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·Original Article·

Isolation and detection of label-retaining cells in a nasopharyngeal carcinoma cell line

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[**Abstract**] **Background and Objective:** Detection of label-retaining cells (LRCs) has been a method to confirm existence of stem cells, and bromodeoxyuridine (BrdU) has commonly been used for labeling. In this study, to verify stem cells in nasopharyngeal carcinoma (NPC), LRCs were established and detected in NPC cell line 5-8F. **Methods:** The 5-8F cells were cultured with BrdU and inoculated subcutaneously into nude mice. By immunohistochemistry, immunocytochemistry, and immunofluorescence, BrdU was detected in 5-8F cells and xenograft tumors. **Results:** BrdU was strongly positive in cells on the 2nd and the 7th day after being added BrdU, while negative when cells were cultured without BrdU. However, only sporadic cells were positive on the 14th day after BrdU being washed-out, and these cells were thought to be LRCs. The average percentage of LRCs was (0.67 ± 0.32)%. After being cultivated with BrdU for 48 h, 5-8F cells were inoculated into nude mice subcutaneously. After chasing 8 weeks, only sporadic LRCs were detected in xenograft tumors, with a proportion of (0.55 ± 0.36)%, and these LRCs were located at cancer margin. **Conclusion:** The existence of LRCs in 5-8F cells indicates the existence of cancer stem cells in NPC.

Key words: Label-retaining cells (LRCs), nasopharyngeal neoplasm, BrdU, stem cells

Normal stem cells and cancer stem cells share the same way to divide, asymmetry division, for self renewal, which means one stem cell can divide into one stem cell and one progenitor cell. Therefore, stem cells have immortalized DNA strands, which can be labeled for a long time. These cells are called label-retaining cells (LRCs). In this study, we used non-radioactive label bromodeoxyuridine (BrdU) to label nasopharyngeal carcinoma (NPC) 5-8F cells and detected whether there were LRCs in 5-8F cells through in vitro cell culture and in vivo subcutaneous inoculation in nude mice to validate the existence of nasopharyngeal carcinoma stem cells in NPC.

Materials and Methods

Materials

Poor differentiated NPC cell line 5-8F was established by Sun Yat-sen University Cancer Center, and cells were cultured in RPMI-1640 with 10% fetal bovine serum (FBS). 5-8F cells were epithelial and adherent. Female BALB/c nude mice (4–6 weeks, specific pathogen-free [SPF] grade) were purchased from Animal Center, Southern Medical University. BrdU and anti-BrdU monoclonal antibody IgG1 (Cat. BU-33) was from Sigma. Goat anti-mouse IgG-FITC was from Santa Cruz.

Methods

In vitro cell culture Cells were cultured to logarithmic phase, and 10 ng/mL of BrdU was added to medium. After 7 days, BrdU was washed out and cells were cultured for another 14 days. Cells were seeded onto slices after the 2nd, 7th and 14th day, respectively.

Tumorigenicity in nude mice Cells were cultured to logarithmic phase, and 10 ng/mL of BrdU was added to medium. After 48 h, 1×10^6 cells were injected subcutaneously into bilateral armpits of two nude mice to observe the tumor formation. After 8 weeks, xenografts were removed, fixed, embedded and made to slices. Immunohistochemistry was used to detect BrdU. BrdU-positive cells were LRCs.

Immunohistochemistry and immunocytochemistry The slices were hydrated and cells were seeded onto slices. Slices were treated with Triton-100 for 5 min and 3% H_2O_2 was added to remove endogenous peroxidase for 10 min. Slices were washed with PBS, treated with 2 mol/L HCl for 30 min at 37℃, and washed with PBS, 2 min per time, 3 times. Then slices were

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incubated with 0.1 mol/L NaB_4O_7 for 8 min and washed with PBS, digested by 0.25% trypsin for 15 min and washed with PBS, then added with anti-BrdU antibody and incubated at 4℃ overnight. After washing with PBS, HRP-goat anti-mouse IgG was added and slides were incubated for 30 min and washed with PBS. Finally, slices were stained with DAB and hematoxyline.

The scoring standards were as follows: according to cell staining, dark brown was scored 3 points, brown-yellow scored 2 points, light yellow scored 1 point and non-staining scored 0; according to the proportion of positive cells in a field of vision, >75% was scored 4 points, 51%–75% scored 3 points, 11%–50% scored 2 points, 1%–10% scored 1 point, and negative scored 0. Two scores were multiplied, 0–2 points was considered as negative, and ⩾ 3 points was considered as positive expression.

Immunofluorescence of BrdU The protocol was as that above mentioned. Slides were incubated with BrdU antibody at 4℃ overnight, then added with goat anti-mouse IgG-FITC and incubated for 30 min at room temperature and observed under fluorescence microscope. Evaluation criteria were as follows: clearly visible fluorescence was recored as +, very weak fluorescence as ±, and no fluorescence as -.

Results

Detection of LRCs cells in 5-8F cells

Immunocytochemistry and immunofluorescence showed that before BrdU was added, cells were negative; on the 2nd and 7th days after BrdU was added, nearly all cells were positive; while on the 14th day after discarding BrdU, only very few cells were positive, these cells were considered as LRCs. Randomly selected 10 high power fields to count, LRCs cells accounted for (0.67 ± 0.32)% of all cells (Figures 1 and 2).

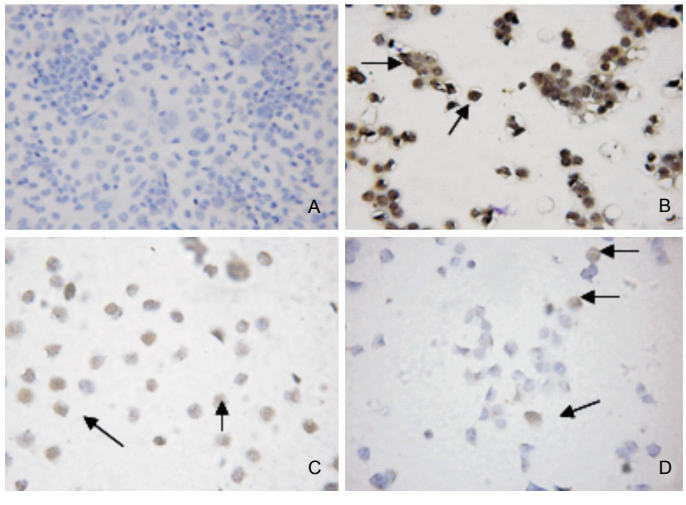

Figure 1 BrdU was detected by immunocytochemistry in 5-8F cells cultured with BrdU (×200)

A, before BrdU was added; B, the 2nd day after BrdU was added; C, the 7th day after BrdU was added; D, the 14th day after BrdU was washed out. Black arrows indicate BrdU-positive cells.

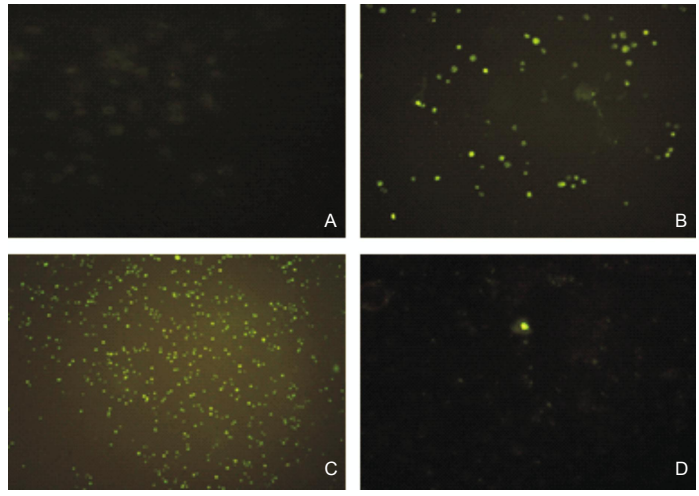

Figure 2 Detection of BrdU by immunofluorescent staining in 5-8F cells cultured with BrdU

A, before BrdU was added (×200); B, the 2nd day after BrdU was added (×200); C, the 7th day after BrdU was added (×200); D, the 14th day after BrdU was washed out (×400).

Detection of LRCs in xenografts

After 8 weeks inoculation, with HE staining, cell morphology of xenografts were consistent and xenografts were poorly differentiated tumors with large areas of necrosis in the tumor nest. BrdU immunohistochemistry results showed that only a few cells were positive, which were LRCs. Randomly selected 10 high power fields to count, LRCs cells accounted for (0.55 ± 0.36)% of all cells (Figure 3).

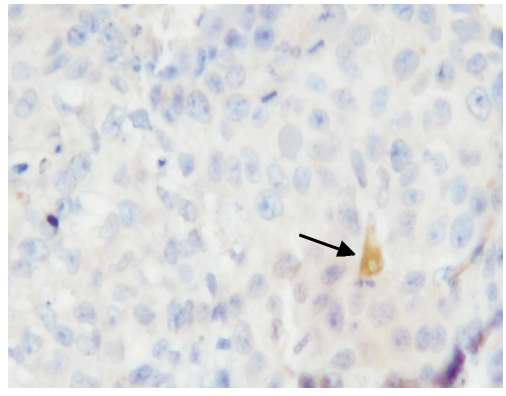

Figure 3 Sporadic BrdU-positive cells in xenograft tumor of 5-8F cells cultured with BrdU (SP ×400)

Black arrow indicates BrdU-positive cell.

Discussion

Compared with non-stem cells, the most prominent characteristic of stem cells is that few stem cells divide and the velocity of division is slow, known as slow-cycling cells. In 1981, Bickenbach *et al*.[1] developed label-retaining method to identify stem cells by applying the characteristic of stem cells. They repeatedly injected tritium-labeled thymidine or thymidine analogs

(BrdU or ^{3}H-TdR, and so on) at the most active cell division phase of newborn mice. Regardless of stem cells or non-stem cells, newly synthesized DNA was incorporated with the markers. After several weeks or months, markers only resided in cellular DNA of those seldom divided cells in adult mice. These cells were named stem cells. In 1990, Cotsarelis *et al.*[2] found that LRCs also existed in hair follicle of mammals. Subsequently, LRCs were detected in multiple organs and tissues, such as the skin[3,4], breast[5], endometrum[6], bronchial epithelium[7], pancreas [8], and so on. LRCs detection has now been considered as an important way for stem cell identification, which is widely used in stem cell research.

There are two commonly used methods for label-retaining cells. ^{3}H-TdR was discovered first, which was used in earlier studies of LRCs. However, ^{3}H-TdR has two drawbacks, one is that it requires for long time autoradiography of tissue sections, and the other is that it is difficult to detect both autoradiography and other stem cell-mediated immune marker together. Later, the emergence of non-radioactive marker BrdU provides a great convenience for LRCs experiments. The mechanism of BrdU incorporating with S-phase cells is the same as that of ^{3}H-TdR. BrdU could compete with endogenous thymidine to incorporate with newly synthesized DNA. In vivo injection or addition BrdU to cell culture medium, and using anti-BrdU monoclonal antibody, immunohistochemical staining displays proliferating cells.

According to the similarity of cancer stem cells and adult stem cells, many studies have showed that cancer stem cells are originated from adult stem cells through gene mutation and other processes. We speculate that adult stem cells have immortalized DNA strands which could be labeled for a long time, thus, BrdU may be used for cancer stem cell labeling in a certain time. The survival time of cancer stem cells is longer than that of common tumor cells. Due to asymmetric division of cancer stem cells, the chromosome containing BrdU-labeled immortal DNA strands will retain in cancer stem cells in a certain time, while other labeled non-immortal DNA strands will be assigned to common tumor cells and lost following cancer cell differentiation. In 2007, Seigel *et al.*[9] cultured retinoblastoma cell line Y79 with BrdU for 7 days, washed away BrdU, and cultured cells for another 14 days. Under immunofluorescence microscopy, nearly all cells were BrdU-positive on the 7th day, but only sporadic cells were BrdU-positive on the 14th day after being washed out BrdU. These cells were considered to be LRCs cells. There is no report using this method to detect LRCs in NPC. In the present study, we cultured 5-8F cells with BrdU for 7 days, applied immunohistochemisty and immunofluorescence staining on the 48th hour and the 7th day respectively, and found that BrdU incorporated into nearly all cellular DNA on the 48th hour, all cells still showed positive staining on the 7th day because of the persistence of BrdU, only a few cells were BrdU-positive on the 14th day after being washed away BrdU. In 10 high power fields, positive cells accounted for 0.67%, which were LRCs. The results are consistent with the those in retinoblastoma report. Zhang *et al.*[10] has proved the existence of LRCs in mouse nasopharyngeal epithelia and NPC cell line by applying BrdU for in situ and xenograft labeling in adult stem cells of mice nasopharyngeal epithelium and NPC cancer stem cells. We cultured 5-8F cells for 48 h with BrdU and injected them into the bilateral armpit of nude mice for tumor formation. After 8 weeks, tumors were extracted and detected. The results showed that there were sporadic LRCs (0.55%) in xenografts, which were similar to that Zhang *et al.*[10] reported. However, whether these cells have the characteristics of stem cells needs to be further confirmed from the aspects of high tumorigenicity, sphere formation and co-expression of BrdU with other stem cell surface markers.

In summary, our study confirms the existence of NPC stem cells in view of LRCs.

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