Abstract. The purpose of this study was to investigate the influence of quinalizarin on the radiosensitivity of nasopharyngeal carcinoma (NPC) cells and the relevant underlying mechanisms. Human NPC cell lines CNE-1, CNE-2 and 5-8F were treated with quinalizarin and then irradiated with different X-rays doses. Cell viability, survival, DNA double-strand breaks (DSB), apoptosis, cell cycle distribution, expression of SHP-1 and other related proteins were detected with MTT assay, colony formation assay, immunofluorescent assay, flow cytometry and western blot analysis, respectively. We also examined how the effects of quinalizarin were affected by SHP-1-overexpression by lentivirus transfection. Quinalizarin at 25 µM enhanced radiosensitivity of NPC cells. This increased radiosensitivity was due to inhibition of cell viability, which delayed DSB repair as seen by significantly increased γ-H2AX foci, promoting apoptosis by 34% in CNE-1 and 9% in CNE-2 cells compared to controls and changing cell cycle distribution in CNE-1, but not CNE-2 cells. Quinalizarin treatment obviously decreased SHP-1 protein expression. Overexpressing SHP-1 partially reversed the radiosensitive effect of quinalizarin. Quinalizarin inhibited binding of p65 and the promoter of SHP-1, and decreased the activities of SHP-1 promoter and SHP-1. Quinalizarin enhanced radiosensitivity of NPC cells partially by suppressing SHP-1 expression.

Introduction

Ionizing radiation (IR) is the primary therapy for nasopharyngeal carcinoma (NPC). Though most NPC cells are sensitive to IR, there are still some tumor cells which are resistant to irradiation. These radioresistant cancer cells often result in local recurrence and metastasis after radiotherapy (1). Thus, finding drugs that can enhance NPC therapy is of great significance.

In recent years, new drugs that target tyrosine kinase have made a breakthrough in tumor therapy (2). Because targeted therapy is highly specific and causes fewer side effects than chemotherapy, targeted therapy has become increasingly widely accepted by patients and doctors (3,4). To date a number of different kinds of tyrosine kinase inhibitors (TKIs) have been approved by the Food and Drug Administration (FDA), such as Imatinib (Gleevec), Gefitinib (Iressa), Erlotinib (Tarceva), and Sorafenib (Nexavar). In addition, there are still many other TKIs undergoing different stages of preclinical and clinical trials (5).

CK2 is an important Ser/Thr kinase that is expressed in most eukaryotes. The CK2 complex is a tetramer composed of two catalytic subunits (αα or αα') and two regulatory subunits (ββ) (6). Using ATP and GTP as phosphate group donors, CK2 phosphorylates the ser/thr residues of its substrates. Substrates of CK2 such as nuclear factor-κB (NF-κB), Dsh/Dvl, the product of the adenomatous polyposis coli gene, transcription elements Lef/Tcf, engrailed, and β-catenin are involved in signal transduction, DNA replication, transcription, translation and many other important events (7). Therefore, CK2 plays a vital role in cell growth, proliferation, differentiation, apoptosis and is considered to be a potential target for regulation of some cell processes such as cell cycle distribution, apoptosis and DNA damage repair (8-10). Some studies have found that CK2 inhibitors can inhibit cancer cell growth and promote apoptosis (10-12). Further research indicated that CK2 took part in DNA damage repair by affecting the binding of DNA-PKcs and DNA (13). Lin et al found that inhibiting CK2 activity increased radiosensitivity of non-small cell lung cancer (NSCLC) cells through decreasing stat3 activation (14). These studies suggest that CK2 may be a potential target to enhance radiosensitivity of tumor cells, including NPC cells.

Traditional CK2 inhibitors include TBB, TBI/TBBz, and DMAT. However, the selectivity of these inhibitors was not found to be as narrow as it was originally believed (15). Therefore, new CK2 inhibitors have been investigated.
Quinalizarin is an ATP-site competitive CK2 inhibitor. It is highly selective and cell-permeable and the selectivity of quinalizarin toward CK2 is superior to any other CK2 inhibitor known so far (16,17). Thus, we used quinalizarin as a CK2 selective inhibitor in this study and assessed its influence on NPC radiosensitivity.

SHP-1, also named PTPN6, PTP1C, HCP, HCPH, HPTP1C, SH-PTP1 (18), is an SH2 domain-containing protein tyrosine phosphatase (PTP) consisting of 17 exons and 16 introns that spans ~17 kb of DNA (19,20). SHP-1 shows high expression in normal hematopoietic cells and low expression in many hematological malignancies (21), including Burkitt lymphomas (22), natural killer cell lymphoma (23), diffuse large cell lymphoma, follicular lymphoma, mantle cell lymphoma, peripheral T-cell lymphoma, adult T-cell lymphoma and leukemia, plasmacytoma (24), and chronic myeloid leukemia (25). Thus, SHP-1 is traditionally regarded as a tumor suppressor. However, some studies have found that SHP-1 is highly expressed in some epithelial carcinoma cells, such as certain types of ovarian cell lines and breast cell lines (26). Despite considerable research into SHP-1 in hematological tumors, the functions of SHP-1 in solid tumors, especially in NPC, are poorly understood. Our previous investigation found that SHP-1 was highly expressed in NPC tissues in contrast to normal nasopharyngeal mucosa and was associated with local recurrence and metastasis after radiotherapy in NPC patients (27). Knocking down SHP-1 by siRNA in the NPC cell line CNE-2 and the non-small cell lung carcinoma (NSCLC) cell line A549 enhanced radiosensitivity, which was an outcome of cell cycle redistribution (28,29).

In this study, we used quinalizarin as a CK2 specific inhibitor and explored its impact on NPC cell radiosensitivity and we also investigated its influence on NPC cell proliferation, apoptosis, cell cycle distribution and DNA damage repair. In addition, we explored how quinalizarin affects the expression of SHP-1.

Materials and methods

Cell culture and irradiation procedure. Human nasopharyngeal carcinoma cell lines CNE-1 and CNE-2 were obtained from the Cell Bank of Sun Yat-sen University (Guangzhou, China). The nasopharyngeal carcinoma cell line 5-8F was obtained from the Cell Bank of The Second Xiangya Hospital of Central South University (Hunan, China). Cells were routinely cultured in RPMI-1640 (Hyclone, USA) medium supplemented with 12% fetal bovine serum (Hyclone), and 1% penicillin/streptomycin (HyClone). The cells were maintained at 37°C in a humidified incubator with 5% CO₂ and 95% air.

Irradiation was performed at room temperature with a single dose of X-rays ranging from 2 to 8 Gy using a linear accelerator (Primus K, Siemens, Munich, Bayern, Germany) with 6 MV photons/100 cm focus-surface distance with a dose rate of 2.0 Gy/min.

Establishment of radioresistant nasopharyngeal carcinoma cell sublines. Radioresistant NPC cells were established according to a previously published method (27). Exponentially growing CNE-1 and CNE-2 cells were irradiated with a dose of 6 Gy x 5. There was a 7-9-day break between the the doses. The surviving sublines (CNE-1R and CNE-2R clones) were then passaged for three months and their radiosensitivity was determined (Table I).

**MTT assay.** Cells (3,000-4,000 cells/well) were seeded into 96-well culture dishes, the next day they were treated with quinalizarin (Merck, Germany) dissolved in DMSO at 50 mM then diluted in RPMI-1640 medium at 0 (0.05% DMSO), 25, 50 and 100 µM. The plates were then incubated for 24, 48 and 72 h, respectively. MTT (20 µl) (Sigma, St. Louis, MO, USA) (5 mg/ml) was added to the wells and incubated in the dark for 4 h. The culture media was removed, and 150 µl DMSO added then the plates were slowly shaken for 15 min. The OD value at 490 nm test wavelength and 630 nm reference wavelength was measured with a microplate reader system (Bio-Tek, USA).

Cell viability rate = OD experimental group/OD control group.

Colony formation assay. Cells were seeded into 6-well culture dishes, at cell densities of 200, 300, 600, 1,500, and 4,000 cells/well, for the 0, 2, 4, 6 and 8 Gy experiments, respectively, but with the same cell density between 25 µM quinalizarin and 0.05% DMSO groups. The cells were treated with 25 µM quinalizarin or 0.05% DMSO the next day for 24 h and then irradiated at the different doses (0, 2, 4, 6, and 8 Gy) the radiotherapy time was based on the dose at 2 Gy/min. Plates were incubated for ~14 days, fixed with methanol, stained with Giemsa, and colonies containing ≥50 cells were counted as a clone. The multi-target click model was used to describe the survival fraction. SF = 1 - (1-e^{-D0N}) (SF, cell survival fraction; D, radiation dose; e, the bottom of the natural logarithm; D0, the mean death dose; N, extrapolate number) was used to fit cell survival curves. The sensitization enhancing ratio (SER) was calculated as a ratio of D0. N reflects the ability of cells to repair damage caused by radiation, if N increases, the dose required to kill the cells increases. Dq represents the quasi-threshold amount required for cell damage, as Dq increases the cell survival curve has a widened shoulder area and enhanced radiation resistance.

Immunofluorescent assay (IFA). Cells (5,000 cells/well) were treated with 25 µM quinalizarin or 0.05% DMSO for 24 h then irradiated with a dose of 2 Gy for 0.5, 3, 6 and 24 h. At specific times after IR, cells were harvested, and fixed in 4% paraformaldehyde for 15 min. After being washed with PBS three times, the cells were permeabilized with Triton X-100 for 15 min on ice. After being washed with PBS again three times, the cells were blocked with 5% BSA for 30 min at room temperature (RT), and then immunostained with anti-γ-H2AX (Abcam, Cambridge, UK) at 4°C overnight. After further washes with PBS three times, the cells were incubated with Dylight 488 labeled secondary antibody (EarthOx Life Sciences, Millbrae, CA, USA) for 1 h, then washed with PBS three times, the nucleus was stained with Hoechst 33258 (Wuhan Google Biological Technology Co., Ltd., China) for 15 min at RT. Photographs were captured by Olympus Laser scanning confocal microscopy (Olympus Optical Co., Tokyo, Honshu, Japan). For each treatment condition, γ-H2AX foci number per cell were counted in ≥50 cells under confocal microscopy with high magnification (x800) by two independent reviewers who were blinded to the grouping.
Flow cytometric analysis of the cell cycle. Cells treated with 25 µM quinalizarin or 0.05% DMSO for 24 h were then irradiated with 6 Gy X-rays. The cells were harvested 24 h after IR, fixed overnight with 70% ethanol, then resuspended in PBS containing 1 mg/ml RNase A and 50 µg/ml propidium iodide (Sigma). The cellular DNA content was determined on a flow cytometer FACScan (Becton-Dickinson, San Jose, CA, USA). Quantifications of cells in the G0/G1, S, G2/M phases were performed using CellQuest software (BD).

TUNEL analysis of apoptosis. Cells (5x10^5 cells/well) were seeded into 6-well plates, treated with 0.05% DMSO or 25 µM quinalizarin for 24 h and then irradiated with X-rays (6 Gy), and 24 h after IR, cells were fixed with 4% paraformaldehyde, treated with Triton X-100. The assay was performed according to the manufacturer's instructions of the TUNEL kit (Roche Applied Science, Shanghai, China). Apoptosis was observed by fluorescence microscopy (Olympus IX71, Olympus Optical Co., Tokyo, Japan). Cells with green fluorescence were regarded as apoptotic cells.

Table I. Parameters of radiosensitivity in the three cell lines treated with different concentrations of quinalizarin.

<table>
<thead>
<tr>
<th>Quinalizarin concentration</th>
<th>CNE-1</th>
<th>CNE-2</th>
<th>5-8F</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µM</td>
<td>N 1.33</td>
<td>1.63</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>SF2 0.47</td>
<td>0.53</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>D0 2.08</td>
<td>2.02</td>
<td>2.25</td>
</tr>
<tr>
<td></td>
<td>Dq 1.02</td>
<td>1.19</td>
<td>1.32</td>
</tr>
<tr>
<td>12.5 µM</td>
<td>N 1.24</td>
<td>2.07</td>
<td>1.65</td>
</tr>
<tr>
<td></td>
<td>SF2 0.44</td>
<td>0.56</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>D0 2.01</td>
<td>1.79</td>
<td>2.09</td>
</tr>
<tr>
<td></td>
<td>Dq 0.92</td>
<td>1.31</td>
<td>1.24</td>
</tr>
<tr>
<td>25 µM</td>
<td>N 1.41</td>
<td>1.19</td>
<td>1.23</td>
</tr>
<tr>
<td></td>
<td>SF2 0.34</td>
<td>0.31</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>D0 1.48</td>
<td>1.53</td>
<td>1.55</td>
</tr>
<tr>
<td></td>
<td>Dq 0.74</td>
<td>0.60</td>
<td>0.64</td>
</tr>
<tr>
<td>50 µM</td>
<td>N2.12</td>
<td>1.14</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>SF2 0.22</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>D0 0.91</td>
<td>0.88</td>
<td>1.06</td>
</tr>
<tr>
<td></td>
<td>Dq 0.66</td>
<td>0.38</td>
<td>0.18</td>
</tr>
<tr>
<td>100 µM</td>
<td>N 0.46</td>
<td>1.09</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>SF2 0.09</td>
<td>0.09</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>D0 1.19</td>
<td>0.79</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>Dq -0.59</td>
<td>-0.15</td>
<td>-0.53</td>
</tr>
</tbody>
</table>

Overexpression of SHP-1. CNE-2 cells were planted at 24-well plates, and incubated with 0.5 ml RPMI-1640 supplemented with 15% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin (HyClone). After 24-h incubation, 50 µl/well lentivirus-mediated SHP-1 overexpression (8.6x10^9 copies/ml) (GeneCopoeia Inc. Guangzhou, China) was added into the CNE-2 cells for 48 h. The stably transfected cells were selected using medium containing 2 µg/ml puromycin (supplemented with 10% heat-inactivated fetal bovine serum, penicillin/streptomycin) for 12 days. Positive clones were obtained and screened by RT-PCR for transfection efficiency. The medium containing puromycin was changed once every three days.

Quantitative real-time RT-PCR. Total RNA was extracted by TRIzol (Invitrogen, Carlsbad, NM, USA) and reverse transcription was used to obtain cDNA, according to the Takara RT-PCR kit manufacturer's instructions (Takara, Japan). Primer sequences were as follows: SHP-1: forward, 5’-ACCAT CATCCACCTCAAGTACC-3’; reverse, 5’-CGAGCAGACA AAGCACGCA-3’; β-actin: forward, 5’-GATGAGATTTGGGCA TGCTTT-3’; reverse, 5’-CACCTTCACGGTTTACAGTTT-3’.

Western blot analysis. Cells were harvested and lysed in RIPA buffer (Wuhan Google Biological Technology Co., Ltd.). Protein concentrations of the lysates were determined by the BCA protein assay system (Gow, Wuhan, China). Equal amounts of protein (40-80 µg) were separated by 12% SDS-PAGE, and transferred to PVDF membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% BSA, and then probed with either anti-SHP-1 (Epitomics, Burlingame, CA, USA), anti-phospho-NF-κB p65 (Cell Signaling Technology, USA), anti-GAPDH (Santa Cruz, Dallas, TX, USA) primary antibodies. After washing, the membrane was incubated with the appropriate horseradish peroxidase secondary antibody (Invitrogen) and visualized by chemiluminescence using a chemiluminescence kit (Invitrogen) and the specific bands were recorded on X-ray film. GAPDH protein levels were used as a control to verify equal protein loading.

EMS5. EMS5 was used to verify the gel-shift assay system using the EMSA kit (Thermo Fisher Scientific, Waltham, MA, USA). Probe sequences of SHP-1 promoter used in EMSA were as follows: forward, 5’-GGCCACGCTTGGCGG CTCC-3’; reverse, 5’-GGAAGCGCCAGGCGTGGCC-3’. To prepare DNA probes, double-stranded DNA probes were incubated at 95°C for 5 min, and slowly cooled down to room temperature. Concentration of probe was 0.5 µM. The
following reagents were added to the tubes and incubated at 37°C for 30 min: ultrapure water 25 µl, 5X TdT reaction buffer 10 µl, unlabeled oligo (1 µM) 5 µl, biotin-11-UTP (5 µM) 5 µl, diluted TdT (2 U/µl) 1U. Then 2.5 µl EDTA (0.2 M) was added to stop the reaction. The labeled DNA probe was collected. The nuclear extracts were prepared according to the manual of the EMSA kit. Then, the nuclear extracts were incubated with the p65 antibodies (Cell Signaling Technology) for 10 min at room temperature. The protein/DNA complexes were resolved on 4-6% polyacrylamide gels and transferred to the membrane (Millipore, Billerica, MA, USA). Subsequently, the complex was visualized by chemiluminescence using a chemiluminescence kit (Invitrogen).

Dual luciferase assay. p-GL3 basic was from Transgene (Beijing, China). Linear vector was constructed using KpnI (10 U/µl) and XhoI (10 U/µl) DNase. The SHP-1 promoter sequence was connected to the linear vectors. DNA plasmids were transiently transfected into NPC cells with 10 µl Lipo2000 (Invitrogen). Cells were harvested 60 h after transfection and lysed in 100 µl 1X cell culture by 513 reagent. Upon adjusting protein concentration, 20 µl of each lysate was assayed in replicates for luciferase activity according to the manufacturer's instructions (Promega, Madison, WI, USA).

SHP-1 activity assay. Cells were treated with DMSO or quinalizarin for 0, 12 and 24 h then total protein were extracted by RIPA lysis buffer containing 1% PMSF. Total protein was incubated with anti-SHP-1 primary antibody overnight at 4°C. Protein A-Sepharose (Merck) were added to each sample and then incubated for 3 h at 4°C with rotation. After immuno-precipitation reaction, the protein-antibody complex was separated from the protein A-Sepharose by centrifugation (4°C, 3,000 rpm, 3 min). Then the complex was resuspended with PBS. SHP-1 activity was determined by RediPlate 96 EnzChek Tyrosine Phosphatase Assay kit (R-22067, Molecular Probes, Invitrogen) according to the manufacturer's instructions.

Statistical analysis. SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Experimental data were expressed as mean ± standard deviation (SD) from at least three independent experiments. Differences in measured variables between experimental and control groups were assessed using t-test and ANOVA with least significant difference (LSD). The criterion for statistical significance was P<0.05.

Results

Quinalizarin (25 µM) enhances radiosensitivity but has little effect on NPC cell proliferation. To investigate the effect of quinalizarin on radiosensitivity of NPC cells, we performed colony formation assays. Fig. 1A indicates that irradiation killed the tumor cells exponentially in CNE-1, CNE-2 and 5-8F. All three NPC of the cell lines showed a lower survival fraction after being treated with quinalizarin at different IR doses. These effects were quinalizarin dose related. D0, D1, N, SF2 were also generally lower in the quinalizarin groups than that in the dimethyl sulfoxide (DMSO) groups, which represents higher sensitivity to IR. Although at some doses N was increased suggesting that the capacity of the cells for repair was increased. At 100 µM quinalizarin, D0 was negative, possibly because the drug alone was enough to kill the cells, and the role of radiotherapy was secondary. At 25 µM overall the enhancement ratio of quinalizarin groups were 1.41, 1.18 and 1.52 for CNE-1, CNE-2 and 5-8F, respectively.

We used different concentrations of quinalizarin to treat NPC cells for 24, 48 and 72 h, respectively. MTT assays were performed to test cell viability. As shown in Fig. 1B, quinalizarin inhibited cell viability in a concentration- and time-dependent manner. At 25 µM, the inhibitory rates of all the three cell lines were <20%, at 24 h (0.839±0.0587 for CNE-1, 0.924±0.0527 for CNE-2 and 0.894±0.0209 for 5-8F compared to the normalized value of 1 for controls). At 48 and 72 h, cell viability decreased sharply. When concentration was increased to 50 µM, the inhibitory efficiency of CNE-2 and 5-8F were ~20% while in CNE-1 was 50%. Consequently, we used 25 µM quinalizarin treatment for 24 h in the later experiments as this value enhanced radiosensitivity, but had little effect on cell proliferation.

Quinalizarin slows down DSB repair. DNA double-strands break (DSB) is one of the most important of DNA damage caused by irradiation and the ability to repair DSB is one of the important influencing factors for radiosensitivity. γ-H2AX foci are vital markers of DSB (1). To detect how quinalizarin influence DSB repair in NPC cells, we used immunofluorescent assays (IFA) to examine the formation of γ-H2AX foci in the DMSO and quinalizarin groups before and at different times after IR. Before IR, quinalizarin treated cells had more γ-H2AX foci than DMSO treated cells. Not surprisingly, 0.5, 3, 6 and 24 h after IR, quinalizarin treated cells still had more γ-H2AX foci than DMSO treated cells. The three cell lines CNE-1, CNE-2 and 5-8F all showed similar results and they were significantly different from the DMSO groups at all time-points (P<0.001, Fig. 2). The results suggested that quinalizarin slowed down the efficiency of DSB repair in NPC cells.

Quinalizarin increases the fraction of NPC cells in G2/M phase and apoptosis. One of the mechanisms that influences radiosensitivity is cell cycle distribution. It is well-known that cells are most sensitive to irradiation in G2/M phase. Thus, we used flow cytometry to detect the cell cycle distribution in the two groups before or after IR. In CNE-1, the cell fractions in G2/M phase and apoptosis.

To examine the effect of quinalizarin on apoptosis before and after IR, we used TUNEL assays to determine the apoptotic rate of NPC cells. Without irradiation by X-ray, the apoptosis rates of the DMSO group was almost equal to the quinalizarin group in both cell lines with rates of 6.6 and 6.7%, respectively,
in CNE-1, 7.6 and 8.3%, respectively, in CNE-2. After irradiation by X-ray, apoptosis rates between the two groups showed significant differences. The apoptosis rates of the quinalizarin group were 34% higher than the DMSO group in CNE-1 and 9% higher in CNE-2 (Fig. 3B). Based on these results, we concluded that quinalizarin promoted apoptosis in NPC cells after IR.

Quinalizarin suppresses SHP-1 expression in radioresistant NPC cells. Radioresistant populations of CNE-1 and CNE-2 cells (Fig. 4A and B) were treated with quinalizarin and this successfully increased their radiosensitivity (Fig. 4C and D). The levels of SHP-1 were then evaluated in the cells by western blot analyses, while the levels in the radioresistant populations were higher than the parent cells (Fig. 4E), the levels in the radioresistant cells that were then treated with quinalizarin were obviously decreased (Fig. 4F).

Overexpression of SHP-1 abolished the radiosensitive effect of quinalizarin. Therefore, we thought that the enhanced radiosensitivity acquired from quinalizarin may depend on its
downregulation of SHP-1. To verify our hypothesis, we used lentivirus to transfect CNE-2 and produced a cell line with high levels of SHP-1 expression (referred to as LP-H802Lv201) and a negative control which only contained the vector (referred to as LP-NegLv201) (Fig. 5A and B). We then performed colony formation assays to test the effect of quinalizarin on radiosensitivity of LP-NegLv201 and LP-H802Lv201. Survival curves showed that quinalizarin treated LP-H802Lv201 had a smaller shoulder area than DMSO treated LP-NegLv201, representing higher radiosensitivity. In LP-H802Lv201, however, being treated with quinalizarin did not display significant enhancement of radiosensitivity, compared with quinalizarin treated LP-NegLv201 (Fig. 5C). Results showed that overexpressing SHP-1 reversed the radiosensitive effect of quinalizarin. Thus, we can conclude, at least partially, that quinalizarin radiosensitized NPC cells by suppressing the expression of SHP-1.

Quinalizarin inhibits binding of p65 and the promoter of SHP-1 and decreases the activity of SHP-1 promoter. Western blot analysis showed that quinalizarin decreased SHP-1 expression, but not p65, a subunit of NF-κB; however, the level of phosphorylated p65 was decreased with quinalizarin (Fig. 6A). To verify whether quinalizarin affected the binding of p65 and SHP-1 promoter, the SHP-1 promoter probe was used for EMSA. The CNE-1 and CNE-2 nuclear proteins were extracted and an anti-p65 Ab was used to precipitate p65. Then the complex of p65 Ab and p65 was used for EMSA to detect its binding to SHP-1 promoter. We noted that treatment with quinalizarin obviously reduced the binding.
of p65 and SHP-1 promoter, both in CNE-1 and CNE-2 cells. However, the effect was not time-dependent (Fig. 6B).

Apart from the binding of p65 and SHP-1 promoter, we also used dual luciferase assay to examine how quinalizarin...
affected the activity of the SHP-1 promoter. As indicated in Fig. 6C, quinalizarin treated cells showed a significant decrease in SHP-1 promoter activity by 36 and 34% for CNE-1 and CNE-2, respectively.

Quinalizarin suppresses the activity of SHP-1 in NPC cells. Besides regulating the expression of SHP-1, we were also interested in the influence of quinalizarin on the phosphatase activity of SHP-1. We incubated the protein extract with anti-SHP-1 antibodies and use immunoprecipitation to gain purified SHP-1 protein. Then a RediPlate 96 EnzChek Tyrosine Phosphatase Assay kit (R-22067) was used to determine SHP-1 activity. As shown in Fig. 6D, basic activity was normalized to 1.0 (treated with quinalizarin or DMSO for 0 h). After treatment with quinalizarin for 12 h, the activity of SHP-1 showed approximately a 10% decrease compared to the DMSO groups. However, prolonging the treatment to 24 h did not cause further decrease in SHP-1 activity, thus, the inhibition was not time-dependent.

Discussion

The purpose of this study was to investigate whether using quinalizarin would increase the radiosensitivity of NPC cells. If this was the case then it may have potential as a method...
of improving radiation therapy of NPC. The results show that quinalizarin inhibited cell viability, and radiosensitized NPC cells. The mechanism of this induced radiosensitivity involved decreasing the efficiency of DSB repair, increasing the fraction of cells in G2/M phase and promoting apoptosis. Quinalizarin decreased SHP-1 expression and when SHP-1 was overexpressed the ability of quinalizarin to induce radiosensitivity was partially suppressed suggesting that reducing expression of SHP-1 is involved in the mechanism of quinalizarin induced radiosensitivity. Firstly, we used MTT assays to determine an appropriate drug concentration. At 25 µM, the drug itself did not show much cytotoxicity so that we could observe its radiosensitive effect. Then using colony formation assays, we found that quinalizarin enhanced NPC cells radiosensitivity. The enhanced radiosensitivity probably results from delayed DSB repair, increasing cell apoptosis, and larger fraction of cells being in G2/M phase. S phase has been shown to provide some degree of radioresistance and G0/G1 are relatively radioresensitive, but G2/M phase shows the most sensitivity to radiation (30,31). Similar results have been seen in other cancers. Kroonen et al concluded that inhibiting CK2 delayed DNA damage repair, but did not radiosensitize malignant glioma cells (32). Liu et al used an RNA interference technique to silence the CK2α gene in NPC cells and that resulted in higher radiosensitivity (33). Another study also found that CK2 inhibitors enhanced the radiosensitivity of human NSCLC cells by inhibiting stat3 activation (14).

Our previous investigation found that SHP-1 was highly expressed in NPC tissues in contrast to normal nasopharyngeal mucosa and was associated with local recurrence and metastasis after radiotherapy in NPC patients (27). Further research showed that SHP-1 overexpression increased the radioresistance of NPC cells by enhancing DSB repair, increasing S phase arrest and decreasing cell apoptosis (34). To assess whether quinalizarin has an influence on SHP-1 expression, we treated NPC cells with quinalizarin and detected the expression of SHP-1. Expression of SHP-1 was obviously inhibited by quinalizarin. The level of SHP-1 was apparently related to the radiosensitivity of the NPC cells (28,29). This encouraged us to try overexpression of SHP-1 and when this was done using lentivirus expression the radiosensitivity induced by quinalizarin was partially suppressed. Thus, it seems likely that quinalizarin radiosensitized NPC cells via a CK2-SHP-1 pathway, at least partially.

It is well known that NF-κB is a downstream target of CK2 and is an important transcriptional factor in many cells. The SHP-1 promoter contains a binding site of NF-κB (35), suggesting that expression of SHP-1 may be regulated by NF-κB. We treated NPC cells with quinalizarin and detected the phosphorylation level of p65, and found that quinalizarin inhibited p65 phosphorylation. Furthermore, EMSA and dual luciferase assay suggested that quinalizarin suppressed the activity of SHP-1 promoter by inhibiting p65 binding to SHP-1 promoter. In addition, we also discovered that quinalizarin directly decreased the phosphatase activity of SHP-1. These results further support the theory that CK2βp65-SHP-1 pathway may have a role in regulating radiosensitivity and cell cycle distribution in NPC cells (Fig. 7).

In this study, we firstly determined the radiosensitive effect of quinalizarin on NPC cells. This demonstrated that quinalizarin induced radiosensitivity on NPC cells, an important finding that may well prove to have therapeutic use in increasing the effectiveness of radiotherapy for NPC. We also discovered the relationship between CK2 and SHP-1 for the first time in this field. However, the details of the interaction between CK2 and SHP-1 need further study to fully elucidate the pathways involved. In conclusion, quinalizarin radiosensitized NPC cells through inhibiting SHP-1 expression.
Acknowledgements

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References


