Ionizing radiation promotes advanced malignant traits in nasopharyngeal carcinoma via activation of epithelial-mesenchymal transition and the cancer stem cell phenotype

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Abstract. Post-irradiation residual mass and recurrence always suggest a worse prognosis for nasopharyngeal carcinoma (NPC). Our study aimed to investigate the malignant behaviors of post-irradiation residual NPC cells, to identify the potential underlying mechanisms and to search for appropriate bio-targets to overcome this malignancy. Two NPC cell lines were firstly exposed to 60 Gy irradiation, and residual cells were collected. In our previous study, colony formation assay detected the radioresistance of these cells. Here, the CCK-8 assay examined the cell sensitivity to paclitaxel and cisplatin. Wound-healing and Transwell assays were performed to investigate cell motility and invasion capabilities. Inverted phase-contrast microscopy was used to observe and photograph the morphology of cells. Expression levels of epithelial-mesenchymal transition (EMT)-related proteins were detected by western blot assay in NPC cells and tissues. The mRNA levels of cancer stem cell (CSC)-related genes were detected via qRT-PCR. The results revealed that residual NPC cells exhibited enhanced radioresistance and cross-resistance to paclitaxel and cisplatin. Higher capacities of invasion and migration were also observed. An elongated morphology with pseudopodia formation and broadening in the intercellular space was observed in the residual cells. Downregulation of E-cadherin and upregulation of vimentin were detected in the residual NPC cells and tissues. CSC-related Lgr5 and c-myc were significantly upregulated in the CNE-2-Rs and 6-10B-Rs radioresistance cells. Higher proportions of Lgr5+ cells were observed in radioresistant cells via immunofluorescent staining and flow cytometry. In conclusion, our study demonstrated that residual NPC cells had an advanced malignant transition and presented with both EMT and a CSC phenotype. This provides a possible clue and treatment strategy for advanced and residual NPC.

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Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common head and neck malignancies in Southeast Asia and Southern China (1). Radiotherapy is a routine treatment for NPC patients (2). With more accurate intensity-modulated radiation therapy and adjuvant chemotherapy, the 5-year survival of NPC patients has reached more than 60% (3). Clinically, post-treatment recurrence and residual mass are still obstacles to successful treatment in a small population of NPC cases. NPC patients with residue are always considered to be refractory to salvage irradiation and chemotherapy and present with higher local or distant metastasis, resulting in a worse prognosis (4). However, the underlying mechanisms and malignant behaviors of residual NPC remains unclear.

Several situations restrict the study of these patients. In routine clinical practice, recurrence and residual NPC tissues are difficult to obtain for the reason that surgery is not the first line therapeutic choice for these NPC patients. Meanwhile, the residues are usually located in the deeper para-pharyngeal space covered with fibrotic scar and necrotic tissue after full course radiotherapy, which also restrict successful biopsy. Thus, we established post-irradiation residual NPC cells by exposure to irradiation in our previous study, which, to some extent, imitate the patients with a residual mass after a full course of radiotherapy (5,6). Using these cells, we detected

their malignant alteration in regards to radioresistance, chemoresistance, motility and invasion capabilities.

Epithelial-mesenchymal transition (EMT) is a process by which cells change their original epithelial morphology and are dispersed with the disappearance of inter-cellular connections, and change into long fibroblast-like cells (7). It is well known that EMT gifts tumor cells with more powerful metastatic potency (7). Recently, compelling evidence indicates that EMT transition in tumor cells also contributes to other malignant behaviors, such as chemoresistance and radioresistance (8,9). On the other hand, cancer stem cells (CSCs) are also considered as another crucial mechanism for the tumor to survive in extreme environments, such as irradiation (10) or chemotherapeutic agents (11). These intra-tumoral cells are generated and are maintained in a small proportion, but have the ability to self-renew and to differentiate in multi-directions which include EMT (10). Thus, we simultaneously focused on cellular morphological changes, detected EMT and CSC biomarker levels, and aimed to identify the underlying mechanisms to reverse the occurrence and residues in NPC.

Materials and methods

Cell culture. The poorly differentiated NPC cell lines CNE-2 and 6-10B were provided by the Cell Center of Central South University (Changsha, China). The radioresistant NPC cells derived from the CNE-2 and 6-10B cells were established as previously described (5) and were termed CNE-2-Rs and 6-10B-Rs, respectively. Cells were cultured in RPMI-1640 medium (Hyclone, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (all from Gibco, MA, USA). Cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37˚C. Cell morphology was monitored with an inverted phase-contrast microscope (Leica, Wetzlar, Germany).

Patients and tissue preparation. Three coupled NPC tissues before/post-radiotherapy were obtained from October 2011 to October 2014 at the Department of Otolaryngology Head and Neck Surgery, Xiangya Hospital of Central South University (Changsha, China). None of the patients had a previous malignancy and did not undergo chemo/radiotherapy. All specimens were snap-frozen immediately and stored in liquid nitrogen for further protein extraction. The present study was approved by the Ethics Committee of Xiangya Hospital of Central South University. Prior patient consent was obtained from all the patients.

Cell viability assay. Cell viability was assessed by the CCK-8 kit (Beyotime, Shanghai, China) as described previously (12). Briefly, the cells were seeded in 96-well plates at 5,000/well and allowed to adhere to the plate overnight. The cells were then exposed to different concentrations of paclitaxel or cisplatin (both from Sigma, USA) for another 48 h. Absorbance values were expressed as percentages relative to the controls. Resistant index was calculated by IC_{50} (resistant cells)/IC_{50} (parental cells) where IC_{50} is the half maximal inhibitory concentration. Each experiment was performed in triplicate.

Wound-healing assay. Wound-healing assay was performed as previously described (13). The cells were seeded in 6-well plates at $2x10^5$/well and allowed to grow to almost confluency. The cells were washed twice with PBS and incubated in serum-free medium for another 24 h. The cell monolayer was wounded with a sterile 10-μl pipette tip. Then the cells were cultured in medium supplemented with 2% FBS for another 48 h. Cell migration was calculated by measuring the distance covered by migrating cells and further divided by that of the original wound. The experiment was carried out in triplicate.

Invasion assay. The invasion assay was also described previously (13). In brief, chambers coated with Matrigel (BD Biosciences, Bedford, MA, USA) were incubated at 37˚C for 30 min. The cells were seeded in the upper well and incubated for 24 or 48 h. The cells in the upper well were removed and the cells that had invaded to the lower side of the filter were fixed with methanol and stained with crystal violet. The number of invading cells was quantified by counting in 5 random fields (x200 field). This experiment was performed in triplicate.

Western blotting. Western blot assay was performed as previously described (12). In brief, the total protein was extracted and separated by SDS-PAGE. Then the separated proteins were transferred to PVDF membranes. The membranes were incubated with the corresponding primary antibodies followed by the relevant secondary antibody. Primary antibodies used in the present study were monoclonal rabbit anti-E-cadherin (1:1,000), anti-vimentin (1:500) (both from Cell Signalling Technology, Danvers, MA, USA) and monoclonal mouse anti-β-actin (1:1,000; Beyotime).

Quantitative real-time reverse transcription-PCR (qRT-PCR). Briefly, cDNA was synthesized from total RNA using a PrimeScript RT reagent kit with a DNA Eraser (Takara, Shiga, Japan). Primers for genes related to cancer stem cells were designed and synthesized. Then, qPCR assays were performed using a Bio-Rad IQ5™ Multicolor Real-Time qRT-PCR detection system (Bio-Rad, Hercules, CA, USA). The mRNA expression levels were detected as previously described (5) using specific primer sequences (Table I). The expression levels were measured in terms of the cycle threshold (Ct) and were then normalized to GAPDH expression using the $2^{-\Delta\Delta Ct}$ method (5,14).

Immunofluorescent staining and flow cytometry assay. The cells were washed with phosphate-buffered saline (PBS, Hyclone, Waltham, MA, USA) and blocked with 0.05% bovine serum albumin (Beyotime, Shanghai, China) in PBS. The cells were diluted to 10^6-10^7/ml and stained with rabbit anti-LGR5-FITC (Bioss, Beijing, China; cat. no: bs-1117R-FITC, 1:100 dilution) at 4˚C for 1 h. After being washed with PBS, the cells were used for a FACScalibur through a flow cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed using WinMDI software.

Statistical analysis. The statistical analyses were performed using SPSS 17.0 software. The quantitative data are presented as the mean ± standard deviation (SD). Statistical comparisons between two groups were performed using the Student's t-test. In all cases, $p<0.05$ was considered statistically significant.

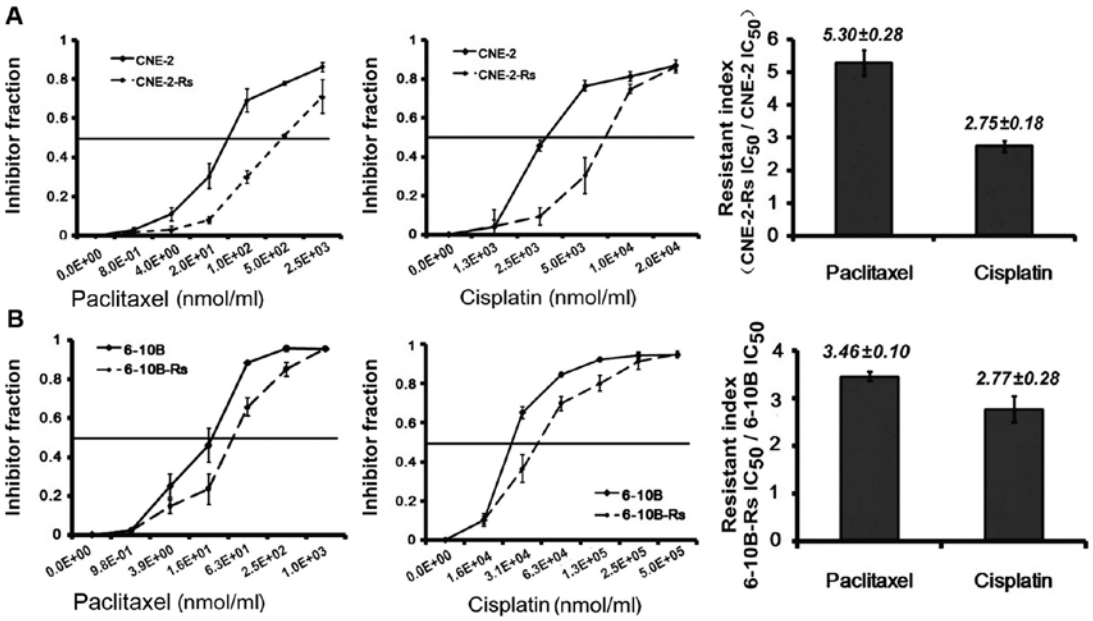

Figure 1. Irradiation induces increasing chemoresistance in the NPC cells. (A) The IC_{50} values for paclitaxel in the CNE-2-Rs and CNE-2 cells were 38.2±6.09 and 7.24±1.29 nmol/ml. The IC_{50} values for cisplatin in the CNE-2-Rs and CNE-2 cells were 8.32±0.83 and 3.02±0.34 (x10^4) nmol/ml. The calculated resistant indices of paclitaxel and cisplatin were 5.28±0.39 and 2.75±0.18 ($p<0.05$), respectively. (B) The IC_{50} values for paclitaxel in the 6-10B-Rs and 6-10B cells were 60.12±3.71 and 17.38±0.59 nmol/ml. The IC_{50} values for cisplatin in the 6-10B-Rs and 6-10B cells were 4.68±0.37 and 1.67±0.24 (x10^4) nmol/ml. The resistant indices of paclitaxel and cisplatin were 3.46±0.10 and 2.77±0.28 ($p<0.05$), respectively.

Table I. Gene primers.

Gene	Primer sequence
Lgr5	F: 5'-CTCTTCCTCAAACCGTCTGC-3'
	R: 5'-GATCGGAGGCTAAGCAACTG-3'
c-myc	F: 5'-GTCAGTATCACGCCCGTTTT-3'
	R: 5'-GCTTCCTTTACGCACTTGGT-3'
CD117	F: 5'-GCACAGCCTTGAGCCTACTC-3'
	R: 5'-TACGAATGCATGGGCAGTAA-3'
Bmi1	F: 5'-CCAGGGCTTTTCAAAAATGA-3'
	R: 5'-CCGATCCAATCTGTTCTGGT-3'
ALDH1	F: 5'-AAGCCAAGTGCTCTATCA-3'
	R: 5'-TCAACATCCTCCTTATCTC-3'
CD133	F: 5'-TTGTGGCAAATCACCAGGTA-3'
	R: 5'-TCAGATCTGTGAACGCCTTG-3'
PD-L1	F: 5'-TATGGTGGTGCCGACTACAA-3'
	R: 5'-TGCTTGTCCAGATGACTTCG-3'
CD24	F: 5'-GCCAGTCTCTTCGTGGTCTC-3'
	R: 5'-CCTGTTTTTCCTTGCCACAT-3'
GAPDH	F: 5'-TCCAAAATCAAGTGGGGCGA-3'
	R: 5'-AGTAGAGGCAGGGATGATGT-3'

F, forward; R, reverse.

Results

Irradiation induces chemoresistance in NPC cells. To investigate the potential impact of irradiation on chemosensitivity, NPC radioresistant cells CNE-2-Rs and 6-10B-Rs, established via exposure to gradually increasing doses of irradiation in our previous study (5), were used in the following experiments. We initially applied the CCK-8 assay to evaluate the sensitivity of radioresistant NPC cells to routine chemoagents including paclitaxel and cisplatin. Our results revealed that both the IC_{50} values of paclitaxel and cisplatin in the radioresistant NPC CNE-2-Rs were increased 5.28-(38.2±6.09 vs. 7.24±1.29 nmol/ml) and 2.75-fold (8.32±0.83 vs. 3.02±0.34 nmol/ml), compared with the parental CNE-2 cells (Fig. 1A). Meanwhile, similar data were obtained in the radioresistant 6-10B-Rs cells, which showed 3.46-(60.12±3.71 vs. 17.38±0.59 nmol/ml) and 2.77-fold (4.68±0.37 vs. 1.67±0.24 nmol/ml) more resistance to paclitaxel and cisplatin (Fig. 1B). Collectively, these results indicate that the radioresistant NPC cells acquired resistance to paclitaxel and cisplatin after surviving from exposure to irradiation.

Irradiation promotes the migration of the NPC cells. The migratory capacity of the radioresistant NPC cells was then investigated by wound healing assay. As indicated in Fig. 2, the migration of NPC cells was observed and photographed at 0 and 48 h, respectively. Our data demonstrated that the migration rate was obviously increased in the radioresistant NPC cells compared to the parental NPC cells (CNE-2-Rs cells: 58.04±7.22% vs. CNE-2 cells: 38.23±6.15%, $p<0.05$; 6-10B-Rs cells: 87.09±10.72% vs. 6-10B cells 44.71±3.98%, $p<0.05$). These results indicate that irradiation can promote the migration of NPC cells *in vitro*.

Irradiation enhances the invasion of the NPC cells. The invasive capacity of the radioresistant NPC cells was also examined by Transwell assay. As indicated in Fig. 3A, the numbers of

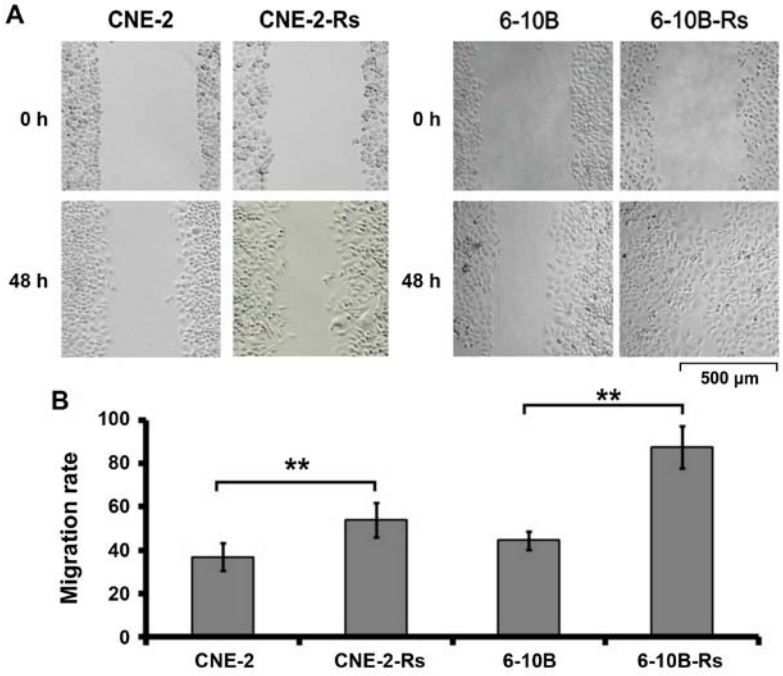

Figure 2. Irradiation promotes the migration of NPC cells. (A and B) The migration of the NPC cells was observed and photographed at 0 and 48 h (upper, x100 field). The calculated migration rates were 58.04±7.22 and 38.23±6.15% ($p<0.05$) in the CNE-2-Rs and CNE-2 cells, respectively, and the calculated migration rates were 87.09±10.72 and 44.71±3.98% ($p<0.01$) in the 6-10B-Rs and 6-10B cells, respectively (lower, x100 field).

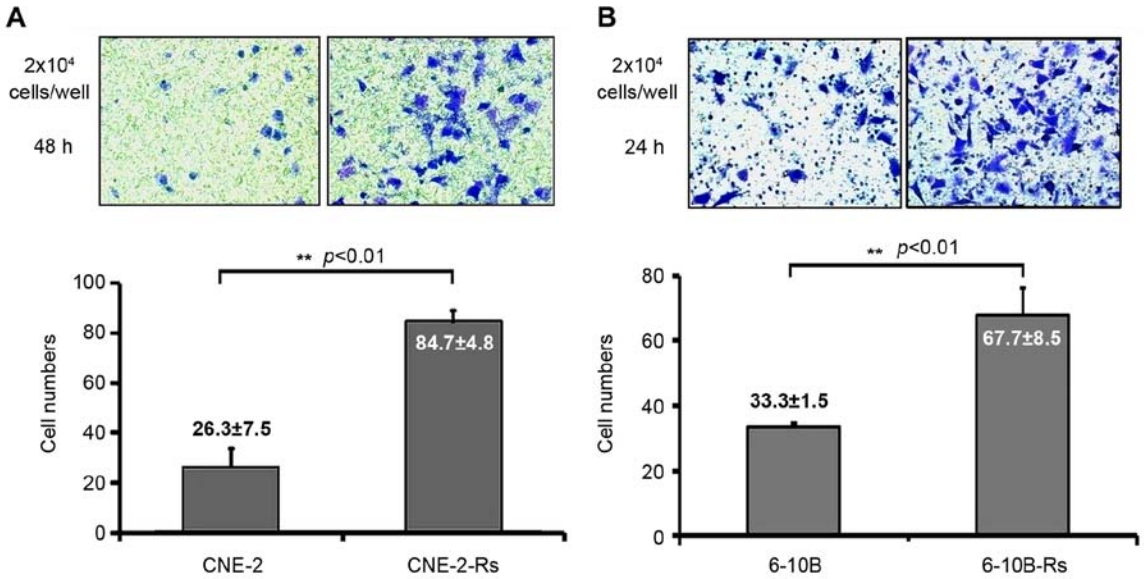

Figure 3. Irradiation promotes the invasion of NPC cells. (A) The number of invading CNE-2 and CNE-2-Rs cells after 48 h were 26.3±7.5 and 84.7±4.8 ($p<0.01$), respectively. (B) The number of invading 6-10B and 6-10B-Rs cells after 24 h were 33.3±1.5 and 67.7±8.5 ($p<0.01$).

invading CNE-2 and CNE-2-Rs cells on the bottom sides of the Matrigel-coated membrane after 48 h were 26.3±7.5 and 84.7±4.8 ($p<0.01$), respectively. Similarly, the numbers of invading 6-10B and 6-10B-Rs cells after 24 h were 33.3±1.5 and 67.7±8.5 ($p<0.01$), respectively (Fig. 3B). Taken together, the above results suggest that irradiation can enhance the metastatic phenotype including migration and invasion in the NPC cells.

Irradiation leads to EMT change in the NPC cells. Our above results revealed that irradiation induces chemoresistance to paclitaxel and cisplatin, and promotes migration and invasion of NPC cells *in vitro*. However, the underlying mechanisms that promote radioresistance, chemoresistance and metastasis in NPC remain to be clarified. In the phase of establishing radioresistant NPC cells, we observed significant morphological changes in the radioresistant NPC cells. Under phase-contrast microscopy, radioresistant CNE-2-Rs and 6-10B-Rs cells were found to become elongated with pseudopodia formation, a decrease in cell-to-cell contact, similar to the shape of fibroblasts (Fig. 4A). These morphological changes were consistent

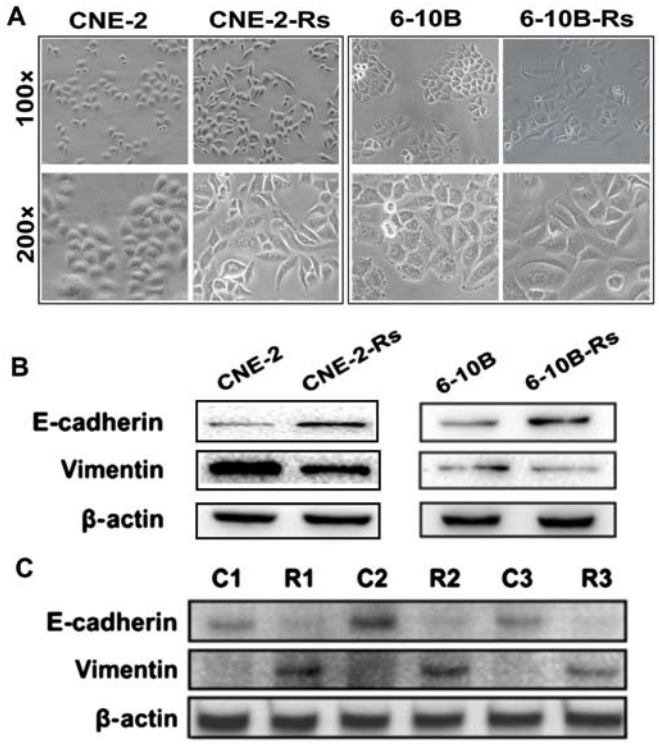

Figure 4. Irradiation induces the EMT phenotype in NPC cells. (A) The morphology of NPC cells was observed and photographed (upper, x100 field; lower, x200 field). The parental CNE-2 and 6-10B cells showed a pebble-like phenotype. The radioresistant CNE-2-Rs and 6-10B cells became elongated with pseudopodia formation, and loose in cell-to-cell contact. (B) Western blot assays showed that the expression of E-cadherin was decreased, while expression of vimentin was increased, in the CNE-2-Rs and 6-10B-Rs cells compared with their parental cells. (C) In three coupled NPC tissues, less E-cadherin expression and more vimentin expression were detected in residual NPC specimens (R1-R3) than in the corresponding tissues (C1-C3) before radiotherapy.

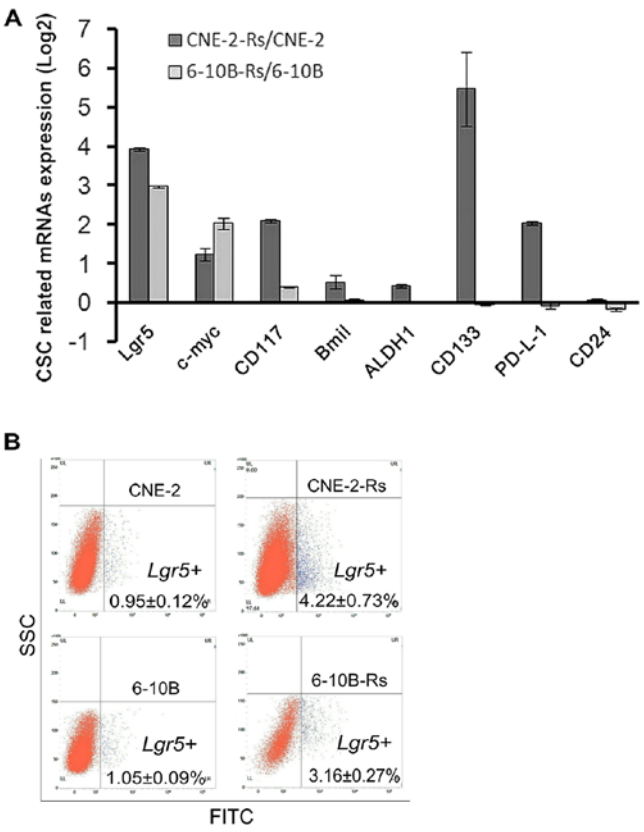

Figure 5. Irradiation induces cancer stem cell (CSC) molecular alterations in the NPC cells. (A) CSC markers Lgr5 and c-myc were upregulated in the CNE-2-Rs and 6-10B-Rs radioresistant cells. (B) B. Higher proportions of Lgr5+ cells were observed in radioresistant cells via immunofluorescent staining and flow cytometry. The Lgr5+ proportion was 4.22±0.73% and 0.95±0.12% ($p<0.05$) in the CNE-2-Rs and CNE-2 cells, respectively, and the Lgr5+ proportion was 3.16±0.27% and 1.05±0.09% ($p<0.05$) in the 6-10B-Rs and 6-10B cells, respectively.

with canonical EMT formation, which has been reported to tightly correlate with radioresistance, chemoresistance and metastasis (15). Therefore, the molecules associated with EMT were further examined. Our data clearly indicated that CNE-2-Rs and 6-10B-Rs cells displayed higher expression of mesenchymal marker vimentin and lower expression of epithelial protein E-cadherin (Fig. 4B). These findings were further validated in clinical NPC samples that experienced irradiation. Three residual NPC specimens post-radiotherapy were obtained. Western blot analysis detected decreased expression of E-cadherin and elevated expression of vimentin in the NPC residues, compared with the NPC samples before radiotherapy (Fig. 4C). These results suggest that irradiation promotes the emergence of EMT, which may be involved in the observed chemoresistance and metastasis in NPC.

Irradiation increases molecules associated with cancer stem cells (CSCs). CSCs persist in tumors as a distinct population and cause cancer progression and relapse by giving rise to new tumors (16). CSCs are involved in diverse cancer malignant behaviors including metastasis, radioresistance and chemoresistance (16). Therefore, we hypothesized that irradiation may also increase the number of CSCs and promote chemoresistance and metastasis. To test this hypothesis, qRT-PCR assays were used to evaluate molecular markers associated with CSCs. Our results showed that Lgr5 and c-myc had consistent changes (fold-change >2) in the CNE-2-Rs and 6-10B-Rs cells, when compared with the parental cells (Fig. 5A). Then, Lgr5+ proportions of the cells were detected by immunofluorescent staining and flow cytometry. The Lgr5+ proportion was 4.22±0.73% and 0.95±0.12% ($p<0.05$) in the CNE-2-Rs and CNE-2 cells, respectively, and the Lgr5+ proportion was 3.16±0.27% and 1.05±0.09% ($p<0.05$) in the 6-10B-Rs and 6-10B cells, respectively (Fig. 5B). The data suggest that irradiation may induce chemoresistance and metastasis via increasing CSCs, which is indicated by increased expression of specific CSC markers.

Discussion

Clinically, the difficulty in obtaining specimens is still an obstacle for the study of NPC post-irradiation residue and recurrence. In order to better understand the underlying mechanism, the establishment of post-irradiation residual NPC cells is the main choice for many laboratories and research teams. Three methods have been widely accepted to obtain post-irradiation NPC cell lines, including low-dose repeated irradiation (17), sublethal dose irradiation (18) and gradient increasing irradiation (6). Compared with the first

two patterns, accumulating doses in a gradient irradiation pattern of up to 60-70 Gy most closely resembles the clinical doses for NPC patients. Therefore, our group chose a gradient increasing irradiation pattern and successfully established two post-irradiation residual NPC cell lines (5,6).

In some cases, post-irradiation residual cells were found to be also accompanied with advanced radioresistance, and were considered to be radioresistant cells (6,17). Similarly, two residual NPC cell lines screened in our previous research were named radioresistant CNE-2-Rs and 6-10B-Rs cells, respectively (6). Previous research has only focused on the mechanism of radioresistance rather than other malignant behaviors. Our results showed chemoresistance as well as aggressive motility and invasion capability in residual NPC cells. These findings, to some extent, may be the answer for treatment failure and the poorer prognosis of NPC patients with post-irradiation residue and recurrence.

Notably, following the total irradiation dose of the gradually accumulation, NPC cells transformed from epithelial cells into a mesenchymal phenotype. Concurrently, a loss of epithelial marker E-cadherin and an increase in the mesenchymal molecule vimentin were also observed in the post-irradiation cells. Thus, we considered that the NPC cells went through an EMT process, which partially explains the higher invasion and metastasis observed in the post-irradiation cells. In addition, in three pairs of patient tissues from pre- and post-irradiation, EMT markers exhibited the same alterations as in the residual cells, further supporting that irradiation may induce EMT in NPC. Several reports suggested the potential connections between the EMT phenotype and post-irradiation residues, in malignancies of the stomach (19), prostate (20) and cervical cancer (21). The mechanisms involved included activation of the WNT/β-catenin (22), PI3K/Akt/mTOR (23), and NF-κB pathways (24). Inspiringly, our group firstly found this connection in NPC cells, and showed that cells with an EMT phenotype not only had a greater radioresistance, but was also accompanied by more aggressive invasion and migration abilities. Our previous studies discovered that the WNT signaling pathway was increasingly activated in radioresistance NPC cells (6,12). Reversely, following WNT2B knockout, the NPC radioresistance and their invasion and migration capacities weakened (data not shown), along with corresponding changes in EMT markers (12). All the above findings suggest that WNT-mediated EMT may be a potential mechanism in NPC malignancy and warrants further investigation.

Chemotherapy is an indispensable method for the treatment of NPC, and our research firstly investigated the chemosensitivity of post-irradiation residual NPC cells. As first-line chemotherapeutics used in NPC treatment, paclitaxel and cisplatin were used in our study and multidrug resistance to both chemotherapeutic agents was observed in the radioresistant cells. Irradiation-induced multidrug resistance or an increase in related genes were also reported in other tumors, such as breast (25), oral (26) and colon (27). Even a low irradiation dose also led to chemoresistance (28,29), suggesting that insufficient irradiation dose and residues may cause present or subsequent chemotherapy failure. In recent years, the role of EMT in drug resistance has attracted attention. In NPC, EMT is necessary for acquired resistance to cisplatin (30). The altered expression of FOXC2 and P53 could also affect NPC chemoresistance via regulation of EMT (31,32).

The cancer stem cell (CSC) is considered as another crucial reason for advanced tumor malignancy. Via self-renewal and multi-direction differentiation, CSCs can survive in extreme situations such as exposure to irradiation or chemo-agents. The related mechanisms attributed to the CSC-mediated survival include high DNA damage repair capacity, cell cycle regulation and enhanced reactive oxygen species (ROS) defenses (8,11). In our study, CSC-related genes Lgr5 and c-Myc were significantly upregulated in both radioresistance cell lines. In addition, higher proportions of Lgr5+ cells were observed in radioresistant cells. Lgr5 is a classic CSC biomarker and is associated with WNT pathway activation in many malignancies (33). c-Myc is reported as a key transcription factor for CSC phenotype maintenance (34,35). Furthermore, during the therapy process, CSC trait transition and self-renewal could also elicit tumor adaptive responses by irradiation and the drug agents themselves (36).

Our present results indicate that co-existence of EMT and CSCs may be the common mechanisms for NPC radioresistance, chemotherapy tolerance, invasion and metastasis. In fact, the EMT phenotype may be the result of CSC multi-directional differentiation and may be a bridge to connect CSCs and treatment resistance (37). Thus, reversing the progression of EMT and CSCs may become a method to restrict multiple malignant bio-behaviors of NPC cells, and become an effective strategy to improve the prognosis and life quality of NPC patients with a residual and recurrent mass. These findings warrant further study.

Acknowledgements

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