Original Paper

Overexpression of β -Catenin Decreases the Radiosensitivity of Human Nasopharyngeal Carcinoma CNE-2 Cells

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Key Words

β -catenin • Overexpression • Nasopharyngeal carcinoma • Radiosensitivity

Abstract

Background/Aims: Nasopharyngeal carcinoma (NPC) is rare worldwide but remains highly prevalent in endemic regions, notably in southern China. Radiotherapy remains the treatment of choice for NPC, but radioresistance has been identified as a major cause of therapeutic failure. The Wnt/ β -catenin signaling has been found to be involved in NPC radioresistance; however, the effect of β -catenin overexpression on radioresistance remains unknown in NPC until now. This study aimed to examine the impact of β -catenin overexpression on the radiosensitivity of human NPC CNE-2 cells. **Methods:** Immunohistochemistry was performed to detect the β -catenin expression in normal nasopharyngeal specimens and NPC specimens. The human NPC CNE-2 cell line overexpressing β -catenin was modeled by transfection with the pcDNA3.1/Hygro(+)/ β -catenin recombinant vector (transfection group), while cells transfected with the pcDNA3.1/Hygro(+) vector served as negative controls and non-transfected cells served as blank controls. The expression of key molecules of the Wnt/ β -catenin signaling pathway was determined using Western blotting and qPCR assays, and the changes of radiation sensitivity were measured with a colony-formation assay. Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. In addition, the cell cycle and apoptosis was detected using flow cytometry and the TCF/LEF transcriptional activity was measured with a Dual Luciferase Reporter Assay System. **Results:** Immunohistochemical staining showed high β -catenin expression in radioresistant NPC specimens, and low expression in radiosensitive NPC specimens and normal nasopharyngeal specimens. Western blotting and qPCR assays detected higher β -catenin expression in the transfection group than in the negative and blank controls ($P < 0.01$). Down-regulation of GSK-

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3β expression ($P < 0.05$) and up-regulation of Cyclin D1 expression ($P < 0.01$) was detected in β -catenin overexpressing NPC cells exposed to X-ray radiation relative to negative and blank controls. Colony-formation assay revealed higher D_0 , D_q and SF in the transfection group than in the negative and blank control groups post-radiation, and the SER in the transfection group was 0.75-fold and 0.68-fold greater than that in the blank and negative control groups, respectively. MTT assay revealed that the viability of CNE-2 cells was significantly higher in the transfection group ($96\% \pm 8.72\%$) than in the negative control group ($74.67 \pm 7.05\%$) and the blank control group ($75.33\% \pm 7.02\%$) 24 h post-exposure to 6 Gy X-ray radiation ($P < 0.05$). X-ray radiation led to a lower proportion of CNE-2 cells at the G2/M phase and a lower apoptotic rate in the transfection group than in the negative and blank control groups ($P < 0.05$). In addition, the TCF/LEF transcriptional activity was higher in the transfection group than in the negative and blank control groups ($P < 0.01$), and 6 Gy X-ray radiation elevated the TCF/LEF transcriptional activity relative to 0 Gy radiation in the transfection group ($P < 0.01$). **Conclusion:** β -catenin overexpression may decrease the radiation sensitivity in NPC CNE-2 cells through activating the downstream transcriptional factors of β -catenin, and reducing G2/M arrest and cell apoptosis.

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Published by S. Karger AG, Basel

Introduction

Nasopharyngeal carcinoma (NPC) is the most common primary malignancy in the nasopharynx [1, 2]. Although NPC is rare worldwide [3], this malignancy remains highly prevalent in endemic regions, notably in southern China [4]. Currently, radiotherapy remains the treatment of choice for NPC, and concomitant chemoradiotherapy has been proved to increase the survival [5, 6]. Although 80% to 90% 5-year local control rates are achieved for NPC, there are still 20% to 30% of patients developing distal metastasis [7], and radioresistance has been identified as a major cause of therapeutic failure [8–10]. Elucidation of the mechanisms underlying the radioresistance in NPC is therefore beneficial for the individualized treatment for NPC.

As a critical component in the Wnt/ β -catenin signaling pathway [11], β -catenin has been linked strongly to the prognosis of NPC [12–14]; however, some studies argued that β -catenin had no associations with survival of NPC patients [15] and did not affect the invasion and metastasis of NPC cells [16]. In addition, the Wnt/ β -catenin signaling has been found to be involved in NPC radioresistance [17, 18]. To the best of our knowledge, however, the effect of β -catenin overexpression on radioresistance remains unknown in NPC until now.

In this study, we modeled β -catenin overexpression in a human NPC CNE-2 cell line by transfection with the pcDNA3.1/Hygro(+)/ β -catenin recombinant vector, and then examined the effects of β -catenin overexpression on the radiosensitivity of human NPC CNE-2 cells and investigated the underlying mechanisms. We detected higher D_0 , D_q , surviving fraction (SF), radiation sensitivity enhancement ratio (SER), viability, TCF/LEF transcriptional activity, lower apoptotic rate, down-regulation of GSK-3 β expression and up-regulation of Cyclin D1 expression in the CNE-2 cells overexpressing β -catenin than in negative and non-transfected controls. Our findings may provide insights into the elucidation of the mechanisms underlying the radioresistance in NPC.

Materials and Methods

Ethical statement

This study was approved by the Ethics Review Committee of Fujian Cancer Hospital (permission no. FJZLYY-201500127). Written informed consent was obtained from all participants following a detailed description of the study purpose.

Tissue samples

Normal nasopharyngeal specimens and NPC specimens were obtained from Fujian Cancer Hospital (Fuzhou, China). All NPC patients were definitively diagnosed with pathological examinations.

Immunohistochemistry

Paraffin blocks that contained sufficient formalin-fixed tumor specimens were cut into 3 μ m sections and mounted on silane-coated slides for immunohistochemical staining analysis. Sections were then deparaffinized with dimethylbenzene and rehydrated consecutively using sequential ethanol (100%, 95%, 90%, 85%, 80% and 75%). Antigen retrieval was done in 0.01 M sodium citrate buffer (autoclaved at 121°C for 2 min, pH 6.0) and endogenous peroxidase was blocked by incubation in 3% H₂O₂ for 10 min at room temperature. The sections were then washed in PBS and incubated with anti- β -catenin (1:1000 dilution; Cell Signaling Technology, Inc.; Danvers, MA, USA) at 4°C for 12 h. Subsequently, sections were washed three times in PBS and incubated with the HRP-conjugated secondary antibody for 30 min at room temperature. All slides were counterstained diaminobenzidine (DAB) solution and 20% hematoxylin, and dehydrated. Slides stained the antibody diluent in the primary antibody step served as negative controls.

Cell line and culture

Human NPC CNE-2 cell line was maintained in Laboratory of Radiation Oncology and Radiobiology, Fujian Cancer Hospital (Fuzhou, China), and cultured in RPMI-1640 medium (Gibco BRL; Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL; Gaithersburg, MD, USA) at 37°C containing 5% CO₂.

Cell transfection

CNE-2 cells were seeded onto 24-well plates (Corning, Inc.; Corning, NY, USA) and cells growing to 80% to 90% confluence were transfected with pcDNA3.1/Hygro(+)/ β -catenin recombinant vector (provided by the Laboratory of Radiation Oncology and Radiobiology, Fujian Cancer Hospital; Fuzhou, China) (transfection group) and pcDNA3.1/Hygro(+) vector (Invitrogen; San Diego, CA, USA) (negative control) using the FuGENE® HD Transfection Reagent (Promega; Madison, WI, USA), while non-transfected cells served as a blank control. Following transfection for 48 h, cells were digested with 0.25% pancreatin (Sigma-Aldrich; St. Louis, MO, USA), re-suspended in RPMI-1640 medium containing hygromycin (Amresco, Inc.; Solon, OH, USA), and seeded onto 6-well plates (Corning, Inc.; Corning, NY, USA) for further 14-day incubation. Then, cells were harvested for the subsequent experiments.

Colony-formation assay

Log-phase CNE-2 cells in the transfection group, negative control group and the blank control group were digested with 0.25% pancreatin and then seeded onto 6-well plates (Corning, Inc.; Corning, NY, USA) at densities of 100, 200, 400, 800, 1600 and 1600 cells per well, which were then exposed to 6 MV X-ray radiation using a linear accelerator (Elekta; Stockholm, Sweden) at single doses of 0, 2, 4, 6, 8 and 10 Gy with the following radiation characteristics: size of the radiation field, 30 cm $\times$ 30 cm; source surface distance, 100 cm; and dose-rate, 285 cGy/min. Four replicates were assigned for each radiation dose. Following radiation, cells were cultured for further 14 days, and then stained with 2% crystal violet. The number of colonies ($\geq$ 50 cells per colony) was counted under a light microscope. The plating efficiency (PE) was calculated using the following formula: PE (%) = number of colonies/original cell-seeding density $\times$ 100%, and the surviving fraction (SF) was estimated using the following formula: SF = number of colonies/(number of cells seeded $\times$ PE). The cell survival curve was plotted using the multi-target single-hit model: SF = 1 - (1 - e^{-D/D₀})^N, and the radiobiological parameters D₀ (the dose that gave an average of one hit per target), D_q (quasi-threshold dose), N (extrapolation number) and SF₂ (the SF following exposure to 2 Gy radiation) were determined using the survival curve. In addition, the radiation sensitivity enhancement ratio (SER) was calculated using the following formula: SER = D₀ of the blank or negative control group/D₀ of the transfection group. All experiments were assayed in triplicate.

Analysis of cell cycle

Log-phase CNE-2 cells at approximately 60% confluence in the transfection group, negative control group and the blank control group were exposed to X-ray radiation at doses of 0 and 6 Gy for 24 h, respectively. Then, cells were digested with 0.25% pancreatin, prepared into single-cell suspensions, and centrifuged at room temperature. The supernatant was discarded, and the sediment was stained with the cell cycle detection kit (BD Biosciences, San Diego, CA, USA) following the manufacturer's instructions. The cell cycle was analyzed with a FACSCanto™II flow Cytometer (BD Biosciences; San Jose, CA, USA). All analyses were repeated in triplicate.

Measurement of cell viability

The viability of the cells was assessed by the MTT assay. Cells were seeded onto 96-well plates (Corning, Inc.; Corning, NY, USA) overnight, and then exposed to X-ray radiation at doses of 0 and 6 Gy. Following 24-h treatment, MTT (2 mg/mL) was added to each well and incubated at 37°C for 4 h. The medium was discarded, and the formed formazan crystals were dissolved in dimethyl sulfoxide (DMSO; 150 μ L/well). The absorbance was read at 570 nm using a Model 680 microplate reader (Bio-Rad Laboratory; Hercules, CA, USA). All measurements were repeated in triplicate.

Detection of apoptosis

Log-phase CNE-2 cells at approximately 60% confluence in the transfection group, negative control group and the blank control group were exposed to X-ray radiation at doses of 0 and 6 Gy for 24 h, respectively. Then, cells were digested with 0.25% pancreatin, prepared into single-cell suspensions, and centrifuged at 1200 r/min at room temperature for 5 min. The supernatant was discarded, and the sediment was washed twice in cooled PBS, suspended in 100 μ L of binding buffer, and mixed evenly with 5 μ L of FITC Annexin V (BD Biosciences; San Diego, CA, USA) and 5 μ L of propidium iodide (PI; BD Biosciences; San Diego, CA, USA) in darkness at room temperature for 15 min. Apoptosis was detected on a FACSCanto™II flow Cytometer (BD Biosciences; San Jose, CA, USA). All measurements were repeated in triplicate.

Measurement of TCF/LEF transcriptional activity

Log-phase CNE-2 cells in the transfection group, negative control group and the blank control group were digested with 0.25% pancreatin and centrifuged, and the supernatant was discarded. The sediment was re-suspended in Opti-MEM® I Reduced Serum Medium (Gibco; Grand Island, NY, USA) containing 10% serum, and prepared into single-cell suspensions. Cells were then seeded onto 96-well plates (Corning, Inc.; Corning, NY, USA) at a density of 2×10^4 cells per well. Subsequently, cells in each group were transfected with 125 ng TCF/LEF luciferase reporter vector (Qiagen; Hilden, Germany) and negative control vector using the FuGENE® HD Transfection Reagent, followed by exposure to X-ray radiation at a dose of 6 Gy. After 24 h of radiation, cells were lysed, and the firefly luciferase activity and *Renilla* luciferase activity were measured using the Dual Luciferase Reporter Assay System (Promega; Madison, WI, USA). The relative luciferase activities were calculated by the ratios of the firefly luciferase activity/*Renilla* luciferase activity in cells transfected with the TCF/LEF luciferase reporter vector to the firefly luciferase activity/*Renilla* luciferase activity in cells transfected with the negative control vector. All measurements were repeated in triplicate.

Effect of X-ray radiation on the expression of key molecules of the Wnt/ β -catenin signaling pathway in CNE-2 cells

CNE-2 cells growing to approximately 50% confluence in the transfection group, negative control group and the blank control group were harvested and exposed to X-ray radiation at doses of 0 and 6 Gy for 24 h, respectively. Then, cells were harvested, and the β -catenin, GSK-3 β and Cyclin D1 expression was determined using Western blotting and qPCR assays.

Western blotting analysis

Cells were digested with pancreatin, harvested, lysed and centrifuged at 12000 r/min, 4°C for 5 min. Nuclear and cytoplasm extracts were prepared using the Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions (Novagen; Darmstadt, Germany). The protein concentration was qualified with a BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.; Waltham, MA, USA). Total protein was separated with 10% SDS-PAGE, and electro-transferred to Nitrocellulose (NC) membranes

(GE Healthcare; Pittsburgh, PA, USA) at 100 V for 1.5 h. The NC membrane was blocked with 3% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA), washed in TTBS (20 mM Tris-HCl, 150 mM NaCl and 0.1% Tween-20, pH 7.2), and incubated with primary antibodies (1:1000 dilution; Cell Signaling Technology, Inc.; Danvers, MA, USA) for 4 h, while β -actin (1:1000 dilution; Cell Signaling Technology, Inc.; Danvers, MA, USA) or lamin B1 (1:3000, Proteintech, USA) served as a loading control. Then, the blots were incubated with the HRP-conjugated secondary antibody (1:5000 dilution; Thermo Fisher Scientific, Inc.; Waltham, MA, USA) for 3 h, visualized with the ECL Chemiluminescent Substrate Reagent Kit (Thermo Fisher Scientific, Inc.; Waltham, MA, USA) and analyzed with the Carestream IS4000MM Pro Molecular Imaging System (Carestream Health, Inc.; Rochester, NY, USA). The expression of the target protein was expressed as the ratio of the absorbance of the target protein band to the absorbance of the loading control band. All determinations were repeated in triplicate.

qPCR assay

Total RNA was extracted from CNE-2 cells using the TRIzol Reagent (Invitrogen; San Diego, CA, USA) and reversely transcribed into cDNA with the cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.; Waltham, MA, USA). The mRNA expression of the target genes was quantified using qPCR assay in a 20 μ l system containing 6 μ l of ddH₂O, 1 μ l of the forward and reverse primers (10 μ M each, Table 1), 10 μ l of Master Mix and 2 μ l of cDNA template. qPCR assay was performed on a Roche LightCycler 480 real-time PCR system (Roche; Basel, Swiss) under the following conditions: at 95°C for 10 min; followed by 40 cycles of at 95°C for 10 s, at 60°C for 10 s, and at 72°C for 20 s. Following completion of the PCR assay, the program for the melting curve was recorded by holding at 95°C for 10 s, at 60°C for 1 min; then heating slowly until 95°C; and finally cooling to 40°C for 30 s. The relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method. All PCR assays were repeated in triplicate.

Statistics

All measurement data were expressed as mean $\pm$ standard deviation (SD), and all statistical analyses were performed using the statistical software SPSS version 18.0 (SPSS, Inc.; Chicago, IL, USA). One-way ANOVA was used for multiple comparisons, and stepwise multiple comparisons were done with LSD test. A P value of < 0.05 was considered statistically significant.

Results

β -catenin is overexpressed in the radioresistant NPC specimens

Immunohistochemical staining revealed high β -catenin expression in radioresistant NPC specimens, and low β -catenin expression in radiosensitive NPC specimens, which was similar to that in normal nasopharyngeal specimens (Fig. 1).

β -catenin is overexpressed in the transfected CNE-2 cells

qPCR assay detected higher relative β -catenin mRNA expression in the transfection group than in the blank control group (3.89 ± 0.27 vs. 1.09 ± 0.20 , $P < 0.01$) and negative control group (3.89 ± 0.27 vs. 1.08 ± 0.2 , $P < 0.01$), while no significant difference was seen between the blank and negative controls (1.09 ± 0.2 vs. 1.08 ± 0.2 , $P > 0.05$) (Fig. 2A). Similarly, Western blotting analysis revealed higher β -catenin protein expression in the transfection group than in the blank and negative controls ($P < 0.05$) and no significant difference between the blank and negative control groups (Fig. 2B). Taken together, our data indicate the successful modeling of β -catenin overexpression in CNE-2 cells.

Table 1. Sequences of primers used for qPCR assay

Gene	Sequence
β -catenin	F: 5'-GCAGCAACAGTCTTACC-3', R: 5'-ACAGGACTTGGGAGGTAT-3'
GSK-3 β	F: 5'-CTCTTGGAGGCGTAAA-3', R: 5'-GGAGCACCTTCTTGATA-3'
Cyclin D1	F: 5'-CCTTCGTTGCCTCTTGT-3', R: 5'-GGGCTCCTCAGGTTCA-3'
β -actin	F: 5'-GGAAATCGTGCGTGACATT-3', R: 5'-CAGGCAGCTCGTAGCTCTT-3'

Fig. 1. Immunohistochemical staining of β -catenin expression in normal nasopharyngeal specimens and NPC specimens ($\times 20$). Normal tissue, normal nasopharyngeal specimens; IS-NPC, radiosensitive NPC specimens; IR-NPC, radioresistant NPC specimens. Scale bar = 50 μ m.

Fig. 2. The efficiency of β -catenin overexpression is checked. (A) qPCR assay is employed to determine β -catenin mRNA expression, while β -actin serves as an internal control. All error bars are mean $\pm$ s.d. ($n = 3$ independent experiments). * $P < 0.05$, ** $P < 0.01$. (B) Western blotting assay is performed to determine the β -catenin protein expression, while β -actin serves as a loading control.

β -catenin overexpression decreases GSK-3 β expression and increases Cyclin D1 expression following exposure to X-ray radiation

Following exposure to X-ray radiation at 6 Gy for 24 h, the GSK-3 β mRNA expression was lower in the transfection group than in the negative ($0.41\% \pm 0.01\%$ vs. $0.81 \pm 0.1\%$, $P < 0.05$) and blank controls ($0.41\% \pm 0.01\%$ vs. $0.7 \pm 0.08\%$, $P < 0.01$), and no significant difference was seen between the negative and blank controls ($0.81 \pm 0.1\%$ vs. $0.7 \pm 0.08\%$, $P > 0.05$) (Fig. 3A). In addition, higher GSK-3 β protein expression was detected in the transfection group than in the negative and blank controls ($P < 0.05$), and no significant difference was seen between the negative and blank controls ($P > 0.05$) (Fig. 3B and 3C).

Following exposure to X-ray radiation at 6 Gy for 24 h, the Cyclin D1 mRNA expression was higher in the transfection group than in the negative ($2.96\% \pm 0.6\%$ vs. $1.23 \pm 0.03\%$, $P < 0.01$) and blank controls ($2.96\% \pm 0.6\%$ vs. $1.11 \pm 0.3\%$, $P < 0.01$), and no significant difference was seen between the negative and blank controls ($P > 0.05$) (Fig. 3A). Additionally, higher Cyclin D1 protein expression was detected in the transfection group than in the negative and blank controls ($P < 0.05$), and no significant difference was seen between the negative and blank controls ($P > 0.05$) (Fig. 3B and 3C).

β -catenin overexpression decreases the radiosensitivity of CNE-2 cells

There was an increase in D_0 and D_{q_0} in the transfection group relative to the negative and blank control groups (Table 2), indicating a reduction in the radiosensitivity in the transfection group as compared to the negative and blank controls. Following exposure to radiation at doses of 2 to 10 Gy, significantly higher SF values were estimated in the transfection group than in the blank control and negative control groups ($P < 0.05$), while no significant differences were found between the blank and negative controls ($P > 0.05$) (Fig. 4). In addition, the SER in the transfection group was 0.75-fold and 0.68-fold changes from the blank and negative control groups, respectively (Table 2).

Fig. 3. β -catenin overexpression decreases GSK-3 β expression and increases Cyclin D1 expression following exposure to X-ray radiation. (A) The β -catenin, GSK-3 β and Cyclin D1 mRNA expression is quantified using qPCR assay in the transfection group, negative control group and the blank control group pre- and post-exposure to X-ray radiation at a dose of 6 Gy for 24 h, while β -actin serves as an internal control. All error bars are mean $\pm$ s.d. $n = 3$ independent experiments. * $P < 0.05$, ** $P < 0.01$. (B, C) Western blotting assay is performed to determine the β -catenin, GSK-3 β and Cyclin D1 protein expression in the transfection group, negative control group and the blank control group cells pre- and post-exposure to X-ray radiation at a dose of 6 Gy for 24 h, while β -actin serves as an internal control. All error bars are mean $\pm$ SD ($n = 3$ independent experiments). * $P < 0.05$, ** $P < 0.01$.

X-ray radiation reduces the proportion of β -catenin overexpressed CNE-2 cells in G2/M phase of the cell cycle

Prior to X-ray radiation, there were no significant differences among the transfection, negative control and blank control groups in terms of the proportions of CNE-2 cells in the G0/G1, S or G2/M phase of the cell cycle ($P > 0.05$). After 24 h of X-ray radiation at a dose of 6 Gy, the proportion of CNE-2 cells in the G2/M phase was significantly lower in the transfection group than in the blank control group ($17.83\% \pm 3.97\%$ vs. $36.21 \pm 8.38\%$, $P < 0.05$) and negative control group ($17.83\% \pm 3.97\%$ vs. $39.54 \pm 12.07\%$, $P < 0.05$), and no significant difference was observed between the blank control group and the negative control group ($36.21 \pm 8.38\%$ vs. $39.54 \pm 12.07\%$, $P > 0.05$). In addition, the proportion of CNE-2 cells in the G0/G1 phase was greater in the transfection group than

Table 2. The radiobiological parameters of CNE-2 cells exposed to X-ray radiation. N, extrapolation number; b, D_0 of the blank control/ D_0 of the negative control; c, D_0 of the blank control group/ D_0 of the transfection group; d, D_0 of the negative control group/ D_0 of the transfection group

Group	D_0 (Gy)	D_q (Gy)	N^a	SF ₂	SER
Blank control	1.44	2.49	1.73	0.34	-
Negative control	1.31	2.71	2.06	0.28	1.09 ^b
Transfection	1.93	3.17	1.62	0.58	0.75 ^c /0.68 ^d

in the negative control group ($50.34 \pm 3.42\%$ vs. $28.2 \pm 8.46\%$, $P < 0.05$), while no significant differences were detected between the blank control group and the transfection group or the negative control group ($P > 0.05$). In terms of the proportion of CNE-2 cells in the S phase of the cell cycle, no significant difference was found among the three groups ($P > 0.05$) (Table 3, Fig. 5).

β -catenin overexpression promotes viability of CNE-2 cells post-radiation

There was no significant difference in the viability of cells among the transfection group ($108 \pm 5.00\%$), negative control group ($101.33 \pm 8.08\%$) and the blank control group ($100 \pm 5.00\%$) prior to X-ray radiation ($P > 0.05$), and the viability of cells was significantly higher in the transfection group than in the negative control group ($96\% \pm 8.72\%$ vs. $74.67 \pm 7.05\%$, $P < 0.05$) and the blank control group ($96\% \pm 8.72\%$ vs. $75.33 \pm 7.02\%$, $P < 0.05$) 24 h post-exposure to 6 Gy X-ray radiation (Fig. 6).

X-ray radiation reduces the apoptosis of CNE-2 cells overexpressing β -catenin

There was no significant difference in the apoptosis rate of CNE-2 cells among the transfection group ($8.57 \pm 0.38\%$), negative control group ($8.4 \pm 1.35\%$) and the blank control group ($10.8 \pm 2.51\%$) prior to X-ray radiation ($P > 0.05$), and the apoptosis rate of CNE-2 cells was significantly lower in the transfection group than in the negative control group ($8.37\% \pm 1.97\%$ vs. $14.9 \pm 4.08\%$, $P < 0.05$) and the blank control group ($8.37\% \pm 1.97\%$ vs. $14.13 \pm 1.76\%$, $P < 0.05$) 24 h post-exposure to 6 Gy X-ray radiation ($P < 0.05$) (Fig. 7A and 7B).

Fig. 4. β -catenin overexpression decreases the radiosensitivity of CNE-2 cells. (A) Colony-formation assay is performed in the transfection group, blank control group and negative control group upon exposure to X-ray radiation at doses of 0, 2, 4, 6, 8 and 10 Gy. (B) Surviving fraction in the transfection group, negative control group and blank control group following exposure to X-ray radiation at doses of 0, 2, 4, 6, 8 and 10 Gy. Significantly higher SF values are found in the transfection group than in the negative control and blank control groups ($P < 0.05$), while no significant differences were found between the negative and blank controls ($P > 0.05$). * $P < 0.05$, ** $P < 0.01$, the blank control group was compared with the transfection group. # $P < 0.05$, ## $P < 0.01$, the negative control group vs. the transfection group.

Table 3. Proportions of CNE-2 cells in the G0/G1, S or G2/M phase of the cell cycle pre- and post-exposure to X-ray radiation

Dose of X-ray radiation	Group	G0/G1 phase	S phase	G2/M phase
0	Blank control	65.76 $\pm$ 5.34	31.65 $\pm$ 11.95	2.59 $\pm$ 0.49
	Negative control	63.67 $\pm$ 6.89	33.38 $\pm$ 7.9	2.95 $\pm$ 1.14
	Transfection	65.21 $\pm$ 13.04	30.77 $\pm$ 5.71	4.02 $\pm$ 1.32
6	Blank control	39.60 $\pm$ 13.97	24.19 $\pm$ 8.86	36.21 $\pm$ 8.38
	Negative control	28.2 $\pm$ 8.46	32.14 $\pm$ 22.64	39.54 $\pm$ 12.07
	Transfection	50.34 $\pm$ 3.42	31.83 $\pm$ 0.9	17.83 $\pm$ 3.97

Fig. 5. β -catenin overexpression reduces G2/M arrest of CNE-2 cells induced by X-ray radiation. NPC cells are exposed to X-ray radiation at a dose of 6 Gy for 24 h, and then the cell cycle distribution is examined using flow cytometry. Each experiment is repeated in triplicate.

Fig. 6. The viability of CNE-2 cells was assessed by MTT assay. Cells in the transfection, blank control and negative control groups are subjected to the MTT assay. * $P < 0.05$ vs. the blank control and negative control groups. All measurements are repeated in triplicate. All error bars are mean $\pm$ SD ($n = 3$ independent experiments).

X-ray Radiation Increases the TCF/LEF Transcriptional Activity of CNE-2 Cells Overexpressing β -catenin

Prior to X-ray radiation, the TCF/LEF transcriptional activity was significantly higher in the transfection group than in the blank control group ($8.79\% \pm 0.89\%$ vs. $0.94 \pm 0.22\%$, $P < 0.01$) and the negative control group ($8.79\% \pm 0.89\%$ vs. $1.09 \pm 0.6\%$, $P < 0.01$), while no significant difference was observed between the blank control and negative control ($0.94\% \pm 0.22\%$ vs. $1.09\% \pm 0.6\%$, $P > 0.05$). Following exposure to 6 Gy X-ray radiation, the TCF/LEF transcriptional activity was significantly higher in the transfection group than in the

Fig. 7. β -catenin overexpression decreases the apoptotic rate of CNE-2 cells induced by X-ray radiation. (A) NPC CNE-2 cells are exposed to X-ray radiation at a dose of 6 Gy for 48 h. Then, cells are stained with Annexin V-FITC and propidium iodide and analyzed using flow cytometry. Each experiment is repeated in triplicate. (B) Histogram shows the total apoptotic rate (late + early phase) of NPC cells. All error bars are mean $\pm$ SD (n = 3 independent experiments). * $P < 0.05$.

blank control group ($13.05\% \pm 0.71\%$ vs. $0.88 \pm 0.11\%$, $P < 0.01$) and the negative control group ($13.05\% \pm 0.71\%$ vs. $1.12 \pm 0.32\%$, $P < 0.01$), while no significant difference was found between the blank control and negative control ($0.88\% \pm 0.11\%$ vs. $1.12\% \pm 0.32\%$, $P > 0.05$). In addition, TCF/LEF transcriptional activity was significantly higher in the transfection group post-radiation relative to prior to radiation at a dose of 6 Gy ($P < 0.05$) (Fig. 8A).

To investigate the nuclear accumulation of β -catenin in the transfection group, NPC cells were exposed to 6 Gy X-ray radiation for 24 h, and then, nuclear and cytoplasmic fractions were isolated. Each fraction was examined by Western blotting. Western blotting analysis revealed significantly higher cytoplasmic β -catenin expression in the transfection group than in the negative (0.65 ± 0.036 vs. 0.4 ± 0.017 , $P < 0.01$) and blank control groups (0.65 ± 0.036 vs. 0.39 ± 0.025 , $P < 0.01$), and no significant difference was seen between the negative and blank controls (0.4 ± 0.017 vs. 0.39 ± 0.025 , $P > 0.05$). Following exposure to X-ray radiation at 6 Gy for 24 h, higher nuclear β -catenin expression was detected in the transfection group than in the negative (0.77 ± 0.028 vs. 0.58 ± 0.04 , $P < 0.01$) and blank controls (0.77 ± 0.028 vs. 0.59 ± 0.043 , $P < 0.01$), and no significant difference was seen between the negative and

Fig. 8. Effect of β -catenin overexpression on TCF/LEF transcriptional activity and β -catenin accumulation in CNE-2 cells. (A) Following 6 Gy X-ray radiation for 24 h, the TCF/LEF transcriptional activity is measured. All error bars are mean $\pm$ SD ($n = 3$ independent experiments). ** $P < 0.01$. (B, C) Western blotting assay is performed to determine the cytoplasmic and nuclear β -catenin protein expression pre- and post-exposure to X-ray radiation at a dose of 6 Gy for 24 h, and β -actin (for cytoplasmic protein) and Lamin B1 (for nuclear protein) serve as loading controls. * $P < 0.05$, ** $P < 0.01$.

blank controls (0.58 ± 0.04 vs. 0.59 ± 0.043 , $P > 0.05$). In addition, the nuclear β -catenin accumulation was higher in the transfection group post-radiation than pre-radiation ($P < 0.05$) (Fig. 8B and 8C).

Discussion

Currently, radiotherapy is the first therapeutic option for NPC [6]. With the great advances in imaging and radiotherapy techniques, a clear-cut reduction is seen in the local recurrent rate and distal metastatic rate in NPC patients after radiotherapy [19]. Notably, following the introduction of intensity-modulated radiotherapy techniques [20–22], a 3-year local recurrence-free survival rate of over 90% and a 3-year distal metastasis-free rate of more than 80% are achieved; however, there are still patients developing regional recurrence and distal metastasis [23–25]. Radioresistance has been identified as a major factor restricting the therapeutic efficacy of NPC [26]. Elucidation of the mechanisms of radioresistance is therefore of great value to improve the therapeutic efficacy of NPC.

It has been shown that the Wnt/ β -catenin signaling pathway is strongly associated with the development and progression of multiple human cancers [27–32]. β -catenin, a critical component of the Wnt/ β -catenin signaling pathway [11], has been found to contribute to the radiosensitivity of cancers [18, 33]. siRNA-induced knockdown of β -catenin expression was found to enhance the radiation sensitivity in head and neck cancer AMC-HN-9 cells [34, 35], and treatment with BC-23 (C21H14ClN3O4S), a novel inhibitor of β -catenin/T-cell factor 4 (Tcf4) interaction, was reported to enhance the radiation sensitivity in human p53-null non-

small cell lung cancer (NSCLC) H1299 cells [36]. Although the Wnt/ β -catenin signaling has been linked to the radiation resistance in NPC [17, 18], there is no knowledge regarding the effect of β -catenin overexpression on radiation resistance in NPC to date. In addition, the associations of β -catenin expression with the biological behaviors of NPC cells and the prognosis of NPC patients remain controversial until now [12–16].

To examine the correlation between β -catenin and radiation sensitivity in NPC, a human NPC CNE-2 cell line overexpressing β -catenin was modeled with the pcDNA3.1/Hygro(+) vector in this study, which overexpressed the target gene and contained hygromycin-resistant genes. By adding hygromycin into the culture of transfected CNE-2 cells, the CNE-2 cells with failure in transfection of the pcDNA3.1/Hygro(+)/ β -catenin recombinant vector died because of intolerance to hygromycin, while the successfully transfected cells that expressed hygromycin-resistant genes survived due to tolerance to hygromycin. After continuous passaging, a single cell clone that was resistant to hygromycin and overexpressed β -catenin was yielded. Western blotting and qPCR assays determined up-regulation of β -catenin expression in CNE-2 cells transfected with the pcDNA3.1/Hygro(+)/ β -catenin vector, indicating the successful modeling of the CNE-2 cell line overexpressing β -catenin.

It is considered that X-ray radiation induces DNA damage in cancer cells, and DNA damage results in cell cycle arrest, which then initiates the DNA repair system of cancer cells; if the cellular repair system is insufficient to complete DNA repair, cells will progress towards apoptosis [37]. Intrinsic cellular radiosensitivity has been shown to strongly correlate with radiation-induced cell cycle distribution, DNA damage repair and apoptosis [38], and a stronger ability of DNA damage repair indicates lower radiation sensitivity [39]. In the present study, colony-formation assay revealed higher D_0 , D_q and SF values in the transfection group than in the negative and blank control groups following radiation at each dose, and the SER in the transfection group was 0.75-fold and 0.68-fold greater than that in the blank and negative control groups, respectively, indicating that β -catenin overexpression reduced the radiation sensitivity in CNE-2 cells. It is reported that an increase in the ability of DNA repair results in radiation resistance and severely limits the efficacy of radiotherapy [40]. In this study, an increase was seen in D_q in β -catenin overexpressed CNE-2 cells, as revealed by the cell survival curve, indicating the enhanced ability in the repair of sublethal cellular damages, and this may be associated with the overexpression of β -catenin. It was found that activation of the β -catenin pathway effectively promoted the repair of DNA double strand breaks (DSBs) in irradiated osteoblasts [41], and up-regulation of β -catenin expression increased the capacity of DSB repair in cancer cells [42]. It is therefore considered that the role of β -catenin in DNA damage repair may be a factor affecting cellular radiation sensitivity.

Cell-cycle regulation is an important mechanism affecting radiation sensitivity [43]. Following radiation-induced DNA damages, DNA damage-related genes trigger the mechanism of cell-cycle regulation, and allow the cell cycle arrest at G1/S and G2/M checkpoints, resulting in cell cycle arrest at G1 and G2 phases [44]. Since the G1/S checkpoint is deficient in most types of cancer cells, the G2/M checkpoint arrest is a major determinant governing the radiation sensitivity in cancer cells [45]. In the cell cycle, cells in the G2/M phase are most sensitive to radiation, and a reduction in the proportion of cells at the G2/M phase indicates the insensitivity to radiation [44]. In the current study, our data showed a decrease in the proportion of CNE-2 cells at the G2/M phase of the cell cycle in the transfection group relative to the blank and negative control groups 24 h post-exposure to X-ray radiation, suggesting the relief of the cell cycle arrest at the G2/M phase, and the reduction in the proportion of CNE-2 cells at the G2/M phase resulted in relative radiation resistance in CNE-2 cells overexpressing β -catenin. Inhibition of β -catenin expression was found to significantly increase the proportion of hepatocellular carcinoma stem cells in the G2/M phase [45], and treatment with polyphyllin I (PPI), a major active constituent extracted from *Paris polyphyllin*, caused down-regulation of β -catenin expression in multiple myeloma cells and cell cycle arrest at G2/M phase [46]. These findings demonstrate the close correlation between β -catenin and cell cycle. Cyclin D1, an important cell cycle-related

molecule, is involved in the regulation of cell cycle checkpoints [47]. Cyclin D1 expression has been found to strongly correlate with cancer radiosensitivity and inhibition of β -catenin and Cyclin D1 expression was reported to result in enhanced sensitivity to radiation [48, 49]. Our findings showed up-regulation of Cyclin D1 expression in CNE-2 cells overexpressing β -catenin. It is therefore hypothesized that the effect of β -catenin on the sensitivity of CNE-2 cells to X-ray radiation may be associated with mediating the expression of its downstream gene Cyclin D1; however, the exact mechanisms require further investigations.

To date, there is no consensus on the correlation between apoptosis and radiation sensitivity [50]. It is reported that radiation mainly leads to programmed cell death [51], and apoptosis, a form of cell death [52], may occur after programmed cell death and have no associations with cell survival [51]. It is therefore considered that apoptosis does not correlate with radiation sensitivity [51]. In another study, however, ionizing radiation was found to induce apoptosis of the human osteosarcoma SAOS-2 cells that were deficient in the expression of the product of the retinoblastoma tumor suppressor gene (pRb), while SAOS-2 derivatives expressing wild-type pRb exhibited increased viability and decreased apoptosis post-exposure to radiation [53]. β -catenin has been linked to apoptosis in cancer cells [54]. siRNA-mediated knockdown of β -catenin was found to inhibit the proliferation and invasion and induce apoptosis of the colon cancer SW480 cells [55], and blocking the T-cell factor (Tcf)/ β -catenin complex was reported to suppress the Wnt/ β -catenin signaling pathway in adrenocortical tumor cells triggering increased apoptosis [56]. In the present study, the apoptotic rate of CNE-2 cells was significantly lower in the transfection group than in the negative and blank control groups post-radiation, and β -catenin overexpression led to down-regulation of GSK-3 β , a major component of the Wnt/ β -catenin signaling pathway. Since down-regulation or inactivation of GSK-3 β , an important apoptosis-related molecule, may inhibit apoptosis, it is therefore assumed that β -catenin may affect the radiation sensitivity in NPC through mediating other Wnt/ β -catenin signaling pathway molecules.

In the classical Wnt signaling pathway, β -catenin translocates to the nucleus and binds with the downstream transcriptional factors TCF/LEF to form the transcriptional complex, thereby activating the TCF/LEF transcriptional activity [57]. In this study, our findings showed that the TCF/LEF transcriptional activity was significantly higher in the transfection group than in the blank and negative control groups, indicating that β -catenin overexpression increased the TCF/LEF transcriptional activation. In addition, 6 Gy X-ray radiation elevated the TCF/LEF transcriptional activity relative to 0 Gy radiation, which may be associated with the nuclear localization and accumulation of β -catenin caused by ionizing radiation and the resulting activation of the downstream transcriptional factors TCF/LEF [58]. To test this hypothesis, the β -catenin protein expression was determined in the cytoplasm and nucleus of CNE-2 cells, and Western blotting analysis revealed an increase in the nuclear accumulation of β -catenin in the transfection and negative control groups. In a recent study, ionizing radiation was found to induce nuclear translocation and accumulation of β -catenin and enhance the TCF/LEF transcriptional activities in glioblastoma U87 cells, and degrading β -catenin significantly reduced cell invasion [59], which is in agreement with our findings.

Conclusion

The results of the present study demonstrate that β -catenin overexpression may decrease the radiation sensitivity and lead to radiation resistance in human NPC CNE-2 cells through up-regulating intracellular β -catenin expression and the resultant nuclear translocation and accumulation of β -catenin, thereby up-regulating Cyclin D1 and down-regulating GSK-3 β expression, activating TCF/LEF transcriptional activities, promoting the relief of the cell cycle arrest at the G2/M phase and decreasing the ability to induce apoptosis.

Acknowledgements

This study was supported by the Natural Science Foundation of Fujian Province (grant no. 2014J01299 and 2018J01732), Fujian Provincial Key Sci & Tech Project (grant no. 2014Y0014), the grant from the National Natural Science Foundation of China (grant no. 81472907 and 81341131) and National Key Clinical Specialty Construction Project of China (grant no. 2013-554).

Disclosure Statement

The authors declare that they have no competing interests.

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