Oral Oncology 50 (2014) 991–999

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Development and characterization of salivary adenoid cystic carcinoma cell line

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ARTICLE INFO

Article history:
Received 27 March 2014
Received in revised form 4 June 2014
Accepted 12 June 2014
Available online 30 July 2014

Keywords:
Adenoid cystic carcinoma
Cell line
Salivary gland tumors

SUMMARY

Objective: To develop in vitro adenoid cystic carcinoma cell line as a surrogate for functional studies.
Materials and methods: Cells obtained from a primary ACC of the base of tongue were cultivated in vitro and immortalized with h-TERT. Morphologic, cytogenetic and functional studies were performed.
Results: Tumor cells were verified by positive reactions to keratin and smooth muscle actin and phenotypic cellular and nuclear features. In-vitro cell growth and colony formation assay supported their tumor nature.
Conclusion: We authenticated an ACC cell line with hybrid epithelial–myoepithelial feature as a resource for functional experimentation.

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Introduction

Adenoid cystic carcinoma

Adenoid cystic carcinoma (ACC) is a rare salivary gland malignancy characterized by distinctive phenotypic features and indolent and variable biological behavior [1]. Histologically, ACC displays three morphologic patterns and dual cell composition of peripheral myoepithelial and inner ductal of the tubular and cribriform patterns. Invariably, two of these patterns are present in any given tumor [2–5]. In both of these patterns, cellular polarity and the extra-cellular matrix deposition have been attributed to the presence of myoepithelial cells. This is empirically supported by the coincident loss of myoepithelial cells and the lack of structural polarization and accelerated clinical progression of the solid form of ACC [6–8]. The primary treatment of ACC is complete surgical excision with post-operative radiotherapy for tumor with adverse pathologic features. Approximately 45% of patients with ACC experience either recurrence or metastatic disease within 10 years [9].

The lack of effective therapy for patients with non-surgical recurrent and metastatic disease mandates the development of novel approaches to advance research and clinical management of this entity. Current efforts to investigate the biological and genomic characteristics of these tumors have been constrained by the lack of authenticated cell line that faithfully represents at least some of the primary tumor characteristics. This deficiency has largely been due to the rarity, slow growth rate and the tediousness of cultivating cells from these tightly adherent tumors [10–13]. Notwithstanding, only few ACC cell lines have been reported and experimentally used, the authenticity of these cell lines have been seriously questioned [14–18]. Central to the validation and verification of cell lines in general and ACC in particular, is the availability of primary tumors from which these cell lines are developed.

Our aim is to establish a well-characterized cell line(s) that faithfully represents at least some of the properties of tumors from which they are derived.

Materials and methods

Tumor

A fresh tumor tissue fragment from adenoid cystic carcinoma of the base of tongue from a 48 year old male was resected. Under approved Institutional Review Board protocol and obtained consent (Protocol ID # 709540), a portion of the tumor was collected in sterile RPMI medium and processed for research.

Tissue culture

The fresh tumor tissue was minced with cross scalpel technique, gently squashed and suspended in rich RPMI-1640 medium

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http://dx.doi.org/10.1016/j.oraloncology.2014.06.012
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(Life Technologies), containing 15% fetal bovine serum (Life Technologies), 1% penicillin–streptomycin liquid (10,000 IU/ml penicillin G sodium, 10 mg/ml streptomycin sulfate in 0.85% saline) (Life technologies), 5 ng/ml epidermal growth factor (Sigma–Aldrich), 10 mg/ml hydrocortisone (Sigma–Aldrich) and 10 mg/ml insulin (Sigma–Aldrich). The minute fragments and cell suspension were transferred to T25 cell culture flasks (Corning) and incubated at 37 °C humidified incubators with an atmosphere of 95% air and 5% CO_2.

Maintenance of primary cell culture

A mixture of cells growing out from primary tumor pieces were trypsinized, suspended in rich RPMI-1640 complete medium and plated into T25 cell culture flasks, then asynchronous log-phase cell growth was maintained in 37 °C humidified 5% CO_2 incubators. Cells were fed by medium change to fresh medium 2–3 times a week. Cells were harvested in log-phase growth phase at 70–80% confluence level and plated in 1:5 ratios for new passage, keeping the cells in continuous log-phase cell growth. Cells from every 5th passage were cryopreserved by mixing the cell suspension in complete medium with freezing solution (20% DMSO + 80% FBS) in 1:1 ratio. Cryopreserved cells were frozen in Nalgene cryo box then stored in liquid nitrogen freezer.

Fibroblasts filtration

Fibroblasts have been positively selected and depleted by a fibroblast-specific antigen using Anti-Fibroblast MicroBeads from Miltenyi Biotec (MACS) following the manufacturer's instructions. In brief, collected cells were filtered to remove cell clumps and obtain single cell suspension. Cells were centrifuged and re-suspended in working buffer (MACS BSA stock solution and rinsing solution by 1:20 ration) and incubated with Anti-fibroblast Microbeads at 4 °C. After washing, cell suspension went through magnetic separation. The follow-through was collected containing cells without fibroblasts.

Mycoplasma testing

Cells were tested for Mycoplasma infection by both MycoAlert® Assay (Lonza Rockland, Inc.) using the cell culture growth medium or fluorescent microscopy of the cells using Mycoplasma Hoechst Stain Assay (MP Biomedicals).

Morphological assessment

The morphology of the asynchronous, exponentially growing cells in monolayer was reviewed and documented using Nikon Eclipse TE300 inverted phase contrast/fluorescence microscope (Nikon Instrument Inc.) and Photometrics Coolsnap camera using RS-Image Version 1.9.2 (Proper Scientific Inc.) capturing software.

Cell Growth Kinetics

Cells were plated in a series of triplicates at the density of 2×10^3 cells/well onto 6-well tissue culture plate (Corning Incorporated) and incubated in 37 °C humidified 5% CO_2 incubators for 24 h, then daily a plate was trypsinized and viable cells were counted using coulter counter (Beckman). The cell numbers were plotted and population doubling time was calculated from the resulting growth curve.

Purification and amplification of human telomerase transcriptase (hTERT) containing plasmid

pBABE-puro-hTERT plasmid (Addgene) was recovered from stab culture. Briefly, the constructed plasmid was grown in LB agar plate containing ampicillin. Single clone was isolated and plasmid DNA was recovered from bacteria cells. Recovered plasmid was verified and amplified following manufacturer's protocols.

Retroviral vectors and transduction

The full length hTERT cDNA was transfected into Phoenix Retroviral Packaging Eco cell line (Allele Biotechnology) using FuGENE6 transfection protocol (Promega). The viral supernatant was collected after 48-h culturing and was used to infect the amphotropic packaging cell line PA317 (ATCC). Infected PA317 were selected with puromycin and verified by reverse transcription-PCR. The retroviral particles containing supernatant produced by infected PA317 was used to infect tumor cells in the presence of polybrene (4 μg/mL). Tumor cells were selected in 1 mg/mL of puromycin 48 h after infection.

Reverse transcription-PCR

Total RNA was extracted using the mirVANA miRNA isolation kit (Ambion), according to the manufacturer's instructions. 2 μg total RNAs were reverse-transcribed into single stranded cDNA using Super Script III First-strand Synthesis System (Life Technologies). Primer designed to amplify the hTERT fragment: hTERT-F (5′-CGTGGTTTCTGTGTGGTGTC-3′) and hTERT-R (5′-TGGAACCCAGAAAGATGGTC-3′). The amplification was performed using the following thermal program: denaturation at 94 °C for 4 min; 30 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, and extension at 72 °C for 45 s; and final extension at 72 °C for 10 min. β-actin was used as a control. Expected length of the amplification product is 303 bp.

MTT proliferation assay

Cell viability and cell growth were measured by MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) assay. Briefly, cells were plated in 96-well plates (1×10^3 cells/well) in triplicates and incubated for up to 7 days, and then MTT solution (2 mg/ml) was added. After incubation, the blue dye was dissolved with dimethyl sulfoxide (100 μg/ml) and absorbance was measured at 595 nm using a 96-well multiscanner (SPECTROstar Nano, BMG Labtech).

Serum free cell culture

Serum containing a complete medium of mid-exponentially growing cells at passage 66 were converted by adaptation using a conditioned medium process to serum free human NeuroCult NS-A Basal Medium (StemCell Technologies) containing basic fibroblast growth factor (FGF-2) (20 ng/ml), epidermal growth factor (80 ng/ml), (Sigma–Aldrich), B27 supplement (1%), Gentamicin and Antibiotic Antimicotic mixture (0.1%) (Life Technologies). The cells were inspected daily under inverted microscope and then at 70% cell density the plates were trypsinized and passed into a new flask using serum free condition. When sphere formation was observed, the old serum free medium was centrifuged at $100\times g$ for 5 min and discarded and pelleted spheres were suspended in fresh serum free medium and added back to the original flask to continue to grow a mix of attached cell and sphere populations.

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