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Development and Characterization of Salivary Adenoid Cystic Carcinoma Cell Line

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Abstract

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.

Keywords

Adenoid Cystic Carcinoma; Cell Line and Salivary Gland Tumors

Introduction

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

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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] Not with standing, 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

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 (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% CO₂.

Maintenance of Primary Cell Culture

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% CO₂ 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% CO₂ incubators for 24 hours, 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-hour 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 (4µg/mL). Tumor cells were selected in 1 mg/mL of puromycin 48 hours 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 minutes; 30 cycles of denaturation at 94°C for 45 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 45 seconds; and final extension at 72°C for 10 minutes. 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 (1×10³ 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 sulfide (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 complete medium of a mid-exponentially growing cells at passage 66 were converted by adaptation using 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 new flask using serum free condition. When sphere formation was observed, the old serum free medium was centrifuged at 100 × g for 5 minutes 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.

Soft Agar Colony Formation Test

Asynchronous exponentially growing cells in monolayer were harvested, adjusted to 2.5×10³ concentration and were re-suspended in 20% RPMI 1640 complete medium in a final agarose concentration of 0.3%. The suspension was overlaid onto a solidified 0.6% agarose base in triplicate plates and incubated in a humidified incubator at 37°C for 3 – 6 weeks. Plates were stained with 0.05% crystal violet, and the colonies with a diameter greater than 0.2 mm were counted under a 7× measuring magnifier and photographed.

Immunohistochemistry

Monolayer of asynchronous cells in logarithmic growth phase were fed with fresh complete medium two hours before harvesting, then trypsinized and suspended in cold 1×PBS solution. An approximate 1×10^5 cells were cytocentrifuged (Shandon Scientific) on to glass slides and fixed in acetone for 10 min, then air dried. Multiple primary antibodies were used for staining: a) Lineage-related Markers: Cytokeratin Cocktail (Dako), CAM 5.2 (BD Bioscientifics) and anti- α -SMA (Sigma-Aldrich). b) Biomarkers: Cytospin preparation of acetone fixed cells were prepared and stained with Anti-EGFR (Life Technologies-Dako, CA), Anti-c-Met (Ventana, CA), Anti-c-Kit (Dako), Anti-p63 (Santa Cruz) and Anti- Myb (Abcam) antibodies. Slides were processed for immunostaining using the DAKO Autostainer.

Cytogenetic Analysis

G-banding and Spectral Karyotyping (SKY) analysis were conducted from primary culture and subsequent passages were characterized by G-banding. In brief, the cell cultures from different passages were harvested and G-banded using standard protocols. Clonal chromosomal abnormalities identified by G-banding were described according to an ISCN, 2013. Clonality was described by detection of two cells with the same chromosome aberration and loss of the same chromosome in three cells. The metaphase spreads were aged at room temperature for a week for SKY analysis. The SKY analysis was conducted using the cocktail of human chromosome paints which was obtained from Applied Spectral Imaging (ASI). Hybridization and detection were carried out according to the manufacturer's protocol, with slight modifications. Chromosomes were counterstained with DAPI. For each case, a minimum of 10 metaphase cells was analyzed by SKY. Images were acquired with a SD300H Spectra cube (ASI) mounted on a Zeiss Axioplan II microscope using a custom designed optical filter (SKY-1) (Chroma Technology), and analyzed using SKY View 2.1.1 software (ASI, Vista, CA).

Array Comparative Genomic Hybridization (aCGH)

The genomic DNA from different passages of the primary cell cultures and its parental fresh-frozen tumor tissues were extracted using the Gentra Puregene Tissue Kit (Qiagen, Inc.) following the manufacturer's instructions. The Affymetrix 250k Nsp SNP array (Affymetrix, Inc.) was conducted by Sequencing and Microarray Facility at MD Anderson Cancer Center as previously described. ^[19]

Short Tandem Repeat (STR) Profiling

The genomic DNA was extracted from the primary cell cultures at different passages and parental fresh-frozen tumor tissues using the Gentra Puregene Tissue Kit (Qiagen, Inc.) following the manufacturer's instructions. STR DNA fingerprinting assay was conducted by the CCSG Characterized Cell Line Core at MD Anderson Cancer Center as previously described. ^[15, 20]

In Vivo Studies in Nude Mice

In vivo studies are being conducted on male 4–6 weeks old NSG mice (Jackson Laboratory). The study is approved by the Institutional Animal Care and Use Committee at the University of Texas MD Anderson Cancer Center and were conducted in accordance with guidelines issued by the National Institutes of Health. Briefly, cell suspensions are being prepared in 100 μ l of 1 $\times$ PBS in 5 $\times$ 10⁶ dilution and are injected directly into left flank with 1 ml U-100 syringe and 26-gauge hypodermic needle (BD Biosciences). Equal numbers of cells prepared in 50 μ l of 1 $\times$ PBS with 50 μ l of Matrigel Matrix Growth Factor Reduced (BD Bioscience) are injected into the right flank of the same mouse. Subcutaneous flank injection is performed on anesthetized male nude mice (4–6 weeks old). Injected mice are being monitored and examined for tumor development once a week.

Results

Primary Tumor

The primary tumor originated from minor salivary gland of the base tongue of a 48-year-old male. Histologically the tumor manifested mainly a cribriform pattern and showed perineural invasion (Fig. 1A). G-banding and spectral karyotypic analysis (SKY) identified t(6;14)(q25;q13) as sole structural aberration and loss of chromosome 16. [21]

Genomic characterization and immortalization

We performed G-Banding, SKY, and array comparative genomic hybridization (aCGH) on primary cells and subsequent passages to identify genomic changes in this cell line. G-Banding and SKY analysis of primary culture revealed a t(6;14)(q25;q13) translocation as the sole structural chromosomal change in the genomic complement. [21] This translocation was maintained till passage 7 and subsequently lost in later passages (>10). (Fig. 1B) aCGH analysis revealed gain of chromosome 8 and focal deletion of 14q in tumor tissue. (Fig. 1C) In passage 5, chromosome 8 gain was disappeared but it retained 14q focal deletion. Both these clonal changes were disappeared in subsequent passages. Furthermore, a prolongation of the population doubling times (PDT) was observed in MDA-ACC-01 cultured cells (p5, PDT=63 hr; p10, PDT=75 hr; p18, PDT = 102 hr) and cell senescence appeared around cell subculture at passage 20. The prolonged population doubling time and stopped growth in the subculture of MDA-ACC-01 made the establishment of the cell line unfeasible. Based on these observations and testimonies, we decided to immortalize the primary cell culture MDA-ACC-01 at of passage 7 by human telomerase transcriptase (hTERT) gene overexpression to stabilize the cell line and presence the structural transformance. According to the PCR result, cells in primary culture MDA-ACC-01 did not contain hTERT gene. But the transformed cells of MDA-ACC-01 (hTERT) achieved the successful expression of hTERT and stayed stable through passages (Fig. 2A). The transformed cell line MDA-ACC-01(hTERT) had consistent PDT= 60 hrs and underwent up to 100 passages, approximately 300-400 population doublings without sign of senescence. (Fig. 2B)

In vitro evaluation

Cells were periodically examined for their morphology by phase contrast microscopy. Both non-immortalized and immortalized cells showed polygonal morphology with mesenchymal (spindle-like) shaped cells and abundant cytoplasm (Fig. 2C, 2D). Both non-transform and transformed cell cultures contained multinucleated cells with pleomorphic nucleoli (upper right insert in Fig. 2C, upper left insert in Fig. 2D). The population doubling time of MDA-ACC-01 and MDA-ACC-01(hTERT) was 102 and 60 hours respectively. Slow growth and cellular senescence was observed in non-transformed cells from MDA-ACC-01 after passage 18. Cellular senescence with prolonged PDT made primary cell culture MDA-ACC-01 hard to grow and harvest at passage 20. However transformed cell line MDA-ACC-01 (hTERT) retained the consistent PDT among passages and cells grow fast with no sign of senescence through 100 passages in vitro. (Fig. 2B) Cell viability and proliferation rate was significantly increased in immortalized cells upon MTT cell proliferation assay (data not shown). These morphologic and nuclear features were consistent with epithelial tumor growth.

Colony formation assay

Adherent population of MDA-ACC-01 (hTERT)-P66 cells propagated in complete medium was transferred to serum free medium for colony formation testing. Cells achieving 3rd passage in serum free medium were evaluated for adherence and semi-sphere formation. These features were observed within 6 days duration. Subsequent observation showed few of the loosely attached tumor spheres completed the sphere formation, detached and started to grow as spheres in suspension together with the compacted attached islands of cells and continued to form spheres (Fig 2D, upper right insert). Upon partially disaggregated spheres were replated into fresh serum free defined medium, the majority of the spheres continued to grow as spheres, but a portion of the spheres reattached and formed sphere producing colonies. When a portion of serum free medium selected cell population was plated to grow in serum containing medium, cells attached and formed a large number of multinucleated cells. (Fig. 2D, lower right insert).

Characterization of hTERT transfected cells

Transformed cell line MDA-ACC-01(hTERT) showed surface independent growth when cells were cultured on solid agarose-coated plates (supplementary figures). Non-transformed cells from early passage of MDA-ACC-01 showed the same characteristics but later passages decreased the cell surface independent growth. However, the immortalized MDA-ACC-01 (hTERT) cells did not grow strictly in monolayer, oriented growth pattern and focus formation were detected. (Fig. 2E) Cells from MDA-ACC-01 (hTERT) were also observed continued growth without senescence during the course of extended culture (> three weeks). The ability of loss of contact inhibition and surface dependence growth indicated transformed cell type. Both immortalized and non-immortalized cells possessed in vitro tumor-initiating ability to form typical colonies in soft agarose. (Fig. 2F) The overall morphology of the tumor cell colonies in soft agarose, were very similar to the morphology of the tumor spheres formed in serum free defined medium (Fig 2D, upper right insert).

Cell Lineage Evaluation

Cells from different passages of MDA-ACC-01 and MDA-ACC-01 (hTERT) were stained with antibodies to α -smooth muscle actin (α -SMA) and a cocktail of low and high molecular weight cytokeratins. Strong expression of α -SMA was observed in early passage of MDA-ACC-01 cells and in cells of MDA-ACC-01 (hTERT) up to passage 25, but became weaker when subpopulation reached passage 50. The expression pattern of cytokeratin did not change during the course of cell culture (Fig. 3). The positive staining for both markers indicated epithelial/myoepithelial nature, whereas the myoepithelial makers were noted to be stronger than keratin supporting myoepithelial dominance. No evidence of Mycoplasma infection was observed by both MycoAlert® Assay using the cell culture growth medium or fluorescent Mycoplasma Hoechst Stain Assay on cells as described in materials and methods.

Cell Line Authentication

Short tandem repeat (STR) DNA fingerprinting analysis was performed to compare cultured cells with the original tumor tissue. STR result revealed that all those molecular markers from transfected cell line MDA-ACC-01(hTERT) and non-transfected MDA-ACC-01 cells were identical to the parent tumor (Table 1, 8 core loci were shown) indicating unique and authentic genotype.

Biomarkers

Immunohistochemical screening of the cell line for the MYB, EGFR, c-kit, p63 and c-Met protein expression showed strong cytoplasmic and membranous expression of EGFR, c-Met and negative staining for c-kit, and p63 (Fig. 4). For MYB expression, although strong nuclear staining mainly myoepithelial cells was noted in primary tumor (Fig. 4A), only weak cytoplasmic staining was found in cell line. (Fig. 4B)

Tumorigenicity

In-vivo cell line injection into nude mice is being conducted. No evidence for tumor growth has yet been detected.

Discussion

We report the development of an adenoid cystic carcinoma cell line and discuss the phenotypic features and the growth characteristics. The cell line is derived from primary untreated predominantly cribriform adenoid cystic carcinoma of the base of tongue. Cytogenetic analysis of the primary tumor revealed clonal translocation involving chromosomes 6q25 and 14q13 and sporadic cytogenetic events including loss of chromosomes 14. [21] This could either be due to loss of progenitor cell(s) or to a finite proliferative capacity of cells with translocations. Alternatively, the loss of this translocation could have resulted from clonal selection shortly after early passages. That later passages devoid of the t(6;14) demonstrated chromosome 8 gain support this possibility. Further genetic testing using array CGH technique revealed limited DNA alterations in both primary tumor and early passages of the cell line. Similar findings have been observed by our group

and others suggesting that cells with chromosomal translocations cannot be sustained in prolonged propagation. [19, 22-24, 29, 30]

Due to the slow growth and the loss of cells with translocation, we immortalize early passage with t(6;14) using h-TERT transfection. [25-28] Although successful transfection was achieved, cytogenetic analysis of subsequent passages of transfected cells lacked the t(6;14). Comparative analysis of h-TERT immortalized cells with primary tumor resulted in concordant random alterations including gain of chromosome 8 and focal 14q loss. Contrary to previous findings in cell lines of different tumor entities, however, we did not observe any genetic alteration in cell line passages that was not present in the primary tumor. [23] The availability of a reference fresh frozen tissue from the original tumor and the use of fingerprinting and lineage related markers serve the constant authentication and validation of the cell line for investigators.

The current cell line manifests hybrid mesenchymal and epithelial markers expression supporting an origin from either a transitional or fully committed myoepithelial cells. This view is supported by early studies of pleomorphic adenoma cells demonstrating that transitional cells with ductal and myoepithelial features is capable of mutual conversion between the two cell types. [31, 32] The biomarkers analysis of our ACC cell line revealed the absence of MYB. [33-35] Interestingly, our analysis of the MYB protein expression revealed marked difference in intensity and location of the marker. In contrast to the nuclear location in primary tumor cells, only weak but consistent MYB in the cytoplasm of cultured cells. The factors associated with the nuclear dislocation of MYB to cytoplasm are unknown. However, MYB results are consistent with the lack of the t(6;9) and genomic alterations of the MYB and the NFIB genes in this tumor (data not included). [33-35] The present cell line, therefore, presents a valuable resource to investigate the role of induced MYB and other growth factors on the biological function and serves as a model to study different growth factors in ACC. [34] However, the cell line highly expressed EGFR and C-Met and was negative for C-kit and p63 expression. These findings resemble those reported in primary ACC except for the lack of p63 which is typically positive in myoepithelial cells. The myoepithelial lineage of the cell line together with the biomarker features characterized provide potential biological targets for further investigations of ACC tumorigenesis. [39-41]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We developed Adenoid Cystic Carcinoma cell line with epi-myoepithelial derivation.
- Morphologic, motility and colonization characteristics support tumor cells.
- The cell line is critically needed for functional experimentation.

Figure 1.

(A) A photomicrograph of the primary base tongue adenoid cystic carcinoma with cribriform pattern from a 48-year-old man. (B) G-Banding result of MDA-ACC-01 cell line revealed the t(6;14) translocation maintained in this cell line at passage 7. (C) Array comparative genomic hybridization (aCGH) result revealed gain of chromosome 8 gain and of focal deletion 14q in tumor tissue; In cell line passage 5 the chromosome 8 gain disappeared, 14q focal deletion remained.

Figure 2.

(A) hTERT transformation of MDA-ACC-01 cell lines: hTERT gene has been transduced into MDA-ACC-01 cell line and stayed stable through passages. hTERT plasmid DNA was used as a positive control, non-transformed cell line (MDA-ACC-01-P8) was used as a negative control, β -actin was used as a loading control. (B) Comparison of the PDT among different cell passages from MDA-ACC-01 and MDA-ACC-01 (hTERT) showed consistent PDT among passages of transformed cells, but prolonged in non-transformed cells. Non-transformed cells stopped growth at passage 20 and no PDT generated after. Phase-contrast photomicrographs of MDA-ACC-01-P5 (C) and MDA-ACC-01 (hTERT)-P50 cells (D) isolated from adenoid cystic carcinomas were captured using 4 $\times$ objectives. Cells from both cell lines showed multi nuclei and pleomorphic nucleoli (right upper insert in C, left upper insert in D, 40 $\times$). Transfected cells cultured in serum free stem cell medium showed higher percentage of multinucleated cells (right lower insert in D) and formed spheres after

transferring to serum free medium(right upper insert in D). (E) The loss of cell contact inhibition was perceived in cell line MDA-ACC-01 (hTERT). Oriented growth pattern and focus formation (right upper insert) were detected in extended culture. (F) Anchorage-independent growth was detected in both MAD-ACC and MDA-ACC-01 (hTERT) cells. Cells were incubated in soft agar for 3 weeks and single colonies were photographed (magnification 10×). Colony numbers formed by MDA-ACC-01 and MDA-ACC-01 (hTERT) cells were determined by the cell numbers.

Figure 3.

Immunohistochemical staining of different passages of MDA-ACC-01 and MDA-ACC-01 (hTERT) cells: MDA-ACC-01-P7 (SMA, upper left; Cam5.2, upper right); MDA-ACC-01 (hTERT)-P2 (SMA, middle left; Cam5.2, middle right); MDA-ACC-01 (hTERT)-P50 (SMA, lower left; Cytokeratin Cocktail, lower right) (4× image with 10× image on right upper corner of each picture)

Figure 4.

Immunostaining of biomarkers. (A) Strong positive nuclear staining in nuclei of mainly myoepithelial cells in primary tumor. (B) Weak and occasionally strong cytoplasmic staining of MYB in cell line cells. (C) Strong cytoplasmic and membrane staining for c-Met. (D) Uniform cytoplasmic and membrane staining for EGFR. (E) Negative staining of cells for c-Kit. (F) Negative staining for p63.

Short tandem repeat (STR) DNA fingerprinting analysis of MDA-ACC-01 and MDA-ACC-01(hTERT) cells.

Table 1

Sample	AMEL	CSF1PO	D16S539	D5S818	D7S820	TH01	TPOX	vWA	Uniqueness
Primary	X,Y	12	11,12	11	11,12	7,9,3	9	16	NMF
Cell Line	X,Y	12	11,12	11	11,12	7,9,3	9	16	NMF
	X,Y	12	11,12	11	11,12	7,9,3	9	16	NMF

* Untransfected
** hTERT Transfected
NMF= No Match Found