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Identification and characterization of a highly metastatic epithelial cancer cell line from rat tongue cancer

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ABSTRACT

Objectives: Tongue squamous cell carcinoma (TSCC) is a clinically devastating disease. However, most established TSCC cell lines currently show undesirable malignant behaviours. The purpose of this study is to establish a highly metastatic TSCC cell line to serve as a useful tool for basic research.

Materials and methods: TSCCs were induced by 4-nitroquinoline-1-oxide (4NQO) in Sprague-Dawley rats. Tumor cells were obtained from the cancer tissues by primary culture and were then purified by an *in vitro* invasion assay and a limiting dilution assay. The growth rate, cell cycle distribution, apoptotic rate, tumorigenicity and distant metastatic phenotypes of the rat tongue cancer cells were fully investigated and characterized.

Results: To date, the rat tongue cancer cell line, named Rca-T, has been continuously cultured *in vitro* for over 210 passages and exhibit a long spindle-shaped morphology, adherent growth, and a stable epithelial phenotype. The population doubling time of Rca-T cells is 23.35 h. Approximately 39.8% of these cells are in S phase, and the apoptosis rate of Rca-T cells is 7.46%. Furthermore, in immunodeficient nude mice, both the xenograft rate and the incidence of experimental lung metastasis are 100%. The *in vitro* assays further reveal the highly malignant and epithelial-mesenchymal transition-like properties of Rca-T cells.

Conclusion: In this study, the tumorigenic and highly distant metastatic TSCC cell line Rca-T was established. The malignant features of this cell line, especially its metastatic potential, will enable a wealth of functional studies on the molecular mechanisms of TSCC metastasis in the future.

1. Introduction

Globally, oral squamous cell carcinoma (OSCC) is a common malignancy that remains a significant burden to patients in terms of diagnosis, treatment and prognosis (Mignogna, Fedele, & Lo Russo, 2004; Parkin, Pisani, & Ferlay, 1999; Rodrigues, Moss, & Tuomainen, 1998; Sinevici & O'Sullivan, 2016). Tongue squamous cell carcinoma (TSCC), a major component of OSCC, typically occurs in older individuals (50–80 years old) and is always associated with exposure to common risk factors such as tobacco and alcohol (Krolls & Hoffman, 1976). The overall incidence of TSCC in younger patients has recently increased and studies have shown that TSCC in younger patients presents poorer clinical outcomes than those in older patients (Bodner, Manor, Friger, & van der Waal, 2014; Goldstein & Irish, 2005; Hollows, McAndrew, & Perini, 2000; Park et al., 2010; Sasaki, Moles, Imai, & Speight, 2005). TSCC is a devastating disease with highly metastatic potential, and

Abbreviations: TSCC, tongue squamous cell carcinoma; 4NQO, 4-nitroquinoline-1-oxide; OSCC, oral squamous cell carcinoma; PBS, phosphate-buffer saline; FBS, fetal bovine serum; PDT, population doubling time; EMT, epithelial-mesenchymal transition

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patients suffering from this disease typically die as a result of local metastatic tumor growth (Ferlito et al., 2002; Leemans, Tiwari, Nauta, van der Waal, & Snow, 1994). Hence, the exploration of the aetiology and the mechanisms of TSCC progression, especially tumor metastasis, remain a hot topic in the field.

Chemical carcinogen-induced OSCC in animal models is a well-known system designed to assist us in further understanding the events leading to the development of OSCC in human patients. In addition, tumor cell lines derived from these animal models are valuable for investigating the molecular mechanisms and therapeutic targets of OSCC. Moreover, at present, most established TSCC cell lines are used only for xenograft assays in immunocompromised nude mice, and the cell lines that can form lung metastasis are rare (Bindels & van den Brekel, 2005; Hawkins et al., 1994). Hence, the establishment of several TSCC cell lines with high metastatic potential is needed for the clinical and experimental study of TSCC.

The 4-nitroquinoline 1-oxide (4NQO) TSCC model was considered as the most reliable simulator of OSCC development in human patients (Mognetti, Di Carlo, & Berta, 2006; Vered, Allon, Buchner, & Dayan, 2007). In our previous study (Qin, Yan, Zhang, Xu et al., 2016), we successfully generated an animal model of OSCC by adding 4NQO into the drinking water of Sprague-Dawley (SD) rats, and we established a buccal SCC cell line named Rca-B (Qin, Yan, Zhang, Xu et al., 2016). During this study, we found that a large number of SD rats suffered from TSCC, and we established a malignant TSCC cell line with high metastatic potential.

In the present study, cancer tissues were collected from the 4NQO-induced TSCC model, and then, primary culture and other purification techniques were performed to establish a highly metastatic TSCC cell line named Rca-T. Rca-T cells were continuously cultured *in vitro* for over 210 passages and its malignant biological characteristics, especially the highly metastatic potential, were extensively characterized. This cell line shows promise as a useful tool that will provide valuable approaches for metastasis-associated investigation and treatment in TSCC.

2. Materials and methods

2.1. Ethics

The Ethics Committee of Shanghai Jiao Tong University approved this study. All animal studies were approved by the Shanghai Jiao Tong University Institute Animal Care and Use Committee, and all mice were kept in the Shanghai Jiao Tong University School of Medicine animal facilities.

2.2. Establishment of the 4NQO TSCC model

4NQO (Sigma, USA) powder was dissolved in the rats' drinking water to a final concentration of 0.02 g/L, according to previously published rodent studies (Hawkins et al., 1994; Jiang et al., 2007; Ohne, Satoh, Yamada, & Takai, 1985). Eighty age-matched adult SD rats (Shanghai Laboratory Animal Center), weighing 200–220 g and aged 6–7 weeks, were housed in a temperature- and light-controlled environment with a 14/10-h light/dark cycle and fed 4NQO-added drinking water for 36 weeks. Another 20 SD rats received pure water (control group).

2.3. Primary culture

Using aseptic technique, the fresh TSCC samples were collected immediately after the animals were sacrificed under general anaesthesia and were incubated in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO-BRL, USA) supplemented with 300 units/mL penicillin and 300 μg/mL streptomycin for 3 h at 4 °C. After washing 3 times with phosphate-buffered saline (PBS), the tumor tissues were cut into sections (0.5 mm^3). The tissue fragments were then transferred into a culture flask (25 cm^2) containing 5 mL of DMEM supplemented with 10% fetal bovine serum (FBS; GIBCO-BRL, USA), 100 units/mL penicillin and 100 μg/mL streptomycin and were maintained at 37 °C in 5% CO_2.

2.4. Establishment of cell line and cell culture

As previously described (Kudo et al., 2004; Qin, Yan, Zhang, Xu et al., 2016), an *in vitro* invasion assay was performed to select the tumor cells with highly metastatic features from the mixed tumor cell population. Then, a limiting dilution assay was implemented to obtain a mono-clone cell line of TSCC. After continuously passaging cultures for 3 months (30 passages), the TSCC cell line was considered "established".

The TSCC cells were cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. To identify the distinct malignant behaviours of this TSCC cell line, the SD rat-derived buccal SCC cell line Rca-B (Qin, Yan, Zhang, Xu et al., 2016) was used as the comparison OSCC cell line. Control cells were also cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO_2 atmosphere.

2.5. Morphological characteristics

After 30 passages, the cell morphology of TSCC cell line cells in culture became very stable; as such, the morphological characteristics of these tumor cells were observed and recorded using an inverted phase-contrast microscope. Briefly, tumor cells were seeded onto climb slides and incubated for 24 h; the cells were then fixed with 4% paraformaldehyde, stained with haematoxylin, and observed under a light microscope.

2.6. Immunohistochemical staining

To analyze the expressions levels of different proteins in tumor cells, cells were seeded on preplaced climb slides, fixed with 4% paraformaldehyde and incubated with 3% H_2O_2. For the immunohistochemical staining of tissue sections, formalin-fixed, paraffin-embedded tissues were cut into 4 μm tissue sections. Then, the sections were deparaffinized with xylene, rehydrated in graded ethanol, submerged into Tris-EDTA buffer and microwaved for antigen retrieval.

The slides or tissue sections were blocked with 2.5% goat serum and incubated overnight at 4 °C with primary antibodies. The slides were developed with a DAKO ChemMate Envision Kit/HRP (Dako-Cytomation, USA) according to the manufacturer's protocol, followed by counterstaining, dehydration, clearing and mounting with neutral gums. Paraffin-embedded TSCC tissue sections were stained with hematoxylin and eosin (H&E) after dewaxing and hydration. Three randomly selected tumor areas of every specimen under the same conditions were captured for further analysis. Protein expression was quantitatively determined using Image J software, and calculated using the following formula: MOD = IOD SUM/area SUM (IOD: integrated optical density; MOD: mean optical density; IOD SUM: the accumulative IOD of targeted areas in one photo; area SUM: the sum of targeted areas). Primary antibodies were used at the following dilutions: mouse polyclonal Pan-CK antibody (Ascend, China; 1:100); mouse monoclonal Vimentin antibody (Sigma, USA; 1:200); mouse monoclonal Ki-67 antibody (eBioscience, USA; 1:200) and rabbit polyclonal S-100 antibody (Ascend, China; 1:500). PBS was used as the primary antibody in the blank control.

2.7. Cell proliferation assay

Briefly, tumor cells were collected and seeded onto 96-well plates in

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