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Establishment of a highly metastatic tongue squamous cell carcinoma cell line from New Zealand White rabbit

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ABSTRACT

Objective: Prior to this study, the widely used tongue squamous cell carcinoma cell lines could only initiate tumours in immunodeficient mice, which greatly delayed studies on immune function during carcinogenesis. This study established a new tongue squamous cell carcinoma cell line named 'RSCC-1', which can initiate tumours in both immunocompetent rabbits and immunodeficient nude mice and has high metastatic ability.
Design: Primary tongue cancer was induced by DMBA and local mechanical stimulation in New Zealand White rabbits. The induced cancer was serially transplanted into homogeneous rabbits to establish transplanted models. At the same time, cancer samples were collected, cultured and passaged in vitro. Finally, a cell line named 'RSCC-1' was established. Its growth behaviour, cell cycle distribution and tumourigenicity in rabbits and nude mice were investigated.
Results: RSCC-1 cells were cultured continuously in vitro for 19 months (165 passages). They contain between 54 and 196 chromosomes, with a modal number of 75. Tumourigenicity rates were 100% in both homogeneous rabbits and nude mice, with 20% lung metastasis and 50% cervical lymph node metastasis in homogeneous rabbits.
Conclusion: RSCC-1 is a poorly differentiated, highly malignant rabbit tongue squamous cell carcinoma cell line. Its behaviour in the inoculated animal model closely resembles human tongue cancer, and could metastasise to local lymph nodes and remote organs.

1. Introduction

Animal models of human tongue squamous cell carcinoma have played an important role in gaining understanding of the mechanisms of carcinogenesis, and accelerated the search for new molecular targets of cancer therapy. However, due to technical obstacles, most established tongue squamous cell carcinoma cell lines can only initiate tumours in immunocompromised nude mice.[1–10] No cell lines that can initiate tongue cancer in immunocompetent large animals have been reported. As such, the authors used DMBA and mechanical stimulation in New Zealand White rabbits to induce tongue cancer. The induced tongue cancer was transplanted serially into homogeneous rabbits to establish transplanted cancer models. Cancer samples were collected and primary culture was performed. Finally, a highly metastatic cancer cell line

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named 'RSCC-1' was established. RSCC-1 cells were cultured continuously in vitro for 19 months (165 passages). This is the first cell line known to initiate tongue cancer in immunocompetent animals.

2. Materials and methods

2.1. Establishment of animal models

Tongue cancer models were established by repeated local mechanical stimulation (with endodontic nerve broaches) and DMBA application on the front two-thirds of the tongue mucosa of 100 New Zealand White rabbits (provided by the Experimental Animal Centre, Chinese Academy of Sciences, Shanghai, China), twice a week for 32 weeks. Pathological examination revealed that the induced cancers were of epithelial origin. Cancer samples were collected, cut into 1-mm^3 pieces and passaged serially into 500 additional New Zealand White rabbits, 5–10 rabbits each time, to establish transplanted cancer models. After 70 passages, the tumour take rate reached 100%. Lymph node and lung metastases were examined in 20 rabbits at the 70th passage. The animal studies were approved by the Animal Welfare Committee of Fudan University, and all animals received optimal care and treatment.

2.2. Primary culture

Tongue cancer-bearing rabbits were anaesthetised with Sumaxin (0.1 mg/kg) (provided by Changchun Veterinary Institution, Changchun, China). Aseptic fresh cancer samples (1 cm^3) were collected in the operating room, and maintained in normal saline with 100 U/ml penicillin, 100 μg/ml streptomycin and 2 μg/ml amphotericin for 2–3 h at 4 °C. Samples were washed three times with normal saline and DMEM medium to eliminate blood. After that, cancer samples were cut into small pieces (1 mm^3) and placed evenly at an average distance of 2–2.5 mm into transwell chambers (12.0 μm pores) in six-well plates (Corning Co., USA), according to the manual for cell migration assay.[2] DMEM (with 20% New Zealand White rabbit serum, 200–1000 U/ml penicillin, 200 mg/l streptomycin and 2 mg/l amphotericin) medium was added. Samples were cultured in a 5% CO_2 incubator at 37 °C. All the reagents, unless specifically mentioned, were bought from Gibco, Auckland, USA.

2.3. Establishment of cell line

Cancer cells began to grow slowly 5 days after seeding. According to the growth speed, cultures were examined every 3–5 days to observe cell growth, the pH of the medium and to rule out infections. The medium was changed when necessary. Passage was carried out when cells in the lower chamber reached 80% confluence. Rapidly proliferating fibroblasts were eliminated by trypsin digestion and repeated anchoring,[4] and the culture was purified after 15 passages.

2.4. Morphology characteristics

After 28–30 passages, the cell morphology and population doubling time of RSCC-1 cells became very stable in culture, so the authors began to record the morphology characteristics of RSCC-1 cells. At the 30th passage, cells were observed and recorded using an inverted phase-contrast microscope. At the 35th passage, cells were spread on slides, fixed with 10% formalin, stained with haematoxylin and eosin (HE), and observed under a light microscope. At the 60th passage, cells were inoculated in 25-cm^2 culture bottles and six-well dishes with pre-placed slides. On the second day, cells were pipetted, fixed with 2.5% glutaraldehyde and 1% osmic acid to prepare ultra-thin sections, and observed with a transmission electron microscope. Cell climb slides were also fixed, coated in gold and observed with a scanning electronic microscope.

2.5. Immunohistochemical staining

At the 28th passage, cells were cultured in dishes with pre-placed slides at 37 °C, 5% CO_2 for 36–48 h. Slides were washed three times with phosphate-buffered saline (PBS) and fixed (ice-cold ethanol:ice-cold acetone 1:1). Traditional two-step EnVision (DAKO, Glostrup, Denmark) staining methods were carried out to investigate the expression of cytokeratin and vimentin. The primary antibodies were polyclonal rabbit anti-cow cytokeratin wide spectrum (dilution 1:500, DakoCytomation, CA, USA) and monoclonal mouse anti-vimentin (clone Vim3B4, dilution 1:200, DakoCytomation, CA, USA). Normal oral epithelial cells (primary culture of normal rabbit oral mucosa) were used as the normal control and the primary antibody was substituted by PBS in the blank control.

2.6. Growth characteristics

At the 57th passage, cells were collected and resuspended at a concentration of 1×10^4/ml, and seeded in 24-well plates with 1-ml suspension per well. The cell number of three random wells was counted during the following 1–8 days, and a growth curve was plotted using the seeding day as the horizontal axis and the cell number as the vertical axis. Doubling time was calculated using the following formula: $PDT = t \times [\log 2/(\log N_t - \log N_0)]$, with N_0 and N_t representing the cell number at the beginning and at t hours after seeding.

2.7. Distribution of cell cycle

At the 57th passage, cells in logarithmic growth phase were collected and fixed with 75% ice-cold ethanol. One millilitre of PI staining solution (with 50 mg/l PI and 50 mg/l Rnase) was added per 2×10^6 cells. The mixture was incubated for 30 min in the dark at room temperature, and analysed using flow cytometry (BD FACSCalibur).

2.8. Chromosome analysis

At the 70th passage, cells in logarithmic growth phase were treated with 3 μl colchicine 0.04 mg/l and cultured at 37 °C, 5% CO_2 for 3 h. Cells were collected, treated with 0.075 mmol/l KCL hypotonic solution, fixed in methanol and glacial acetic acid (3:1), and spread on slides. After air-drying and Giemsa staining, 100 well-dispersed and intact nuclei in metaphase were chosen to count the number of chromosomes under an oil lens for karyotype analysis.