**MYC Regulation of CHK1 and CHK2 Promotes Radioresistance in a Stem-Cell-Like Population of Nasopharyngeal Carcinoma Cells**

**Wen-Jun Wang\(^1\)**, **Si-Pei Wu\(^1\)**, **Jia-Bin Liu\(^2\)**, **Yong-Sheng Shi\(^3\)**, **Xue Huang\(^1\)**, **Qian-Bing Zhang\(^1\)**, and **Kai-Tai Yao\(^1\)**

**Abstract**

Radiotherapy is the most successful nonsurgical treatment for nasopharyngeal carcinoma (NPC). Despite this, the prognosis remains poor. Although NPCs initially respond well to a full course of radiation, recurrence is frequent. The cancer stem cell (CSC) hypothesis provides a framework for explaining the discrepancy between the response of NPC to therapy and the poor survival rate. In this study, a stem-cell-like subpopulation (PKH26\(^+\)) was identified in NPC cell lines using a label-retention technique. PKH26\(^+\) cells were enriched for clonogenicity, sphere formation, side-population cells, and resistance to radiotherapy. Using genomic approaches, we show that the proto-oncogene c-MYC (MYC) regulates radiotolerance through transcriptional activation of CHK1 (CHEK1) and CHK2 (CHEK2) checkpoint kinases through direct binding to the CHK1 and CHK2 promoters. Overexpression of c-MYC in the PKH26\(^+\) subpopulation leads to increased expression of CHK1 and CHK2 and subsequent activation of the DNA-damage-checkpoint response, resulting in radioresistance. Furthermore, loss of CHK1 and CHK2 expression reverses radioresistance in PKH26\(^+\) (c-MYC high expression) cells *in vitro* and *in vivo*. This study elucidates the role of the c-MYC-CHK1/CHK2 axis in regulating DNA-damage-checkpoint responses and stem cell characteristics in the PKH26\(^+\) subpopulation. Furthermore, these data reveal a potential therapeutic application in reversal of radioresistance through inhibition of the c-MYC-CHK1/CHK2 pathway. *Cancer Res; 73(3); 1219-31. ©2012 AACR.*

**Introduction**

Nasopharyngeal carcinoma (NPC) is a rare form of epithelial cancer that occurs in most parts of the world. However, its occurrence is particularly high in Southeast Asia and southern China, where its incidence rate is approximately 25 to 50 cases per 100,000 individuals, which is 25-fold higher than that in Western countries (1). Surgical approaches to treat NPC are limited by the inaccessibility of the anatomic location. However, NPC is sensitive to radiation and, therefore, treatments primarily rely on radiotherapy. About 30% of patients presenting with localized tumors develop recurrent disease, and 30% to 60% of patients with metastatic NPC die within 5 years of diagnosis (2).

In recent years, the concept of cancer stem cell (CSC) has been proposed, which is defined as a cell within a tumor that possesses the capacity to self-renew and to generate the heterogeneous lineages of cancer cells that comprise the tumor (3). This concept has been extensively investigated following the identification of CSCs in diverse cancers including breast, brain, lung, and liver (4–7). Analyses are based on exploitation of CSC surface markers, elevated levels of aldehyde dehydrogenase (ALDH1\(^+\)), and enhanced PKH26 dye-retaining capacity (8, 9). This has raised the prospect of human CSC isolation. Recent data suggest that CSCs are more resistant to chemo- and radiotherapy than non-stem cells (10). These key properties enable CSCs to initiate tumors and promote cancer progression and may account for the failure of current therapies to eradicate malignant tumors. A recent study showed that CSCs contribute to radioresistance through preferential activation of the CHK1 and CHK2 checkpoint response and an increase in DNA-repair capacity (11).

The c-MYC oncoprotein is a well-characterized transcription factor, and deregulation of c-MYC contributes to the genesis of most human tumors (12). As a result, c-MYC is considered to play an important role in carcinogenesis and tumor progression due to its influence on all basic cellular processes (13). MYC can activate cyclin E/Cdk2 in quiescent fibroblasts (14) by inhibiting the cdk2 inhibitor p27 (15). MYC was reported for cooperative actions of p53 and Pten in the regulation of normal and malignant stem/progenitor cell differentiation, self-renewal, and tumorigenic potential (16). Previous research has also indicated that c-MYC network
accounts for similarities between embryonic stem and cancer cell transcription programs (17).

The aim of this study is to determine the capacity of PKH26 dye retention to successfully identify slow-cycling PKH26+ cells (18). We determined that these cells were radioresistant. It has also been shown that c-MYC can directly regulate CHK1/2. Here, we explore the role of c-MYC in the radioresistance of nasopharyngeal carcinoma, and the molecular mechanisms of DNA-damage repair that are implicated in this process.

Materials and Methods

Cells and culture conditions

NPC cell lines (CNE1, CNE2, SUNE1, and HONE1) and a non-small cell carcinoma of the lung cancer cell line (A549) were available from the Cancer Institute of Southern Medical University (Guangzhou, China). The authenticity of cell lines in our study have verified with the DNA fingerprinting method. All cell lines used in this study were maintained in RPMI-1640 medium (Invitrogen) with 10% FBS (HyClone), 100 U/mL penicillin, 100 mg/mL streptomycin (Invitrogen), and incubated at 5% CO₂ at 37°C.

PKH26 labeling of cells and sorting of cell populations

CNE1 and CNE2 cells were labeled using PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich) before cell culture, according to the instructions provided by the manufacturer. PKH26 is a lipophilic marker that intercalates into the membranes of viable cells (18) and is not transferred between cells. PKH26 labeling of cells and sorting of cell populations in 70% ethanol, and stored at -80°C. PKH26+ cells were cultured for about 4 weeks. PKH26+ and PKH26− fractions were identified by fluorescence-activated cell sorting (FACS). Freshly PKH26-labeled cells and unlabeled cells were used as positive and negative controls, respectively. Cells were sorted for subsequent analyses. After sorting, PKH26+ cells were cultured in serum-free stem cell medium (MEBM; Clonetics division of Cambrex BioScience) to maintain stem cell characteristics. PKH26− and unsorted cells were cultured in RPMI-1640 medium.

Cell-cycle analysis by FACS

Flow cytometry was carried out as previously described (19). Briefly, cells were trypsinized, washed with PBS, resuspended in 70% ethanol, and stored at −20°C overnight. Cells were subsequently centrifuged, washed in PBS, resuspended in 450 μL PBS and 10 μL 10 μg/mL DNase-free RNase (Roche), and incubated at 37°C for 45 minutes. Following RNase treatment, 50 μL of propidium iodide (PI: Boehringer Mannheim Corp.) was added, and cells were incubated at room temperature for 10 minutes protected from light. Cell aggregates were removed by filtration before analysis. Cell-cycle analysis was carried out using the BD FACSDiva. The population of cells in each of the G₁, S, M, and G₂ phases was determined for at least 250,000 cells with doublerejection. Analysis of cell-cycle position was carried out using the BD FACSDiva software.

Tumor spheroid assay

Spheroid-forming assays were conducted as previously described (20). In brief, single cells were plated in 6-well ultralow attachment plates (Corning Inc.) at a density of 1,000 cells/mL. Tumor spheroids were generated in Dulbecco’s Modified Eagle’s Medium, DMEM+F12 supplemented with 1% N2 Supplement (Invitrogen), 2% B27 Supplement (Invitrogen), 20 ng/mL human platelet growth factor (Sigma-Aldrich), 100 ng/mL epidermal growth factor (Invitrogen), and 1% antibiotic–antimycotic (Invitrogen) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cultures were fed weekly and passage every 2 weeks. When passaged, tumorspheres were harvested. Spheroids were dissociated with Accutase (Innovative Cell Technologies, Inc.).

Antibodies, Western blotting, and immunofluorescence analysis

Western blot and immunofluorescence analyses were conducted according to standard protocols (21) using the following antibodies: mouse anti-human cyclin D1, cyclin A, and cyclin B (1:300; Santa Cruz Biotechnology), mouse anti-human ABCG2, CD-44, CHK1, CHK2 pCHK1, pCHK2 GAPDH (1:1,000; Cell Signaling), and γ-H2AX (1:1,000; Abcam). Peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) secondary antibody (1:500; Cell Signaling) was used as the secondary detection antibody. Quantification of blots was done using Image J software (National Institutes of Health). Chamber slides were analyzed using a Nikon Eclipse fluorescence microscope.

Xenograft experiments

Animal studies were conducted in strict accordance with the principles and procedures approved by the Committee on the Ethics of Animal Experiments of Southern Medical University. Nude mice (BALB/C nu/nu) were fed autoclaved water and laboratory rodent chow. Freshly sorted PKH26+ and PKH26− cells suspended in 200 μL PBS were inoculated into the flanks of 6- to 7-week-old nude female mice on the afternoon of the day of sorting. The mice were monitored twice weekly for palpable tumor formation and euthanized 4 weeks after transplantation to assess tumor formation. Tumors were measured using a Vernier caliper, weighed, and photographed. A portion of the subcutaneous (s.c.) tumor tissue was collected, fixed in 10% formaldehyde, and embedded in paraffin for hematoxylin & eosin (H&E) staining to assess tumor pathology.

To analyze the effect of ionizing radiation (IR) and si-c-MYC as combination therapy, 5 × 10⁵ PKH26+ derived from CNE2 cells were subcutaneously injected into athymic nude mice, and tumor volume was monitored every 5 days as calculated by the equation V (mm³) = (a × b²)/2, where “a” is the largest diameter and “b” is the perpendicular diameter. When the tumors reached a size of 70 mm³, mice were randomly distributed to 6 groups (4 mice per group) and treated with IR (8 Gy and 2 × 4 Gy), si-c-MYC, or the combination of IR and si-c-MYC. There were 4 cycles (days 10, 24, 38, and 52) of IR, and tumor volume was monitored at various times up to 55 days. In addition, cells obtained from the treated tumors were analyzed for the percentage of PKH26+ and CD44+ by flow cytometry.

Details of the patients and tissue samples, ethics statement, immunohistochemistry (IHC), c-MYCS prediction and chromatin immunoprecipitation (ChIP) assay, EdU labeling,
luciferase assay alkaline comet assay, quantitative real-time (qRT)-PCR, and clonogenic survival assay are presented in the Supplementary Material and Methods.

Statistical analysis

Unless stated otherwise, all experiments were conducted in triplicate. Data are expressed as the mean ± SD of at least 3 independent experiments. The significance of differences between mean values was determined using 2-way ANOVA. P less than 0.05 was considered significant.

Results

Cell sorting and PKH+ cell characterization

After labeling NPC cells with PKH26 and culturing for 30 days, we found that nondividing or slowly dividing cells remain brightly labeled with membrane intercalating dyes (Fig. 1A). We also found that these cells maintain adhesion and morphological integrity. After that, we used flow cytometry to isolate healthy PKH-negative (PKH−) and PHK-positive (PKH+) cells. Approximately 97.3% of NPC CNE1 cells and 94.7% of CNE2 cells were positive within the first week. However, at 2 and 4 weeks, respectively, there was a reduction in the number of PKH26+ cells (38.6% and 2.2% in CNE1; 47.9% and 2.8% in CNE2; Fig. 1B; Supplementary Fig. S1B). The other human NPC cell lines, SUNE-1 and HONE-1, also contained a small subpopulation of PKH+ cells, and, after 4 weeks of labeling, they displayed a positive staining rate of 2.5% and 1.9%, respectively (data not show). We sorted the top 2% from the total cell population in CNE1 and CNE2 cells after labeling with PKH26 for 4 weeks. Then, we used FACS to determine cell-cycle distribution. PKH26 dye has been previously used to trace cell divisions and identify slow-cycling populations, such as long-term culture-initiating cells (22). As seen in Fig. 1C, PKH+ cells showed higher fractions of cells in the G1 phase and lower fractions of cells in S and G2–M phase. We next examined the expression of cell cycle-related proteins (Fig. 1D).

PKH26+ cells are enriched for the stem cell-like subpopulation and resistant to radiotherapy

The in vivo DNA-binding dye Hoechst 33342 was used to stain PKH26+ and PKH26− NPC cells. Simultaneous detection of the wavelengths of red and blue fluorescent dyes allowed selection of the 2 groups of specific subpopulations: the main group of highly fluorescent cells (main population, MP) and a low fluorescence side-population of cells (side-population, SP; ref. 23). SP cells were sorted on the basis of minimal nuclear staining by the fluorescent dye Hoechst 33342 due to cellular efflux (23). The proportion of SP cells was analyzed in CNE1 and CNE2 cells. Verapamil blocks efflux of Hoechst 33342 from SP cells and was used for detection of these cells. The data show that, in sorted CNE1 cells, the PKH26+ population contained 32.1 ± 5.2% SP cells, whereas the PKH26− population contained 0.5 ± 0.3% SP cells. Using the same methods, we sorted

Figure 1. NPC cells were incubated and sorted with the PKH26 reagent. A, image of labeled CNE2 cells after overnight and 30 days of culture under transmitted light and fluorescent light (magnification, ×20). B, purity of the freshly sorted PKH26+ cell populations was analyzed by FACS staining of PKH26+ cells cultured for approximately 4 weeks and used for subsequent experiments. (Data represent the mean ± SD; n = 3). C, PKH26− and PKH26+ cells were analyzed by FACS to determine the percentage of cells in the indicated cell-cycle phase. Data are based on 3 independent experiments conducted in duplicate. Error bars represent SD from the mean. D, Western blot showing reduced cyclin D1 and cyclin A in PKH26− cells and increased cyclin B in PKH26+ cells derived from NPC cell lines. Glyceraldehyde-3-phosphate dehydrogenase served as a loading control.
CNE2 cells and found that the PKH26+ population contained 37.1 ± 7.1% SP cells, and the PKH26− population contained only 0.7 ± 0.5% SP cells (Fig. 2A). Recent studies have reported that SP cells within NPC cells exhibit CSC characteristics (24). High expression of the adenosine triphosphate-binding cassette superfamily G member-2 transport protein (ABCG-2) has been closely related with SP cell phenotype (25). Western blotting analysis of representative stem cell markers, ABCG2 and CD44, revealed expression of these proteins in all PKH26− and PKH26+ cells (Fig. 2B). CD44 has also been associated with

Figure 2. PKH26+ cells represent an enriched cancer stem cell-like subpopulation and correlate with radioresistance. A, sorting by flow cytometry and detection of PKH− and PKH+ cells containing SP cells. The percentages of SP cells following preincubation of CNE1 and CNE2 cells with verapamil to block the ATP transporter are indicated. (Data represent the mean ± SD; n = 3; *, P = 0.000; ***, P = 0.000). B, PKH26− and PKH26+ cell lysates were analyzed by Western blotting with anti-ABCG-2 and anti-CD44. GAPDH served as a loading control. C, colony-formation assay for PKH26+ and PKH26− cells using irradiated (8 Gy). These cells formed fewer colonies (right) compared with the untreated control cells. D, dose-survival curves of irradiated PKH26− and PKH26+ cells (range, 0–10 Gy). E, representative images of irradiated (8 Gy) PKH26− and PKH26+ cells were seeded in tumorsphere culture medium at a density of 1,000 cells/well for 7 days. Data shown represent the average sphere count from a representative experiment conducted in triplicate wells. Data represent the mean ± SD; n = 3; *, P < 0.05; ***, P < 0.05. F, The indicated amounts of PKH− and PKH+ cells from CNE2 cells were transplanted into 21-day-old nude mice. G, immunohistochemical detection of Ki-67 expression in PKH+ and PKH− cells.
cancer stem cell characteristics and has been specifically used as a marker for NPC stem cells (26). In order to verify that the PKH26+ cells also expressed CD44, we used flow cytometry and found that 85 ± 3.4% and 93 ± 1.4% of CNE1 and CNE2 PKH26+ cells, respectively, contained PKH26/CD44 double-positive cells (Supplementary Fig. S1A). Clonogenic survival has been defined as a characteristic of radiation-induced cell proliferation and death. Therefore, the cloning properties of PKH26+ and PKH26− were evaluated. PKH26+ and PKH26− subpopulations of CNE1 and CNE2 cells were harvested and cultured for 2 weeks. PKH26+ cells gave a rise to significantly more colonies than PKH26− cells (Fig. 2C). Clonogenic survival of PKH26+ and PKH26− cells was determined after increasing doses of irradiation (P < 0.001; Fig. 2D). The association of enhanced self-renewal properties with CSC properties was then investigated in tumor spheroid assays using single-cell suspensions derived from sorted PKH26+ and PKH26− cells. PKH26+ cells formed spheres that were significantly greater in number and larger than those formed by PKH26− cells (Fig. 2E). PKH26+ cells sorted from NPC cell line CNE2 and NSCLC cell line A549 were tested for the formation of xenografts in nude mice. We found that the PKH26+ cells grew faster (Fig. 2F; Supplementary Fig. S4A and B). In addition, IHC of Ki-67 detected increased expression in PKH26+ cells in our xenograft model (Fig. 2G).

**PKH26+ cells promote radioresistance through c-MYC overexpression**

DNA-damage-checkpoint responses play essential roles in cellular radiosensitivity (27–30). To determine the role of DNA-damage-checkpoint responses in PKH26+ subpopulation, we compared the expression of early DNA-damage-checkpoint regulators in PKH26+ and PKH26− NPC subpopulations. We irradiated (8 Gy) PKH26− and PKH26+ cells and measured protein expression of c-MYC, CHK1, pCHK1, CHK2, and pCHK2 (Fig. 3A). Irradiation increased levels of CHK1 and CHK2 phosphorylation in both PKH26− and PKH26+ cells. However, the activating phosphorylation of these checkpoint proteins was significantly higher in PKH26+ cells than in PKH26− cells, indicating that PKH26+ cells show greater checkpoint activation in response to DNA damage. In addition, we also found that PKH26+ cells expressed increased c-MYC compared with PKH26− cells. Immunofluorescence staining revealed that c-MYC, pCHK1, and pCHK2 were also localized to the nucleus in PKH26+ cells (Fig. 3B). As mentioned above, c-MYC is highly expressed in PKH26− NPC cells. Thus, we hypothesize that c-MYC is involved in DNA-damage responses in NPC. To elucidate the relationship between c-MYC and the DNA-damage response, we constructed a lentiviral vector of c-MYC to overexpress the transcription factor in the NPC cell lines CNE1 and CNE2. We found that expression levels of DNA-damage-related protein γ-H2AX in the c-MYC overexpressing group is greatly reduced Fig. 3C. Using comet assays, we found that the percentage of cells displaying DNA damage decreased by approximately 2.8-fold in both CNE1 and CNE2 cells overexpressing c-MYC (Fig. 3D). EDU assay showed that following irradiation, cells overexpressing c-MYC showed improved proliferative capacity (Fig. 3E). This result suggests that c-MYC overexpression can significantly reduce DNA damage and improve proliferative capacity in CNE1 and CNE2 cells after IR.

**c-MYC targets CHK1 and CHK2**

Experiments indicate that c-MYC may rapidly respond to IR in PKH26+ cells to allow increased DNA repair. In order to find whether c-MYC directly regulates CHK1 and CHK2, we used the Gene Regulation website and Patch software (http://www.biobase-international.com/gene-regulation) to predict c-MYC binding sites within the promoter region of CHK1 and CHK2. According to the Patch prediction, we found 3 potential c-MYC binding sites (c-MYCBSs) within the 2,000 bp promoter upstream of the CHK1 and CHK2 genes, respectively (Fig. 4A). To validate direct association of c-MYC with CHK1 and CHK2 promoters, we conducted ChIP analysis in NPC cells for all the putative c-MYCBSs (A–F) using a c-MYC antibody. The ChIP results suggest that c-MYC is most significantly bound to sites C and E within the CHK1 and CHK2 promoters (Fig. 4B). Knockdown of c-MYC diminished the amount of DNA c-MYCBSs C and E that could be immunoprecipitated with the c-MYC antibody (Fig. 4C), suggesting that c-MYC directly associates with these promoter regions. Next, we constructed a luciferase reporter gene vector containing CHK1 c-MYCBSs C and CHK2 c-MYCBSs E to verify the predicted results. After being overexpressed in NPC cells, c-MYC increases luciferase expression from the promoter reporter. To illustrate interaction between c-MYC and c-MYCBSs C and E from CHK1 and CHK2 promoters, we mutated the c-MYCBSs C and E in CHK1 and CHK2 luciferase reporter gene vectors. The results showed that mutated c-MYCBSs did not display increased luciferase activity after c-MYC overexpression, thus, confirming that c-MYC acts through CHK1 c-MYCBSs C and CHK2 c-MYCBSs E (Fig. 4D). We also examined CHK1 and CHK2 protein and mRNA levels. The results showed that, when overexpressed, c-MYC causes increased expression of CHK1 and CHK2 at both the protein and mRNA levels (Fig. 4E and F). Together, these results suggest that c-MYC targets CHK1 and CHK2 directly by interaction with their promoter regions via sites c-MYCBSs C and E.

**PKH26+ subpopulation promotes DNA-repair capacity and stemness by activation of c-MYC expression**

Previous experiments have confirmed the following: (i) high expression of c-MYC is consistent with CHK1 and CHK2 in PKH26+ cells; (ii) PKH26+ cells display greater DNA-damage-repair capacity and radiation resistance; and (iii) c-MYC overexpression significantly improves DNA-damage repair after IR and increases stemness in NPC CNE1 and CNE2 cells. We hypothesize that c-MYC is also an essential factor in PKH26+ cells to maintain their cancer stemness and DNA-damage-repair capacity following IR. To investigate the role of c-MYC in the DNA-damage response and cancer stemness of PKH26+ subpopulation cells, we used lentivirus-expressing short hairpin RNA (shRNA) specific to c-MYC (sh-c-MYC1 and sh-c-MYC2; ref. 31), which constitutively expresses shRNAs that specifically silence c-MYC expression (Fig. 5A). Depletion
of c-MYC levels in PKH26+ cells resulted in a decreased proportion of the stem cell-like side populations (SP) in CNE1 PKH26+ cells (from 32.8 ± 6.5% to 2.4 ± 1.2%) and in CNE2 PKH26+ cells (from 35.6 ± 7.1% to 2.0 ± 3.3%; Fig. 5B). To further confirm our hypothesis, we sorted PKH26+ cells from CNE1 and CNE2 cell lines for tumor sphere-formation assays. Inhibition of c-MYC by sh-c-MYC reduced the tumor-sphere-formation proportion in CNE1 PKH26+ cells (from 128/1,000 to 42/1,000 cells) and CNE2 PKH26+ cells (from 122/1,000 to 38/1,000 cells; Fig. 5C). To examine the effect of c-MYC in IR-induced DNA damage of PKH26+ subpopulation cells in nasopharyngeal carcinoma, we conducted alkaline comet assays. The percentage of cells with comet tails increased 3.1 to 3.7 times more rapidly in vector control-treated PKH26+ cells than in sh-c-MYC-treated PKH26+ cells in the NPC cell lines (Fig. 5D). Moreover, EdU assays revealed that c-MYC inhibition reduces the proliferative capacity of PKH26+ cells after irradiation (Fig. 5E). Inhibition of c-MYC in PKH26+ cells also decreases expression of CHK1 and CHK2 (Supplementary Fig. S3B), and increases the expression of DNA-damage-related factor γ-H2AX after IR (Fig. 5F). Taken together, c-MYC plays an important role in PKH26+ cells to...

Figure 3. Reduced c-Myc expression in NPC cells after irradiation-induced DNA damage. A, Western blot detection of c-MYC, pCHK1, CHK1, pCHK2, and CHK2 in PKH26+ and PKH26− cells sorted from CNE1 and CNE2 cells assessed 24 hours after irradiation (8 Gy). B, Immunofluorescence staining reveals expression of pCHK1, pCHK2, and c-MYC in irradiated (8 Gy) CNE1 and CNE2 cells compared with untreated controls (magnification, ×200). C, overexpression of c-MYC by transient transfection in CNE1 and CNE2 lines. c-MYC and γ-H2AX were analyzed by Western blotting. D, top, the presence of DNA damage was assessed in irradiated (8 Gy) CNE1 and CNE2 cells by single-cell gel electrophoresis assays under alkaline conditions (alkaline comet assay); bottom, quantification of the percentages of CNE1 and CNE2 cells with comet tails following irradiation at different doses. E, EdU incorporation by CNE1 and CNE2 cells after 8-Gy IR treatment for 24 hours. Chart shows EdU cell staining compared with Hoechst 33342 stained nuclei (±SE). A minimum of 500 cells was counted for each treatment (magnification, ×100, with a 20 ms exposure time for image acquisition).
c-MYC mediates DNA-damage response and radioresistance in PKH26+ cells through modulation of CHK1/2

To further understand how c-MYC regulates the DNA-damage response by modulating CHK1/2, we inhibited CHK1/2 expression in PKH26+ cells with shRNA. Although depletion of CHK1/2 does not affect expression of c-MYC (Fig. 6A; Supplementary Fig. S2A), it does impair DNA repair in c-MYC overexpressing PKH26+ cells after IR. The percentage of cells with comet tails increased 3 to 4.5 times more rapidly in vector control PKH26+ cells than in CHK1/2 shRNA-infected PKH26+ and PKH26− cells (Fig. 6B). To test whether the survival of PKH26+ cells is influenced by c-MYC after IR, we compared cell survival in vector control and sh-c-MYC lentivirus-infected PKH26+ cells at different doses of IR. We found that inhibition of c-MYC reverses the IR resistance in PKH26+ (Fig. 6C). To further identify c-MYC-mediated PKH26+ subpopulations that contribute to NPC radioresistance, we studied the radiosensitivity of PKH26− and PKH26+ tumor cell subpopulations. Colony-formation assays confirmed that PKH26− cells were more resistant to IR treatment than PKH26+ cells. In addition, inhibition of c-MYC resulted in sensitization of PKH26− and PKH26+ cells to IR (Fig. 6D).

To further confirm that c-MYC regulates the DNA-damage response through CHK1/2 in NPC, we compared survival of c-MYC knockdown cell with the cotreated (sh-c-MYC+ CHK1/2 overexpression) cells after IR using colony-formation assays (Fig. 6E). Significantly, CHK1, CHK2, and the DNA damage marker histone variant H2AX (γH2AX; ref. 32) are dramatically induced by IR in the MYC knockdown PKH26+ cells, but not in the control knockdown group or in the cotreated (sh-c-MYC+ CHK1/2 overexpression) PKH26+ cells (Fig. 6F). Moreover, CHK1/2 could reverse the effect of c-MYC in radioresistance in PKH26− cells (Supplementary Fig. S2B and C). This result implies that c-MYC-mediated DNA repair depends on activation of CHK1/2. Together, our results show that the c-MYC-CHK1/2 pathway plays an important role in mediating resistance of the PKH26+ subpopulation to IR.

c-MYC-CHK1/2 pathway is important for PKH26+ cell formation and maintenance of radioresistance in vivo

To verify the role of the c-MYC-CHK1/2 pathway for PKH26+ cell function in vivo, we conducted xenograft experiments in which PKH26+ subpopulations derived from NPC cells were subcutaneously injected into nude mice. As expected, different IR treatments caused significant regression of the tumor, but relapse of the disease occurred after approximately 30 days. Treatment with si-c-MYC was slightly more effective than IR in inhibiting tumor growth, presumably
because these treatments did not affect the PKH26+ cells. Furthermore, combination of IR with si-c-MYC caused even stronger regression of tumor growth, and relapse was prevented (Fig. 7A and B; Supplementary Fig. S5A). Thus, these observations indicate that c-MYC is important for PKH26+ cell formation and radioresistance in vivo.

To determine the basis for why combinatorial therapy of IR with si-c-MYC was more effective than IR alone, we examined the populations of cells from tumors. After treatment, the PKH26+ and CD44+ population were nearly absent from mice subjected to combinatorial therapy, while they were easily observed in tumors from mice treated with IR alone (Fig. 7C). Furthermore, tumors treated with IR and si-c-MYC showed reduced levels of c-MYC, CHK1/2, and pCHK1/2 as compared with untreated tumors (Fig. 7D). We also analyzed Ki-67- and BrdU-positive cells decreased in the tissues from the combination group of IR with si-c-MYC (Supplementary Fig. 5B).

To further verify the pathological correlation among c-MYC, CHK1/2, and CD44 in nasopharyngeal carcinoma, we conducted a correlation analysis of c-MYC, CHK1/2, and CD44 protein expression levels using IHC, respectively, in primary nasopharyngeal carcinoma tissue consisting of 62 primary nasopharyngeal carcinoma samples (Fig. 7E and F). Consistently, high expression of c-MYC positively correlated with increased CHK1/2 and CD44 expression levels (Fig. 7E). The data suggest that expression of c-MYC could lead to upregulation of CHK1/2 and activation of stemness signature that contribute to nasopharyngeal carcinoma progression, and the c-MYC-CHK1/2 pathway is essential to maintain the formation and radioresistance of PKH26+ cells.

Figure 5. c-MYC expression in PKH26+ cells. A, CNE1 and CNE2 cells after treated with empty vector and 2 different shRNAs targeting c-MYC were lysed and analyzed by Western blotting with anti-c-MYC. GAPDH served as a loading control. B, flow cytometric profiles of SP cells within the PKH26+ and PKH26+ CNE2 cell populations after treatment of c-MYC or sh-c-MYC. The percentages of SP cells are indicated. Data represent the mean ± SD; n = 3. C, images showing tumor sphere formation in PKH26+ and PKH26+ NPC cells expressing the indicated constructs (magnification, ×20). Columns in chart show number of spheres counted in 10 fields for each group; *, P < 0.05. D, alkaline comet assay of vector control and c-MYC-infected irradiated (8 Gy) PKH26+ and PKH26+ NPC CNE2 cells. Quantification of the percentages of cells with comet tails following irradiation at different doses. E, EdU incorporation by irradiated (8 Gy) PKH26+ and PKH26+ NPC cells at 24 hours. Columns in chart show EdU cell staining compared with Hoechst 33342-stained nuclei (Data represent the mean ± SD; n = 3). (magnification, ×40, with a 20 ms exposure time for image acquisition). F, Western blot detection of γ-H2AX in whole cell lysates of irradiated (8 Gy) NPC cells collected after 24 hours.
Radiotherapy is the most commonly applied treatment for NPC, but tumor recurrence is essentially universal due to marked radioresistance. Cancer stem cells play an important role in radioresistance and tumor repopulation through preferential checkpoint response and DNA repair. Thus, the targeting of the checkpoint response in cancer stem cells may overcome tumor radioresistance. PKH26 is a fluorescent membrane dye used in combination with other cell surface antibodies for flow cytometric sorting of stem cells (32, 33). PKH26 label retention/quenching characteristics were used in a recent study by Dembinski and colleagues to identify a subpopulation of stem cell-like, slow-cycling tumor cells in pancreatic adenocarcinoma (34). These cells were resistant to chemotherapy and exhibited the highest self-renewal potential (35). In the present study, we have...
discovered and identified a rare population of PKH26+ cells in the NPC cell population that exhibit a delay of cell-cycle progression and are enriched for cancer stem cells that are resistant to radiation in comparison with matched PKH26− cells in vitro and in vivo.

IR leads to cell death by production of irreparable DNA double-strand breaks (DSB; ref. 37). A hallmark of DNA DSB recognition and repair is histone H2AX phosphorylation (γ-H2AX), a marker of DNA damage (38). Cells commonly respond to DNA-damaging agents by activating cell-cycle checkpoints, and cell-cycle regulation is, perhaps, the most important determinant of IR sensitivity. Our studies show that overexpression of c-MYC in NPCs reduces the expression of γ-H2AX, which may indicate that c-MYC prevents DNA damage by IR in NPCs. In addition, the current report shows that nonirradiated CSCs are predominantly in the G0 phase of the cell cycle, while radiation mobilizes CSCs from a quiescent to a proliferative state (G1 phase; refs. 18, 39). Ataxia telangiectasia mutated (ATM) is the most proximal signal transducer initiating cell-cycle changes after DNA damage induced by IR (40; Supplementary Fig. S1C). Studies in glioblastoma have shown that CD133+ glioma cells promote radioresistance by activation of the CHK1 and CHK2 checkpoint kinases, thus contributing to DNA-damage repair (11). In our study, after PKH26+ cells are exposed to radiation, DNA damage activates the phosphorylation of CHK1/CHK2. Thus, CHK1 and CHK2 also play an important role in DNA-damage repair in PKH26+ in NPC.

c-MYC is a proto-oncogene that encodes a transcription factor that regulates cell proliferation, growth, and apoptosis.
subpopulation directs positive regulation of CHK1/2, leading to increased DNA-damage-repair capacity in these cells. Moreover, an increased proportion of SP cells and tumor-sphere formation capacity were observed in NPC cell lines expressing CD44, a CSC marker. It can be speculated that overexpression of c-MYC not only increases the stemness of these cells, but also increases the DNA-repair capacity through overexpression of CHK1/2, thereby increasing the radiation resistance of tumor cells. This suggests that the c-MYC-CHK1/2 pathway is a strong causal factor in DNA repair and a driving force in maintaining the stemness in PKH26+ cells. Possibly, targeting the c-MYC-CHK1/2 pathway in PKH26+ cancer cells can overcome NPC radiosensitivity in vitro and in vivo, which may provide a therapeutic advantage to reduce recurrence.

The findings presented here have allowed us to reach a number of important conclusions. First, we have identified a rare population of PKH26+ cells in the NPC cell population that have many stem cell properties, including cell-cycle arrest, unlimited proliferation potential, self-renewal, resistance to radiation, and strong tumor formation ability in vivo. These findings help support the cancer stem cell hypothesis and tumor maintenance after therapy. Second, we show that PKH26+ cancer cells contribute to radiosensitivity and tumor repopulation through preferential DNA-damage response. Third, we identified that c-MYC binds the CHK1/2 promoters to regulate the DNA-damage checkpoint response and radioresistance of NPCs. Moreover, inhibition of c-MYC or CHK1/2 could overcome NPC radiosensitivity in vitro and in vivo. Thus, therapies targeting the c-MYC-CHK1/2 pathway in preclinical and clinical development may provide novel therapeutics to improve the treatment outcome of radiation treatment in nasopharyngeal carcinoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: K. Yao, W. Wang
Development of methodology: W. Wang, S-P. Wu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Yao, W. Wang, S-P. Wu, J. Liu, Y. Shi, X. Huang, Q. Zhang
Analysis and interpretation of data (e.g., statistical analysis, biosstatistics, computational analysis): W. Wang, S-P. Wu
Writing, review, and/or revision of the manuscript: W. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Yao, J. Liu, Y. Shi
Study supervision: K. Yao, W. Wang

Acknowledgments
The authors thank Prof. Gao from The First Affiliated Hospital of Zhengzhou University for providing human nasopharyngeal carcinoma specimens.

Grant Support
This work was financially supported by a grant from Key Project of Joint Fund of Natural Science Foundation of China and Guangdong Province (grant no. 1060006).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 15, 2012; revised November 2, 2012; accepted November 12, 2012. Published OnlineFirst December 26, 2012.
References


MYC Regulation of CHK1 and CHK2 Promotes Radioresistance in a Stem Cell-like Population of Nasopharyngeal Carcinoma Cells

Wen-Jun Wang, Si-Pei Wu, Jia-Bin Liu, et al.


Updated version
Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-12-1408

Supplementary Material
Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2012/12/20/0008-5472.CAN-12-1408.DC1

Cited articles
This article cites 47 articles, 11 of which you can access for free at: http://cancerres.aacrjournals.org/content/73/3/1219.full.html#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at: /content/73/3/1219.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.