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Establishment and characterization of a novel HPV-negative laryngeal squamous cell carcinoma cell line, FD-LSC-1, with missense and nonsense mutations of TP53 in the DNA-binding domain

Chun-Ping Wu [a], Liang Zhou [a,*], Hong-Li Gong [a], Huai-Dong Du [a], Jie Tian [b], Shan Sun [b,c], Jin-Yan Li [d]

[a] Department of Otolaryngology-Head and Neck Surgery, Eye, Ear, Nose and Throat Hospital, Fudan University, Shanghai 200031, China
[b] Research Center, Eye, Ear, Nose and Throat Hospital, Fudan University, Shanghai 200031, China
[c] Institute of Biomedical Science, Stem Cell and Regenerative Medicine, Shanghai Medical College, Fudan University, Shanghai 200032, China
[d] Department of Cellular and Genetic Medicine, Shanghai Medical College, Fudan University, Shanghai 200032, China

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ABSTRACT

Laryngeal squamous cell carcinoma (LSCC) is a common malignancy in China; however, publically available LSCC cell lines are few and not established from Chinese populations. Hence, novel and well-characterized LSCC cell lines of Chinese origin are urgently needed to provide researchers with a comprehensive database for LSCC research. From 40 cases of LSCC, we established a novel cell line that was maintained for more than 100 passages *in vitro* and was found to have typical epithelial morphology and ultrastructure. In-depth characterization analysis revealed polyploidy in DNA content; a doubling time of some 24 h; high tumorigenicity in immunodeficient mice; higher invasive potential and more sensitive to radiation and cisplatin compared with HeLa cell line; upregulated Ki67, Notch1, EGFR, and CK5 protein levels; negative infection of human papillomavirus (HPV) and mycoplasma; expression of head and neck squamous cell carcinoma (HNSCC) biomarkers; mutations of TP53 in exons 5 and 8; a near-triploid karyotype with complex structural aberrations; and dozens of dysregulated genes and miRNAs. Cell authentication testing by the American Type Culture Collection (ATCC) confirmed the human origin of this cell line. Our findings indicate that a novel and well-differentiated LSCC cell line recapitulating the primary tumor's malignant characteristics is established and well characterized. It does not match any cell lines within the ATCC database and helps to elucidate the molecular pathogenesis of LSCC.

1. Introduction

Laryngeal squamous cell carcinoma (LSCC) is one of the most common malignancies of the head and neck. The advanced stages are still associated with devastating prognosis and real progress in treatment has still not been achieved, despite advanced surgical techniques, new chemotherapeutic drugs and improvements in radiotherapy within the last 30 years [1].

Permanent cancer cell lines are powerful tools for basic and preclinical cancer research and play an important role in elucidating the molecular mechanism of carcinogenesis, and testing effective therapeutic reagents. However, genetic abnormalities in human cancers are largely geographically dependent, and cultural and environmental backgrounds are thought to be closely associated with the carcinogenic process [2]. Therefore, different mechanisms of carcinogenesis and characterizations may occur in cancers from different regions. Although a few LSCC cell lines, represented by HEp-2, are publically available, they are rarely compared with other solid tumors. In addition, these cell lines are not established from Chinese populations, despite LSCC being a common malignancy in China. The HEp-2 cell line was the most widely used cell line in LSCC research; however, it was established some 60 years ago, and was reported to be contaminated by cervical carcinoma cell line HeLa [3,4], which was recently confirmed by the American Tissue Culture Collection (ATCC) (http://www.atcc.org). AMC-HN-3, AMC-HN-7, and AMC-HN-8 were reported in 1997 [5]. SNU-48, SNU-585, SNU-899, SNU-1066, SNU-1076, and SNU-1214 were documented in 1999 [6]. Newly established LSCC cell lines are even less common. To combat this ongoing disease with insufficient, invariable or contaminated cancer cell lines is unwise. Therefore, the ongoing development of newly established LSCC cell lines with different geographical characterizations is urgently needed to respond effectively to not only the changing cancer, but also changes in cultural and environmental factors. By establishing novel LSCC cell lines representative of the Chinese population, a comprehen-

* Corresponding author. Address: Department of Otolaryngology-Head and Neck Surgery, Eye, Ear, Nose and Throat Hospital, Fudan University, 83 Fen Yang Road, Shanghai 200031, China. Tel.: +86 21 64377134; fax: +86 21 64377151.
E-mail address: zhoulent@126.com (L. Zhou).

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sive database would be available to elucidate the etiology and molecular pathogenesis of LSCC.

As a part of this process, we established a new human LSCC cell line, designated FD-LSC-1, from the epiglottis of a Chinese donor, although most of the LSCC cell lines are established from the glottis of the larynx. We subsequently investigated cell morphology, ultrastructure, DNA content, doubling time, tumorigenicity, invasion, therapeutic sensitivity, protein levels (Ki67, Notch1, EGFR, and CK5), human papillomavirus (HPV) and mycoplasma infection, mutation of 53 gene, cytogenetic karyotype, head and neck squamous cell carcinoma (HNSCC) biomarkers, and dysregulated genes and miRNAs involved in HNSCC.

2. Materials and methods

2.1. Ethics statement

Tumor specimens were obtained with the approval of the ethics committee of the Eye, Ear, Nose and Throat Hospital, Fudan University, Shanghai, China. Signed informed consent was obtained from each patient. Animal care and experimental protocols were approved by the Shanghai Medical Experimental Animal Care Committee. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.2. Cell line used as control

The HPV-positive cervical carcinoma cell line HeLa was obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (CBTCCCAS, Shanghai). The cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA), and supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) in a humidified 5% CO_2 incubator at 37 °C. The medium was renewed every 3 days.

2.3. Primary culture and establishment of FD-LSC-1 cell line

A surgically resected tumor specimen, deriving from the epiglottic neoplasm of a 68-year-old Chinese male undergoing laryngectomy, was immediately immersed in cold, triple antibiotic phosphate-buffered saline (PBS) containing 1% penicillin/streptomycin and amphotericin B (10 μg/ml) (Invitrogen). The specimen was then washed three times, and scissored into small fragments, which were then dissociated enzymatically in RPMI 1640 medium containing type IV collagenase (Sigma, Saint Louis, MO, USA) at a final concentration of 200 U/ml for 12 h at 37 °C. After digestion, the cells and remaining fragments were rinsed twice and suspended in BEGM™ Bronchial Epithelial Cell Growth Medium (Lonza, Walkersville, MD, USA), supplemented with 10% FBS, 1% penicillin/streptomycin (Invitrogen), and 1% 200 mM L-glutamine (Invitrogen). The suspension was then seeded in 60-mm Petri dishes in a humidified 5% CO_2 incubator at 37 °C. After 3–4 days of incubation, some of the fragments and cells adhered to the dish; those that did not adhere were washed away before the medium was renewed. Cancer-associated fibroblasts (CAFs) were removed by brief exposure to 0.25% trypsin–EDTA (Invitrogen). Cancer cells near confluence were trypsinized and subcultured. Cells were stored in liquid nitrogen at regular intervals, starting from passage 1. Paired normal laryngeal epithelia (PNLE) of the same patient cultured in the same conditions served as a control.

2.4. Morphological examination and transmission electron microscopy

Cultured FD-LSC-1 and PNLE cells were photographed under a phase-contrast microscope. For Giemsa staining, cytospins of FD-LSC-1cells were washed three times with PBS, fixed in methanol for 10 min and stained with crystal violet for 10 min at room temperature. Cytospins of PNLE cells were stained with cytokeratin (CK) (pan) (1:50; Maixin Biotech, Fuzhou, China) to validate their epithelial origin, as described in the following immunochemical staining section. For transmission electron microscopy, a pellet obtained from the harvested FD-LSC-1cells at passage 26 was fixed with 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide (OsO_4). Dehydration was done through a series of graded alcohols, and the sample was embedded in resin. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and observed under a Philips CM120 transmission electron microscope.

2.5. Flow cytometry for purity and DNA content

FD-LSC-1 cells of passages 30–33 were used. To ensure purity of the assay, cells were trypsinized, washed in PBS, fixed in 4% paraformaldehyde (PFA) for 10 min, and incubated in 1% bovine serum albumin (BSA)/10% normal goat serum/0.3% Triton X-100 (Boster, Wuhan, China) at room temperature for 40 min to permeabilize the cells and block non-specific interactions, followed by the incubation in PBS containing CK(pan)–FITC antibody (1:500, clone C-11; Sigma) for 30 min at room temperature. Cells were then washed twice with PBS and analyzed using a CyAn ADP flow cytometer (Beckman Coulter, Fullerton, CA, USA). For DNA content assay, exponentially-growing FD-LSC-1 and HeLa cells were harvested and processed as previously described [7]. Normal human lymphocytes were processed as an internal standard. Samples were analyzed using a flow cytometer. To determine the percentage of cells in the proliferation phase, the proliferation index (PI) was measured using the formula PI = (G2 M + S)/(G0G1 + S + G2 M). The S-phase cell fraction (SPF) reflects the percentage of cells in the S-phase, and was measured using the formula SPF = S/(G0G1 + S + G2 M).

2.6. Growth rate assay for doubling time

FD-LSC-1 cells of passages 48–50 were seeded in 96-well plates at a density of 5×10^3 and 1×10^4 cells/well in 6 replicates in 0.2 ml medium, and a medium without cells served as a control. At hours 0, 12, 24, 36, 48, 60, and 72 after seeding, the growth rate was assayed in 6 wells using the Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). The incubation lasted 2.5 h, and the assay was conducted in triplicate. The ultraviolet absorbance was measured at the wavelength of 450 nm.

2.7. Heterotransplantation in immunodeficient mice

Six-week-old male non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice, fed in laminar flow cabinets under specific pathogen-free conditions, were purchased from Slac Laboratory Animal Company (Shanghai, China). Approximately 1×10^7 viable FD-LSC-1 cells of passage 47 suspended in 0.2 ml PBS were injected into the subcutaneous space of the right armpit of each animal ($n = 3$). Four weeks after implantation, tumors were removed and fixed in 10% formalin, embedded in paraffin, and sectioned for hematoxylin and eosin (HE) staining.

2.8. Migration and invasion assay

Briefly, 2×10^4 FD-LSC-1 cells of passage 58–60 and HeLa cells were suspended in 200 μl of serum-free medium and plated in the upper chamber of 24-well transwell chambers (pore size, 8 μm) (Corning Incorporated, Corning, NY, USA) coated with Matrigel (BD Biosciences, Bedford, MA, USA). The lower chamber was filled with 600 μl of RPMI 1640 or BEGM, supplemented with 10% FBS, and the plates were then incubated for 24 h. The cells on the top membrane surface were removed with a cotton swab, and those cells that migrated through the Matrigel were fixed with methanol for 15 min, stained with 0.1% crystal violet for 15 min, rinsed with PBS, and counted under microscope. Ten low power fields (×100) were randomly selected for counting.

2.9. Radiation sensitivity assay

FD-LSC-1 cells of passage 58–60 and HeLa cells were seeded at 5×10^3 cells/well in 96-well plates and cultured in BEGM or RPMI 1640 medium, supplemented with 10% FBS. The cells were irradiated with 0, 5, 10, 15, or 20 Gy of X-ray radiation on the day of seeding and each group was repeated in 6 wells. The radiation of 0 Gy served as the control. An additional 6 wells, containing medium only, served as a blank control. After being cultured for 48 and 72 h, the cells were assessed by incubating with CCK-8 for 2.5 h. The radiation inhibition rate (IR) was evaluated using the formula IR = 100% − survival rate (SR), and the SR was measured using the formula SR = (mean absorbance of the test wells/mean absorbance of the control wells) × 100%.

2.10. Drug sensitivity assay

FD-LSC-1 cells of passage 58–60 and HeLa cells were seeded at 5×10^3 cells/well in 96-well plates and cultured in BEGM or RPMI 1640 medium (supplemented with 10% FBS) containing cisplatin (DDP) (Pharmaceutical Factory, Nanjing, China) in a concentration gradient (0, 0.5, 1, 3, 5, 7, 9, 11, 13 μg/ml). Each concentration was repeated in 6 wells. The concentration of 0 μg/ml served as the control. An additional 6 wells, containing medium only, served as a blank control. After being cultured for 24 and 48 h, the drug IR was assessed using CCK-8 and calculated as mentioned previously.

2.11. Western blot for Ki67, Notch1, EGFR, and CK5

About 50 μg of protein samples from sonicated cell lysates of FD-LSC-1 and PNLE cells were analyzed by electrophoresis using sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) (Beyotime, Shanghai, China), electrotransferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA), and probed overnight with primary antibody for Ki 67 (1:500; Epitomics, Burlingame, CA, USA), Notch1 (1:500; Sigma), EGFR (R-1) (1:200; Santa Cruz, CA, USA), and CK5 (1:500; Anbo, San Francisco, CA, USA). Goat anti-mouse/rabbit/rat IgG (H + L)