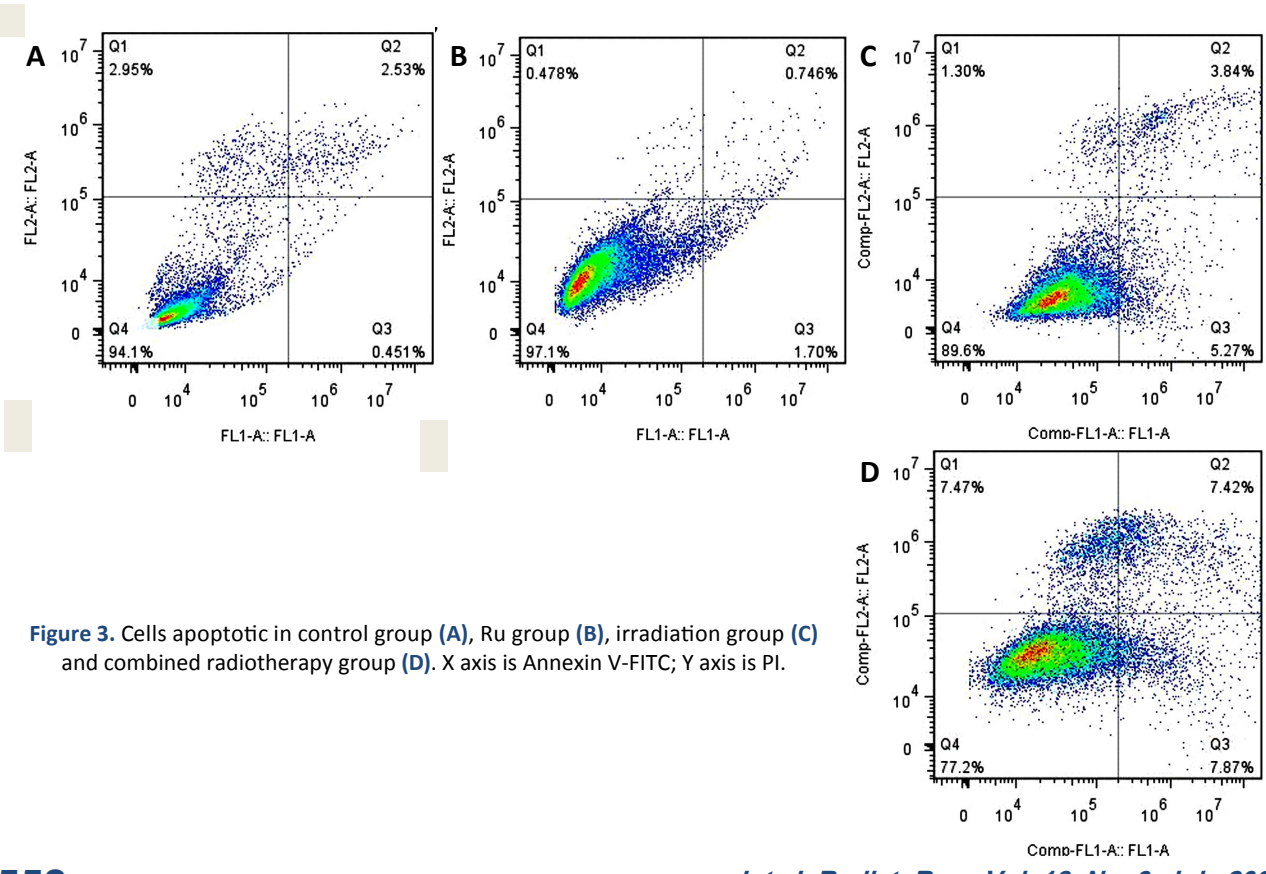

Figure 3. Cells apoptotic in control group **(A)**, Ru group **(B)**, irradiation group **(C)** and combined radiotherapy group **(D)**. X axis is Annexin V-FITC; Y axis is PI.

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Clone formation assay

With the increase in radiation dose, the cell survival fraction of Ru group and control group were decreased gradually, but the former was significantly lower than the latter. At the same dose, Ru group had lower survival fraction than control group (P <0.05). The parameter of multi-target model in SPSS fitted dose survival curve (figure 4): The D_0 value of control group was 3.223 Gy, the Dq value was 1.412 Gy, and the SF2 value was 68%; The D_0 value of Ru group was 2.106 Gy, the Dq value was 1.111 Gy, and SF2 value was 56%; Radiosensitivity ratio was 1.227 (Dq ratio).

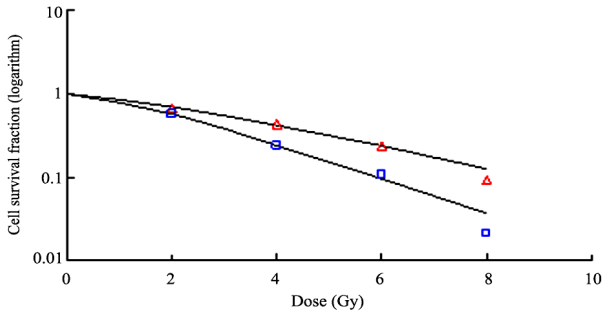

Figure 4. Multi-target model to draw the cell survival curve of control group **(A)** and Ru group **(B)**. The red triangle represents the control group; The blue square represents the Ru group.

DISCUSSION

In southern China, most of NPC was undifferentiated cancer, which was relatively sensitive to radiotherapy and chemotherapy, but had a higher degree of malignancy, and was more prone to lymph node metastasis. In recent years, although a variety of treatment methods were updated and improved, but 5-year survival rate of NPC patients had not improved significantly [13,14]. The early symptoms of NPC were not obvious and often diagnosed late stage, and many even distant metastasis may be occurred. So the initial radiation therapy of NPC is important. Once the treatment fails, the sensitivity of radiation to patient is decreased significantly once more, and side effects of radiotherapy are very obvious. Therefore, radiotherapy sensitizers are developed to improve the killing effect, reduce radiation-related complications, and the treatment of NPC is of great significance.

Metal complexes have been used clinically as a radiotherapy sensitizer, and typical examples are platinum complexes, but they are limited by their severe side effects, activity limitations, and tumor cell targeting [6]. The ruthenium complexes have the following characteristics: 1) Low toxicity: Ruthenium complexes are severed as prodrug before entering the body. They enter into the body and are activated through the hydrolysis or reduction reaction, and then play a role in this function; In addition, metal ruthenium has similar characteristics with iron, and are combined with serum proteins, iron transporters and other biological macromolecules after entering the body [15]. These are the reasons for its low toxicity; 2) Targeting: The target of platinum complex is mainly DNA. In addition that ruthenium-based complexes are combined with DNA, and the combination with protein also plays an important role [16], thus they become the focus of emerging radiotherapy sensitizer. Martin *et al.* synthesized the ruthenium (II) polypyridine complex [Ru(dppz)$_2$(PIP)] $^{2+}$, which has strong DNA embedding ability, prevents DNA replication from advancing, activates DNA replication emergency reaction, and blocks cell growth by cell cycle control. Under the coordination of external beam ionization radiation, it could kill cancer cells, showing a radiosensitizing effect [17]; In addition, Deng Zijin found that 2,6-dibenzothiazole containing metal ruthenium complex with radiotherapy sensitization increased the expression of *P19* [18]. These studies have shown that ruthenium complexes have radiosensitizing effects. In this study, [Ru(bpy)$_2$(phen)]Cl$_2$, a ruthenium-based complex formed with 1,10-phenanthroline as ligand and ruthenium, was selected as the study object, which indicated that it had strong radiotherapy sensitization to NPC.

The results showed that SF2 was cell viability at 2 Gy of dose, and was the most commonly used parameter to reflect the radiosensitivity of cells. It could reflect the effect of enhanced sensitivity compounds on cell sensitivity. The results of this study showed that D_0 value of cell

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survival curve in combined radiotherapy group was significantly lower than that in control group, and the SF2 decreases from 68% to 56%, suggesting that [Ru(bpy)$_2$(phen)]Cl$_2$ can improve the sensitivity of CNE-1 cells to radiation. Compared with the control group, the Dq value of combined radiotherapy group was decreased, indicating that the sublethal damage repair ability of NPC CNE-1 cells was decreased, and the smaller dose change could make it into exponential killing.

In order to further explore its possible mechanism, we have consulted a large number of literatures and found that regardless of what the original target of cancer treatments(such as chemotherapy, radiotherapy, immunotherapy), and substantially apoptosis program of tumor cell is activated to achieve the role of killing tumor cells [19]. *P53* gene regulation of apoptosis is more studied [20]. *P53* gene is a tumor suppressor gene, which plays an important role in preventing cell proliferation and maintaining the integrity of DNA-damaged genomes [21], and can regulate a large number of cell activity. In previous study, Montel et.al found that neutron activation increased activity of ruthenium-based complexes and induced cell death in glioma cells independent of *P19* tumor suppressor gene [22]. In another study, Ru (II)-Arene Schiff-base (RAS) complexes were identified, which could induce P53-independent cytotoxicity and study structural features that were required for their p53-independent activity, indicating that all nine complexes demonstrated P53-independent activity [23]. In combination with the above, we used flow cytometry to detect the apoptotic rate of cells of each group and to explore the difference of *P19* content in each group by fluorescence quantitative PCR, and RT-RCR showed that *P19* gene expression in combined radiotherapy group was significantly higher than that in the other groups.

Through the functional test, it was confirmed that ruthenium complexes selected in this study could inhibit cell growth, promote cell apoptosis and inhibit cell cloning, and have radiosensitizing effect. At the same time, ruthenium complex could also improve expression level of *P19* gene in NPC cell line CNE1, so it was speculated that the mechanism of radiosensitization may be related to the enhanced expression of *P19* and the promotion of cancer cells apoptosis. But the specific pathways and links of apoptosis regulation is not clear, and further studies are needed.

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