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Establishment and Genomic Characterization of Primary Salivary Duct Carcinoma Cell Line

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Abstract

Objectives—To develop and characterize in vitro salivary duct carcinoma as a surrogate for functional studies.

Materials and Methods—Cells were dispersed from tumor tissue fragments under sterile conditions in RPMI media. Disassociated cells were cultivated, immortalized with hTERT and propagated for more than 100 passages. Morphologic, lineage, cytogenetic and genomic analyses were performed on different passages of cell line and primary tumor. Soft agar growth was performed.

Results—Analysis of cytomorphologic features, growth characteristics and lineage specific markers expression confirmed the epithelial derivation and the neoplastic nature of the cell line. DNA STRs analysis showed identical match of both cell line and primary tumor. Cultivated cells expressed Androgen Receptor (AR), PTEN, and EGFR proteins and the V-7 isoform transcript. Comparative exome-sequencing identified common mutated genes in both cell line and primary tumor. In-vitro colony formation of late passages is established.

Conclusion—We report the development of the first human salivary duct carcinoma cell line (MDA-SDC-04) that retains critical biological and genomic features of the donor tumor.

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Keywords

Salivary duct carcinoma; cell line development; androgen receptor; salivary gland carcinomas; cell line validation

INTRODUCTION

Salivary duct carcinoma, a rare highly aggressive epithelial malignancy, commonly presents at advanced stage in elderly patients of both sexes.[1–3] The primary management of SDCs is surgery and/or post-operative radio-therapy while advanced primary and metastatic disease are empirically treated with either conventional chemo-radiotherapy or targeted agents with limited success.[4, 5] SDC, like mammary ductal carcinoma, manifest high expression of HER-2 and AR (Androgen receptors) and frequent alterations of the PI3K signaling pathway.[6–13] Investigations of the biological and therapeutic implications of these, and yet to be identified, targets in patients with SDC have been restrained by the lack of experimental ex-vivo models that faithfully represent primary tumors.

To accelerate cell line developments of this uncommon entity, systematic screening of patients with high grade primary salivary carcinomas for timely in-vitro cultivation is necessary. The National Institute of Dental and Craniofacial Research (NIDCR) has established and sustained the Salivary Gland Tumor Biorepository (SGTB) as a biobanking infrastructure for tumor tissues and patients' materials and to the development of cell lines representing these tumors.[14] Through the SGTB, a prospective protocol for pre-surgical screening of cytologically and/or clinically diagnosed primary salivary gland carcinomas of consented patients has been implemented for tissue acquisition. In-house patients with suspected primary high grade salivary carcinoma were systemically identified for timely tumor tissue acquisition under sterile conditions for cultivation.

We present the genomic and biological characteristics of the first successfully cultured and characterized cell line from primary untreated SDC.

MATERIALS AND METHODS

Primary tumor

Under an approved Institutional Review Board protocol (Protocol ID # 709540), a small portion of the resected fresh tumor mass was promptly harvested from a 5 cm in size primary salivary duct carcinoma of an 89-year-old male patient.

Primary culture and cell line establishment

Tumor tissue was minced and cell suspension was prepared as previously described.[14] Cells were cultured in a DMEM medium (Thermo-Fisher Scientific) containing 15% fetal bovine serum (Thermo-Fisher Scientific), 5 ng/ml epidermal growth factor (Sigma-Aldrich), 10 mg/ml insulin (Sigma-Aldrich), 10 mg/ml hydrocortisone (Sigma-Aldrich) and 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo-Fisher Scientific) in a 37°C incubator supplied with 95% air and 5% CO₂. Cells were fed by fresh medium 2–3 times a week and

sub-cultured at a split ratio 1:2 when they reached 80% confluence. Fibroblasts were depleted with fibroblast-specific antigen attached to anti-fibroblast MicroBeads (Miltenyi Biotec) following the manufacturer's instructions. Cell cultures were tested for the presence of mycoplasma using MycoAlert® assay (Lonza Rockland, Inc.).

Morphological examination and growth kinetics

Morphologic cellular changes were monitored and photographed under a phase contrast microscope (Nikon Instrument Inc.). Cells in early and late passage were studied to calculate the population doubling time (PDT). In brief, 10^5 cells at day 0 were plated onto a 6-well plate (Corning Incorporated, USA) in a series of triplicates. Cell numbers were counted at 24 hour intervals using coulter counter (Beckman). The growth rate was recorded and plotted and the PDT was calculated from the linear curve regression.

hTERT immortalization

Plasmid pBABE-puro-hTERT (Addgene) was recovered and plasmid DNA was amplified as previously described.[14] The hTERT containing retroviral vector was transfected into Phoenix Retroviral Packaging Eco cell line (Allele Biotechnology) using FuGENE6 transfection protocol (Promega). Viral supernatant was harvested after 48 hours of culturing, passed through 0.45 μ m syringe filter and used to infect tumor cells in the presence of polybrene (4 μ g/mL). Tumor cells were selected in 1000 ng/mL of puromycin 48 hours after infection. Total RNA from cells was extracted using the RNeasy Plus Universal Mini Kit (Qiagen, Inc.), according to the manufacturer's instructions. A total of 2 μ g RNAs were reverse-transcribed into single stranded cDNA using Super Script III First-strand Synthesis System (Thermo-Fisher Scientific). Amplification program comprised of 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. Primer designed to amplify the hTERT fragment: hTERT-F (5' - CGTGGTTTCTGTGTGGTGTC-3') and hTERT-R (5' - TGGAACCCAGAAAGATGGTC-3'). Expected length of the amplification product is 303 bp.[14]

RT-PCR

Total RNA was extracted from tissue samples and cell lines using RNeasy Universal Kit (Qiagen, Inc.). First-Strand cDNA synthesis was performed using 2 μ g of total RNA, oligo(dT) primer and the SuperScript III reverse transcriptase (Thermo-Fisher Scientific). AR full-length primers (5' -TGGATAGCTACTCCGGACCTTA -3' and 5' - GGAGTTGACATTGGTGAAGGAT-3') and AR-V7 specific primers (5' - CTACTCCGGACCTTACGGGGACATGCG-3' and 5' - TGCCAACCCGGAATTTTCTCCC-3') were used following previous report.[13]

Immunohistochemical staining

Immunocytochemistry was performed as previously described.[14] Cells were harvested and suspended in cold 1xPBS solution. Cytospin preparation of acetone fixed cells were

prepared and stained with Cytokeratin Cocktail (Dako), Anti-Androgen Receptor (Dako), Anti-PTEN (Dako), and Anti-EGFR (Thermo-Fisher Scientific) antibodies.

Cytogenetic analysis

Chromosome preparations and G-banded karyotypes were performed according to standard protocols. Chromosomal abnormalities identified by G-banding were described according to an International System for Human Cytogenetic Nomenclature (ISCN, 2013). Clonality was determined based on the detection chromosome gains in two or more cells and loss in three or more cells.

Short tandem repeat (STR) profiling

Genomic DNA from passage 22 and 46 samples and parental tumor tissues were isolated using the Gentra Puregene Tissue Kit (Qiagen, Inc.). A total of 14 STR loci (AMEL, CSF1PO, D13S317, D16S539, D18S51, D21S11, D3S1358, D5S818, D7S820, D8S1179, FGA, TH01, TPOX, vWA) were used with matching comparison of DNA fingerprinting analysis (CCSG Cell Line Core).

Exome sequencing

Exome sequencing of primary tumor tissue and different passages of cultures cells from DNA was isolated using the Gentra Puregene Tissue Kit and 2 µg genomic DNA and submitted to MD Anderson Cancer Center Genomic Core Facility. The Illumina HiSeq3000 sequencing system (Illumina Inc., San Diego, CA) was used for analysis.

Soft agar colony formation assay

Cell suspension of 1×10^5 cells in DMEM rich medium containing 0.3% agarose were seeded onto a base agarose concentration of 0.6% in triplicate in 6-well plate. Plates were incubated at 37 °C for 3–6 weeks. Plates were then stained with 0.05% crystal violet. Colonies with diameter greater than 0.2 mm were counted and photographed.

Xenograft transplantation in nude mice

In-vivo cell line injections were conducted on NSG mice (Jackson Laboratory) as previous described.[14] In brief, $1 \sim 5 \times 10^6$ cell suspensions were prepared in 100 µl of 1 X PBS solution with 100 µl of Matrigel Growth Factor Reduced Basement Membrane Matrix (BD Bioscience). Subcutaneous injections were performed on both flanks of anesthetized male 4–6 weeks old mice. Injected mice were monitored and examined for tumor development once a week.

RESULTS

Primary culture

Cells were cultivated, maintained and evaluated at multiple temporal points of more than 100 passages of cultivation. Cells grew at a faster rate in early passages with a PDT (population doubling time) of 55 ± 3 hr. (passage 7, Fig. 1A) and at a slower growth rate in late passages with PDT of 168 ± 3 hr. (passage 43 Fig. 1A). To stabilize cell growth, we

immortalized primary cell culture MDA-SDC-04 at passage 1 with hTERT transfection (Fig. 1B). Transfected cell line MDA-SDC-04 resumed growth rate at consistent PDT = 62 ± 3 hr. and underwent up to 100 passages without signs of senescence (Fig. 1A). MycoAlert® Assay was performed every ten passages with no evidence of mycoplasma organism.

Phenotypic evaluations

Cells characteristics were routinely examined and recorded by phase-contrast microscopy. Both early and late passages showed polygonal and satellite cell morphology and sparse adherent growth. (Fig. 1C). Morphologic and nuclear features included multinucleated cell formation and pleomorphic nucleoli in both non-transformed and transformed cells. (Inserts in Fig. 1C: b, d, f). hTERT transfected cells showed surface independent growth on solid agarose-coated plates and maintained continued growth without evidence of senescence during extended culture (> three weeks).

Karyotyping

Karyotypic analysis of the primary tumor and the first two passages revealed multiple non-clonal structural and numerical chromosomal aberrations. Subsequent multiple passages of both non-transfected and hTERT transfected cells showed normal male karyotypes. (Supplementary Figure)

Expression of epithelial and biological markers

Early cell line passages showed strong keratin CCK5/6 expression while later (>40) passages showed focal cytoplasmic staining (Fig. 2A: b). Analysis of the primary tumor and cultured cells demonstrated strong nuclear androgen receptor expression in both primary and cell line samples (Fig. 2A: c, d). The cell line mirrored primary tumor in strong expression of PTEN (Fig. 2A: e, f), and epidermal growth factor expression in tumor cells (Fig. 2A: g, h). HER2 expression showed faint and heterogeneous staining.

Transcript expression of androgen receptor and its isoforms

RT-PCR analysis showed the full length and the AR isoform AR-V7 in both tumor and of all passages of SDC cells (Fig. 2B).

Short-tandem repeat (STR) DNA fingerprinting profile

Fourteen STRs loci (AMEL, CSF1PO, D13S317, D16S539, D18S51, D21S11, D3S1358, D5S818, D7S820, D8S1179, FGA, TH01, TPOX, vWA) were tested. The STR profiles of both non-transformed and transformed cells were identical to the original tumor (Table 1, 8 core loci were shown). The STR profile showed unique pattern with no documented match in existing databases.

Exome sequencing analysis

Table 2 presents selected set of mutated tumor associated genes in both cell lines and primary tumor. Cell line passages and tumor shared 140 (40%) mutations at passage 40. Cell line specific mutations varied between passages with 121 (37%) mutations. Seventy-three (23%) 40 tumor specific mutations were also found at passage 40. Our exome-seq data was

also compared with cancer related genes in Cancer Gene Census database and some cancer driver genes were found in both primary tumor and all cell lines (Supplementary Figure 2).

Colony formation assay and xenograft

Cultured cells formed numerous colonies within 4 weeks (Fig. A). Subcutaneous injection of up to 1×10^7 cells of late passages into immune-deficient mice is being conducted with no evidence of tumor formation being detected yet after 4 months. Continuous efforts to induce tumor in nude mice are being made.

DISCUSSION

Identification and validation of novel therapeutic targets for patients with SDC are constrained by the lack of faithful ex-vivo surrogate models. Developing thoroughly characterized cell lines, from this rare cancer type is central to accelerating research and development.[15–19] We report the establishment and characterization of the first cell line derived from primary untreated salivary duct carcinoma. Importantly, the cell line shared lineage and molecular characteristics and both the full-length AR and the AR-V7 isoform with the parent tumor. Given the common finding of high AR nuclear expression in the majority of SDCs in both sexes and the lack of AR expression in normal salivary gland, the cell line is fundamental to investigate the mechanism and biological role of AR in patients with SDC.[11, 13, 20–23] In addition to AR, high expression of EGFR and PTEN proteins were also reflected their status in primary tumor providing additional markers for investigations.

The access to advanced second generation sequencing allows for detailed genome analysis of tumors and cell lines and the identification of critical genes and molecular events associated with tumorigenesis.[24] A major limiting factor in the use of available cell line is the lack of primary tumor materials for comparative and authentication purposes.[25–29] The availability of primary tumor for comparative genomic sequencing analysis with the cell line is important to in identifying potential key genes and for investigating their biological and therapeutic implication in SDC. In this study, the finding of common set of shared mutations between cell line and primary tumor not only provide additional novel genes for investigations but also underscore its faithful representation of donor tumor.[30–32] Notably, several cancer associated genes including the MAML2, HOXC13, TRIM24 and the NUTM2B genes were shared by cell line and donor tumor can further be targeted for their role in SDC tumorigenesis.[33–35]

Our findings are in agreement with previous comparative cell line and primary tumor studies of liver, lung and lymphoreticular malignancies. Substantial differences between cell line and corresponding tumors have been reported, however, and can be attributed to heterogeneity and/or tumor context differences.[25] Concordant with other studies, we observed restricted cell line alterations suggesting a selection and/or enrichment for a minor cell population in primary tumor. Failure to detect this population in primary is most likely due either to low depth sequencing or to random occurrence of mutation and/or acquisition of mutation required for sustained all growth.[34, 36] We, however, cannot firmly exclude

the possibility of low coverage sequencing as a cause. Tumor specific mutations were also found and could be explained by either intra- tumoral heterogeneity.

In conclusion, the cell line represents growth and tumorigenic considerable characteristics of the primary tumor and manifests colony forming ability for studies of SDC. Interestingly, the cell line lacked any of the chromosomal alterations identified in primary tumor suggesting that either the selective nature of cell culture on the finite proliferation capacity of cells with these alterations could play a role. The availability of materials from primary donor tumor for periodic verification of unique characteristics in cell line is critical asset in their broad and sustained utilization. The presence of both fresh frozen primary and archival tumor tissues, as well as samples of early cultured cells allows for periodic verification and authentication of this cell line over time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Development of the fully characterized salivary duct carcinoma cell line.
- This cell line retains considerable molecular and genomic characteristics of parent tumor.
- The cell line provides valuable in-vitro resource for investigating SDC.

Figure 1. Population doubling and morphology on hTERT immortalized and non-transformed cells

(A) Cells of late passage (p43, line in diamond) had increased population doubling time than cells of early passage (p7, line in square). hTERT transfected cells resumed the fast growth rate and PDT (p40-hTERT, line in triangle). (B) hTERT transformation of MDA-SDC-04 cell lines. RT-PCR result of hTERT gene expression on transformed cell line (hTERT-p2 and p20) and stayed stable through passages. The hTERT plasmid DNA, non-transformed cell line (MDA-SDC-04-p2) and β -actin were used as positive control, negative control and loading control, respectively. (C) Morphology of non-transformed and transformed MDA-SDC-04 cells. Phase contrast photomicrograph of the non-transformed cells in passage 7 (4x magnification in a and 10x in b), in passage 43 (4x magnification in c and 10x in d), and transformed cells in passage 7 (4x magnification in e and 10x in f). Both non-transformed and transformed cells contain high percentage of multinucleated cells (right upper inserts in b, d and f) and pleomorphic nucleolus cells (left lower inserts in b, d and f).

Figure 2. Photomicrographic of primary tumor and corresponding cell line phenotypic and biomarkers expression

(A) (a) Light-microscopic image of salivary adenocarcinoma. b) Cytokeratin stain of early passage of cell line with strong expression in cytoplasm of cultured cells. c & d) Androgen expression in primary (c) and cell line (d) displaying strong nuclear expression in both primary and cell line. e & f) PTEN expression in primary (e) and cell line (f) strong cytoplasmic and nuclear expression in both primary tumor and cell line. g & h) EGRF expression in primary (g) and cell line (h). High membranous and/or cytoplasmic expression in primary tumor (g) and cell line (h). (B) AR expression detected by RT-PCR. AR-fl and AR-V7 variants were expressed on both tumor and cell line but not on normal tissue. Vcap cells were used as positive control

Figure 3. Soft Agar Colony Formation Analysis of MDA-SDC-04 cells

(A) Both non-transformed and transformed cells formed small colonies after 4 weeks of incubation. Left, magnification 4x; Right, magnification 10x with single colony on upper right insert. Comparison of colony number (B) and size (C) between MDA-SDC-04 cells and malignant salivary gland tumor cell line (RET981).

Table 1
Short tandem repeat result of non-transformed and transformed MDA-ASDC-4 cells and the original tumor

Sample	AMEL	CSF1PO	D16S539	D18S51	FGA	TH01	TPOX	vWA	Uniqueness
Primary	X,Y	10,12	12	18,19	21,22	8	9,10	16	NMF
CL-NT	X,Y	10,12	12	18,19	21,22	8	9,10	16	NMF
CL-T	X,Y	10,12	12	18,19	21,22	8	9,10	16	NMF

NMF: No match found; CL-NT: Cell line non-transformed; CL-T: Cell line transformed

Table 2

Comparative genomic of primary and different passages of the SDC cell line

Category	Cell line			
	NT	T	T-p20	Tp-40
Combined: (Tumor + Cell Line)	127 (33%)	110(35%)	105 (33%)	130 (40%)
Individual: (Tumor only)	76 (20%)	93 (29%)	98 (31%)	73 (23%)
Individual: (Cell Line only)	178 (47%)	114(36%)	116(36%)	121 (37%)

T – Transformed; NT – Non-transformed