Establishment and characterization of novel autologous pair cell lines from two Indian non-habitual tongue carcinoma patients

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Abstract. Oral tongue squamous cell carcinoma (OTSCC) is one of the major causes of fatality in India due to very high percentage of patients with habits of smoking and chewing tobacco and associated products. Being highly heterogeneous in nature, every patient poses a different challenge clinically. To understand disease progression in an improved way, knowledge of cross-talk between tumor stroma and the tumor cells becomes indispensable. Patient-derived *in vitro* cell line models are helpful to understand the complexity of diseases. However, they have very low efficiency of establishment from the tumor samples, particularly the cancer-associated fibroblasts (CAFs). In the present study, two novel autologous pairs were immortalized spontaneously from non-habitual, HPV-positive patients, who presented with OTSCC. The epithelial and fibroblast cell lines had typical polygonal and spindle-shaped morphology, respectively. Positive staining with epithelial specific Pan-cytokeratin (PanCK) and fibroblast specific protein (FSP-1) further confirmed their epithelial and fibroblast origin. Unique Short Tandem Repeat (STR) profile of the cultures confirmed their novelty, while the similarity of the STR profiles between the epithelial and fibroblast cells from the same patient, confirmed their autologous nature. DNA analysis revealed aneuploidy of the established cultures. An increase in the tumorigenic potential of the established epithelial cultures upon treatment with CAF-conditioned medium proved the 'CAF-ness' of the established fibroblast cells. The established cultures are the first of their kind which would serve as a useful platform in understanding the tumor-stroma cross-talk in tongue cancer progression.

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Introduction

Cancer of the oral cavity is ranked among the top five most frequent cancers occurring in India among both men and women. Comprising 10.3% of all cancers, males (16.2%) appear to be affected more by it than females (4.6%) in India (1). Oral tongue squamous cell carcinoma (OTSCC) distresses ~16,000 people annually in USA (2) and is typically related to a long history of smoking and/or heavy alcohol use (3). Even though the smoking rates continue to drop, the incidence of squamous cell carcinoma (SCC) of the oral cavity has remained constant (4) with the increase of OSCC patients who have never smoked or whose habit was non-significant. These individuals are often women in their mid-forties or younger (3,5-8). Oncogenic Human Papilloma Virus (HPV) has been implicated in the recent rise and shown to improve disease-specific survival in oropharyngeal cancers (9,10). Prognosis is particularly improved in the case of tonsillar and base of tongue cancer (11).

Knowledge of the molecular and biological characteristics of cancer cells is essential to develop therapeutic strategies or identify drug targets for cancer treatment. Primary cultures generated from different cell types, enable research into microenvironment-driven characteristics displayed by the parent tumors. However, due to the difficulty in long-term maintenance, cell lines remain desirable source for conducting translational research in laboratory settings (12). Additionally, the scarcity of commercially available cancer-associated fibroblasts (CAFs) imposes the need to establish and characterize novel CAF lines from patient tumors. Microenvironment cross talks can be further studied in detail if the epithelial and CAF cells can be obtained from the same patients (13-15).

In vitro cell-based models of cancers have proven to be an essential tool to enhance the current understanding of tumor heterogeneity and stroma-tumor cross-talk (15-17). For oral cancer, various articles report the establishment of epithelial cell lines from buccal mucosa (18), gingivobuccal mucosa (19), oral cavity (20-22), tongue (23,24), sinonasal (25), pyriform fossa (26), lower alveolus and retromolar trigone (24). Zhao *et al* (27) in 2011 further assembled and characterized 85 cell lines from various head and neck tumor

sites. To elucidate the impact of different mutations on tumor behaviour, 16 cell lines from Head and Neck Squamous Cell Carcinoma (HNSCC) patients were previously established by Hayes *et al* (28).

Earlier attempts were made to establish cell lines from HNSCC patients who had undergone chemotherapy (CT) and radiotherapy (RT) (29,30). More recently, one epithelial line was established from a non-smoking patient, diagnosed with OTSCC (31). Patil *et al* (23) established the first-ever cisplatin sensitive epithelial cell line established from an Indian gutka chewer, diagnosed with SCC of the tongue. Pansare *et al* (19) and Gawas *et al* (18) reported establishing four and three epithelial lines from tobacco chewers, from gingivobuccal and buccal mucosa, respectively. Attempt to establish four epithelial cell lines from patients diagnosed with poorly-differentiated SCC of the tongue, moderately-differentiated SCC of the lower alveolus and well-differentiated SCC of retromolar trigone has been reported by Tatake *et al* in 1990 (24). Other epithelial cells were also established from grade III SCC of the pyriform fossa (26). Though majority of the studies established cells from treatment-naïve samples, Pansare *et al* (19) established cell lines with patients receiving CT or RT after radical surgery. Studies of establishment of cell lines do not display any gender bias.

Although abundant literature is available on the establishment of cell lines from either chewers or smokers, cell lines from non-habitual patients are comparatively few. Additionally, a model system with epithelial and CAF from the same patient, is not available.

The aim of the present study was to establish a novel *in vitro* model system from tongue-tumor of two non-habitual patients. Differential trypsinization assisted in the isolation of two different cellular populations described. Extensive characterization established the novelty of the cells and their autologous nature provides a unique platform to study tumor-stroma cross talk. To the best of our knowledge, such paired cell systems isolated using explant culture (or any other cell establishment methods) have not been reported in the literature so far. The autologous pairs reported in the present study can be used as a model system to identify theragnostic biomarkers as appropriate.

Materials and methods

Tumor specimen and establishment. Tumor samples were collected after obtaining informed consent from patients. The present study was approved [approval no. NHH/MEC-CL-2015-405 (A)] by the ethics committee of Narayana Health City (Bangalore, India). Tissues were collected aseptically in RPMI-1640 (cat. no. AT222A; Himedia Laboratories, LLC) with triple strength penicillin-streptomycin (cat. no. 15140122; Gibco; Thermo Fisher Scientific, Inc.) from two 65-year-old females with no risk habits, diagnosed with OTSCC. Patient MhCT08 had undergone neoadjuvant therapy followed by surgery and CT-RT and patient MhCT12 was a naive surgical sample. Patient MhCT08 also developed lung metastasis (poorly differentiated adenocarcinoma) one year post treatment. The clinical details of the patients are mentioned in Table I. The tissue samples were thoroughly washed 3 times at 5 min interval with 3X penicillin-streptomycin followed by 10% povidone iodine solution (Win Medicare Pvt. Ltd.) and finally with complete growth medium. The tissue was cut into small sections and treated with 0.25% trypsin (cat. no. 25200056; Gibco; Thermo Fisher Scientific, Inc.) for 30 min at 37˚C. The chopped and digested pieces were placed in a serrated 60-mm petri dishes and supplemented with 10 ng/μl each of human recombinant epidermal growth factor (hEGF; cat. no. E9644; Sigma-Aldrich; Merck KGaA), N2 suppliment-1X (cat. no. 17502048; Gibco; Thermo Fisher Scientific, Inc.), Epilife defined growth supplement (EDGS; cat. no. S0125; Gibco; Thermo Fisher Scientific, Inc.) along with 20% FBS (cat. no. RM10434; Himedia Laboratories, LLC), in RPMI-1640 media with 1X penicillin-streptomycin. The media was changed every 48 h to remove the dead cells. The epithelial cells were enriched by differential trypsinization and further sub-cultured. Briefly, the cells were trypsinized for two different time points. After a min of trypsinization, floating cells (fibroblasts) were removed and seeded in a separate flask. Since fibroblasts can detach faster than epithelial cells, this differential trypsinization technique yielded two separate cellular populations. The separated cells were cultured in RPMI-1640 media with 20% FBS and no additional growth supplements. The cells were passaged for more than P50 and were characterized for cell-type specificity at both early and late passages. Later passages of the cells were maintained in RPMI-1640 medium, pH 7.2, supplemented with 20% FBS and 1X penicillin-streptomycin solution. Both the epithelial and fibroblast cells originated from the tongue tissue of the patient. The established cell types were stained with Pan-cytokeratin (PanCK; epithelial specific marker) and FSP-1 (fibroblast specific marker), to verify their identities after differential trypsinization. Isolated epithelial and fibroblast cells were denoted as MhCT08/12-E and MhCT08/12-F, respectively. The names have been arrived at through an acronym: M, Mazumdar shaw medical foundation; h, Human; C, Cancer of; T, Tongue; 08/12, Patient code; E, Epithelial; and F, Fibroblast.

HeLa cell culture. HeLa cells were cultured in DMEM medium, pH 7.2, (cat. no. 11995-065; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 1X penicillin-streptomycin solution at 37˚C.

Characterization

Growth curve. Cells were collected and seeded at a concentration of $1x10^4$ cells per well in a six-well plate. Cell counts of three random wells were conducted every day for up to 5 days using trypan blue (cat. no. T6146; Sigma-Aldrich; Merck KGaA). The doubling time was calculated using the equation: Td=TX log2/log(N/N0), where Td is the doubling time; T is the time interval; N is the final cell number and N0 is the initial cell number. Results are presented as the mean ± standard deviation (SD) of three independent experiments.

Flow cytometry. Cells at a concentration of 10^6 cells/100 μl were washed twice in PBS, permeabilized with 0.1% triton X100 (cat. no. 10655; Thermo Fisher Scientific, Inc.) for 30 min, and incubated with primary antibody (1:50). The cell types were probed with anti-PanCK (cat. no. 4545; Cell

Table I. Clinical and pathological details of the established cell lines.

Cell line	Patient's age (years)/sex/habit	Tumor diagnosis	Pathological staging	Clinical staging	Tumor progression
MhCT08-E MhCT08-F	65/F/None	Tongue Squamous cell carcinoma	pT4N1Mx	cT4aN2c	Presentation: Primary (Post NACT) followed by CT-RT after surgery. Reported lung metastasis one year post surgery (FNAC: Poorly differentiated adenocarcinoma)
MhCT12-E MhCT12-F	65/F/None	Tongue Squamous cell carcinoma	pT3N3bMx	cT4aN2c	Presentation: Primary Patient Expired

F, female; NACT, Neoadjuvant chemotherapy; CT-RT, chemotherapy and radiotherapy; FNAC, Fine Needle Aspiration Cytology.

Signalling Technology, Inc.) and anti-Fibroblast specific protein (FSP-1; cat. no. F4771; Sigma-Aldrich; Merck KGaA) as primary antibodies for 1 h on ice. Cells were then pelleted down by centrifugation at 500 x g for 5 min at 4˚C and washed with PBS (cat. no. 10010-023; Gibco; Thermo Fisher Scientific, Inc.) followed by incubation with the corresponding AlexaFluor488-conjugated anti-mouse secondary antibody (cat. no. A11029; Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at room temperature in the dark. For the marker expression analysis, cell sorting gates were established using an unstained control population. The experiment was performed in duplicates using the BD FACS Canto II system and analyzed using BDFACS Diva software version 8.0.1 from BD Biosciences.

Immunofluorescence. Cells were seeded on coverslips at a density of 20,000 cells per 100 μl of complete medium and were allowed to attach overnight. Coverslips were then treated with ice cold 100% methanol (cat. no. AS059; HiMedia Laboratories, LLC) to fix the cells for 15 min at -20˚C followed by three PBS washes. Prior to staining, the cells were blocked in 1X PBS containing 1% BSA (cat. no. TC194; HiMedia Laboratories, LLC) and 0.3% triton-X 100 for 1 h at room temperature. The cells were then incubated with anti-PanCK and anti-FSP-1 as primary antibodies, overnight, at 4˚C. After three washes with 1X PBS, the cells were incubated in anti-mouse Alexa488-conjugated secondary antibody for 1 h at room temperature in dark. The processed coverslips were then mounted on slides with DAPI histology mount (cat. no. F6057; HiMedia Laboratories, LLC) and visualized under Zeiss Scope A1 fluorescent microscope.

DNA ploidy determination. Cells were harvested following trypsinization and resuspended in PBS containing RNase (10 μg/ml; cat. no. 12091021; Invitrogen; Thermo Fisher Scientific, Inc.) and propidium iodide (40 μg/ml) (cat. no. P4170; Sigma-Aldrich; Merck KGaA) and incubated at 37˚C for 30 min for DNA staining. The DNA content was compared with human mononuclear cells from peripheral blood, which served as a control for diploid human genomic DNA content. The cells were analyzed using a BD FACS Canto II system to determine their fluorescence. The mean channel of cells that were in the G0 phase was divided by that of the lymphocytes to determine the DNA index of each cell line. The DNA index was then used to predict the ploidy of the cells.

Isolation of human lymphocytes. Lymphocytes were isolated from a 28-year old healthy male from Karnataka in late August 2018 by layering whole blood (diluted 1:3 with 1X PBS) above Ficoll-Paque Histopaque (cat. no. LSM-1077; HiMedia Laboratories, LLC) at 1:1 ratio. Subsequently, the Ficoll-Paque with whole blood was centrifuged at 400 x g for 25 min at 20˚C. The resultant buffy coat containing lymphocytes was taken out and washed twice with 1X PBS before further usage (32,33).

Immunocytochemistry. The established cell lines were examined for the presence of HPV infection status by staining with p16 (cat. no. AM540-5M; BioGenex Laboratories) and E7 antibody (cat. no. sc-58661; Santa Cruz Biotechnology, Inc.) via immunocytochemistry. HeLa cells, a cervical cancer cell line, was used as a positive control for the experiment. Cells ($5x10^3$) were cultured on coverslips and fixed in 4% paraformaldehyde for 10 min (cat. no. GRM3660; HiMedia Laboratories, LLC) followed by permeabilization with 0.1% triton X-100 for 10 min, and probed with p16 or E7 overnight at 4˚C. The coverslips were further processed with Dako kit (cat. no. K5007; Dako; Agilent Technologies, Inc.). The presence of target proteins was visualised using DAB as chromogen and the cells were counterstained with haematoxylin (cat. no. S034; HiMedia Laboratories, LLC) for 5 min at room temperature, mounted with DPX mounting medium (cat. no. Q18404; Qualigen; Thermo Fisher Scientific, Inc.) and examined under Nikon Eclipse E200 light microscope. For only haematoxylin staining, the cells were stained directly after permeabilization as aforementioned and the coverslips were mounted with DPX mounting medium for examination under Nikon Eclipse E200 light microscope.

PCR. Both the samples were screened for the presence of HPV infection by PCR to amplify a 450-bp fragment from the conserved region in all HPV variants with MY09/MY11 primers: forward, 5'-CGTCCMARRGGAWACTGATC-3' and reverse, 5'-GCMCAGGGWCATAAYAATGG-3' as the reverse primer (34). PCR was performed using 200 ng of DNA along with dNTPs (0.2 mM each), Taq polymerase

(cat. no. D1806; Sigma-Aldrich; Merck KGaA), 1X reaction buffer, and primers at a final concentration of 0.1 μM each. Forward, 5'-AGCCATGTACGTTGCTATCCA-3' and reverse, 5'-ACCGGAGTCCATCACGATG-3' primers were used to amplify beta actin (amplicon 120 bp). Amplifications were performed using the following thermocycling conditions: 94˚C for 5 min followed by denaturation at 95˚C for 1 min, annealing for 1 min (50˚C for MY09/MY11 and 60˚C for beta actin), and elongation at 72˚C for 1 min. A final elongation step was carried out at the end of the final cycle at 72˚C for 10 min. Genomic DNA extracted from the HeLa cell line was used as the positive control and no template was added to the reaction mix as a negative control. Amplified products were subjected to agarose gel electrophoresis (1.2% gel) for amplicon visualization.

STR profiling. STR profiling of 10 loci was performed to establish the genomic identity, cell line identity and exclude any cross-contamination of MhCT08-E,F and MhCT12-E,F cells. STR multiplex assay was outsourced to TheraCUES Innovations Pvt. Ltd. and performed using GenePrint 10 (Promega Corporation). SoftGenetics GeneMarker_HID version 3.0.0 was used for analyzing the results. STR data were then examined in the reference STR database of ATCC and CLASTR using the standard match threshold of 80%.

Estimation of proliferation. Conditioned media from CAFs was collected 48 h after seeding. Epithelial cells were seeded at a concentration of $1x10^4$ cells per well in a 96-well plate, overnight at 37˚C in 5% CO_2. They were allowed to grow further for 72 h in CAF-conditioned medium, provided neat or supplemented with 20 and 50% of fresh complete RPMI-1640 medium. Proliferative potential of the cells was calculated every 24 h, using Alamar Blue Reagent (cat. no. R7017; Sigma-Aldrich; Merck KGaA), prepared at a final concentration of 0.2 mg/ml in sterile PBS. A total of 20 μl of Alamar Blue was added per 100 μl of the growth medium in a 96-well plate and incubated for 2 h at 37˚C. Optical density at 570 and 600 nm was measured. Proliferation was calculated using the formula (O2 X A1)-(O1 X A2); where O1 is the molar extinction coefficient (E) of oxidized Alamar blue at 570 nm, O2 is the E of the oxidized Alamar blue at 600 nm, A1 is the absorbance of test wells at 570 nm and A2 is the absorbance of test wells at 600 nm. From the table of oxidation coefficients of Alamar Blue, values of O1 and O2 were taken as 80856 and 117216, respectively. The fold proliferation was calculated over the negative control i.e. media and the Alamar Blue reagent without any cells. Results show the mean ± SD of three independent experiments.

Wound healing assay. Conditioned media from CAFs was collected for 24, 48 and 72 h after seeding. Epithelial cells ($0.3x10^6$) were seeded in each well of a six-well plate and cultured till 80% confluency. The cell monolayer was gently scraped with a sterile 200-μl pipette tip and the wells were washed twice with 1X PBS to remove cell debris. The cells were then treated with conditioned medium collected at different time points, as indicated in the figures. The width of the scratch was determined by images captured under a light microscope at 0 and 24 h after creating the wound. During the time of wound closure, the cells were maintained at 1% FBS. Wound closure was then quantified using ImageJ software_1.53k (National Institutes of Health) and the percentage of wound closure was plotted. Percentage wound healing was calculated by the formula: % wound confluence=(A-B)*100%)/A; where A is the width of the initial scratch wound and B is the width of the scratch wound at time 24 h. Results present the mean ± SD of three independent experiments.

Invasion assay. ECM gel (cat. no. E1270; Sigma-Aldrich; Merck KGaA) was prepared at a final concentration of 1 mg/ml in DMEM serum free media. A total of 100 μl of ECM was coated on the Transwell inserts (pore size 8.0 μm) and incubated for 2 h at 37˚C to allow gel formation. Cells ($1x10^4$) were then seeded in serum free medium on the top chamber of the Transwell insert (cat. no. TCP083; HiMedia Laboratories, LLC) with conditioned medium collected at different time points (as indicated in figures) as the chemoattractant in the lower chamber with 10% FBS. The cells were allowed to invade for 48 h. For imaging, the cells on the inside of the Transwell were removed gently using cotton swabs, followed by staining with 2% crystal violet (cat. no. S012; HiMedia Laboratories, LLC) for 10 min at room temperature. The Transwell inserts were washed with 1X PBS twice to remove any unbound crystal violet and then air-dried before imaging. For quantification of invasion, bound crystal violet was eluted by incubating the Transwell inserts in 10% acetic acid (cat. no. Q21057; Qualigen; Thermo Fisher Scientific, Inc.) in water with shaking for 10 min at room temperature. The eluent was then transferred to a 96-well clear microplate, and absorbance was read at 590 nm. Results show the mean ± SD of three independent experiments.

Sphere formation assay. 3D sphere formation was carried out as detailed by Arya *et al*, 2016 (35). Briefly, $0.5x10^6$ cells were encapsulated in 7.5% 3D GelMA hydrogels and cultured for 14 days, with a partial medium change (normal or CAF conditioned medium, as indicated in the figures) on alternative days. After 14 days, the spheroids were harvested using enzymatic degradation of the hydrogel and images were captured. The spheroid size was calculated using the formula $4/3\pi r^3$, where 'r' represents the geometric mean of the two diameters of the spheroids. Results present the mean ± SD of three independent experiments.

Statistical analysis. All the quantitative data is expressed as the mean ± standard error of the mean. Statistical significance was calculated using paired Student's t-test unless stated otherwise. Two-way ANOVA followed by Bonferroni test was performed for proliferation assay and one-way ANOVA followed by Dunnett's test was performed for invasion and migration assays. $P<0.05$ was considered to indicate a statistically significant difference. All the statistical calculations were performed using GraphPad Prism software (version 5.00; GraphPad Software, Inc.).

Results

Tissue samples from both patients, MhCT12 and MhCT08, were sub-cultured for more than 40 passages. Both yielded

epithelial as well as CAF cells and were further characterized in various ways.

Characterization of established cell lines

Morphology. To determine the morphological features of established cell lines, the cells were observed under a light microscope. Both the epithelial cells had typical polygonal morphology while both the fibroblast cell types were observed to have typical spindle-shaped morphology (36,37) as revealed in Fig. 1A. The haematoxylin-stained images were captured after passage 35 for each cell type.

Purity. Explant culture from the tissue yielded two types of cellular populations. To confirm their origin and to rule out any cross contamination, checking the purity of the established cell lines became indispensable to perform further experiments. The epithelial and CAF nature of the cell lines was confirmed by the presence of lineage-specific markers, using flow cytometry (Fig. 1B). The removal of fibroblasts from the epithelial population was confirmed by negative staining with fibroblast specific FSP-1 antibody (38,39). Similarly, a pure population of fibroblast was confirmed by negative staining with epithelial specific PanCK antibody (40). FACS was performed at both early (p8) and late passage (p43) of cells (Fig. 1B) and percentage of total positively-stained cells was represented graphically (Fig. 1C). As revealed in Fig. 1B and C, MhCT12-E and MhCT08-E cells showed 98 and 81% PanCK positivity, respectively, with less than 0.2% FSP positivity, confirming that epithelial populations were not contaminated with fibroblasts. Similarly, MhCT12-F and MhCT08-F exhibited 94.9 and 85% FSP-1-positivity, respectively. Furthermore, fibroblast cultures at later passages revealed exclusively FSP-1 enriched populations as compared with the early passages. The FSP-1 positive population increased from 83.4% at early passage to 94.9% at late passage of MhCT12-F cells. Similarly, MhCT08-F cultures also displayed increase from 53.2% at early passage to 85% at later passage. Immuno-cytochemical analysis also revealed that the established fibroblast cultures stained positively with FSP-1, a fibroblast specific marker, and did not exhibit any staining with PanCK, an epithelial specific marker. Similarly, the established epithelial cells were stained positively with PanCK, but did not show any positive stain with FSP-1 (Fig. 1D). Collectively, these results verified the purity of established cell lines in the present study.

HPV detection. HPV infection status has been known to affect patient prognosis. It has been reported that HPV-positive cancers have a more favourable patient prognosis when compared with HPV-negative cancers (41,42). To determine HPV infection status of the established cell lines, PCR and immunocytochemical analysis were performed. Immunocytochemical analysis revealed that both the epithelial and fibroblast cells were positively stained for p16 and E7 antibodies (Fig. S1A). P16 antibody against cyclin dependent kinase inhibitor 2A, stained the nuclear region thus confirming the HPV positivity of the established lines (Fig. S1A). Fragments of 450 bp were also amplified using MY09/MY11 primers against L1 region of the viral genome (43), thus confirming the HPV positive status of the established cell lines (Fig. S1B) for either HPV 16 or 18.

Ploidy determination. One of the major hallmarks of cancer is flawed DNA amplification cycle which leads to tumor progression (44). The DNA content of the cell lines was determined by performing ploidy analysis (45). Since normal lymphocytes rarely exhibit any altered DNA content (46) and their average DNA value has been designated as diploid (47), normal lymphocytes from a healthy donor were used as a diploid control for the experiment. The DNA indices of MhCT08 and MhCT12 cells, and diploid lymphocytes were 1.1, 0.8 and 1 respectively (Fig. 2A). The results, therefore, indicated that both the patient samples have abnormal DNA content which may be responsible for the immortalization of these cells.

Growth characteristics. The cell lines exhibited different doubling times (Fig. 2B). Doubling time of 23.61 and 25.54 h was observed for MhCT08-E and MhCT12-E cell lines, respectively. Both the fibroblast cell lines had higher doubling time than epithelial cells as revealed with MhCT12-F doubling at every 39.73 h and MhCT08-F doubling every 32.23 h.

STR profiling. The STR profile of the established cell lines was performed to indicate that they are distinct from that of any other cell lines deposited in ATCC and Expasy Cellosaurus STR (CLASTR) database (Table II). STR profiling indicated that both MhCT08-F and MhCT12-F and epithelial cells are novel. According to the American Type Culture Collection, an STR profile match of ≥80% between cell lines indicates their 'relatedness' to each other (48). The percentage match (>80%) between epithelial and fibroblast cells from the same patient distinctly proved the autologous nature of the cells (Table III). No cross-contamination, particularly with Cal27 and HSC3 cell lines (simultaneously handled by author ND) was observed as shown in Table III. Two pairs of autologous cultures were thus established using explant culture method. Differential trypsinization yielded two separate cellular populations-Epithelial and Fibroblast which were extensively characterized in various ways as outlined in Fig. 1E.

Tumorigenic properties of established epithelial cell lines. Furthermore, to assess the tumorigenic potential of the epithelial cell lines, various assays were performed post-treatment with CAF-conditioned medium.

Proliferation. Epithelial cells revealed a significant increase ($P<0.05$) in proliferation when treated with CAF-conditioned medium. Since no significant difference was observed between neat and conditioned mediums supplemented with fresh growth mediums, only neat-conditioned medium was selected for subsequent experiments. A slight decrease in proliferation was observed at 72 h, which may be attributed to the over-confluency of the cells leading to floating cells. Maximum proliferation under the effect of neat CAF-conditioned medium was observed at 48 h for both the cell lines (Fig. 3A and B).

Wound healing assay. Treatment with neat-conditioned media from the autologous fibroblast pair significantly increased the migration potential of MhCT12-E cells from 23 to 38% (Fig. 3C) and 22 to 48% for MhCT08-E cells (Fig. 3E)

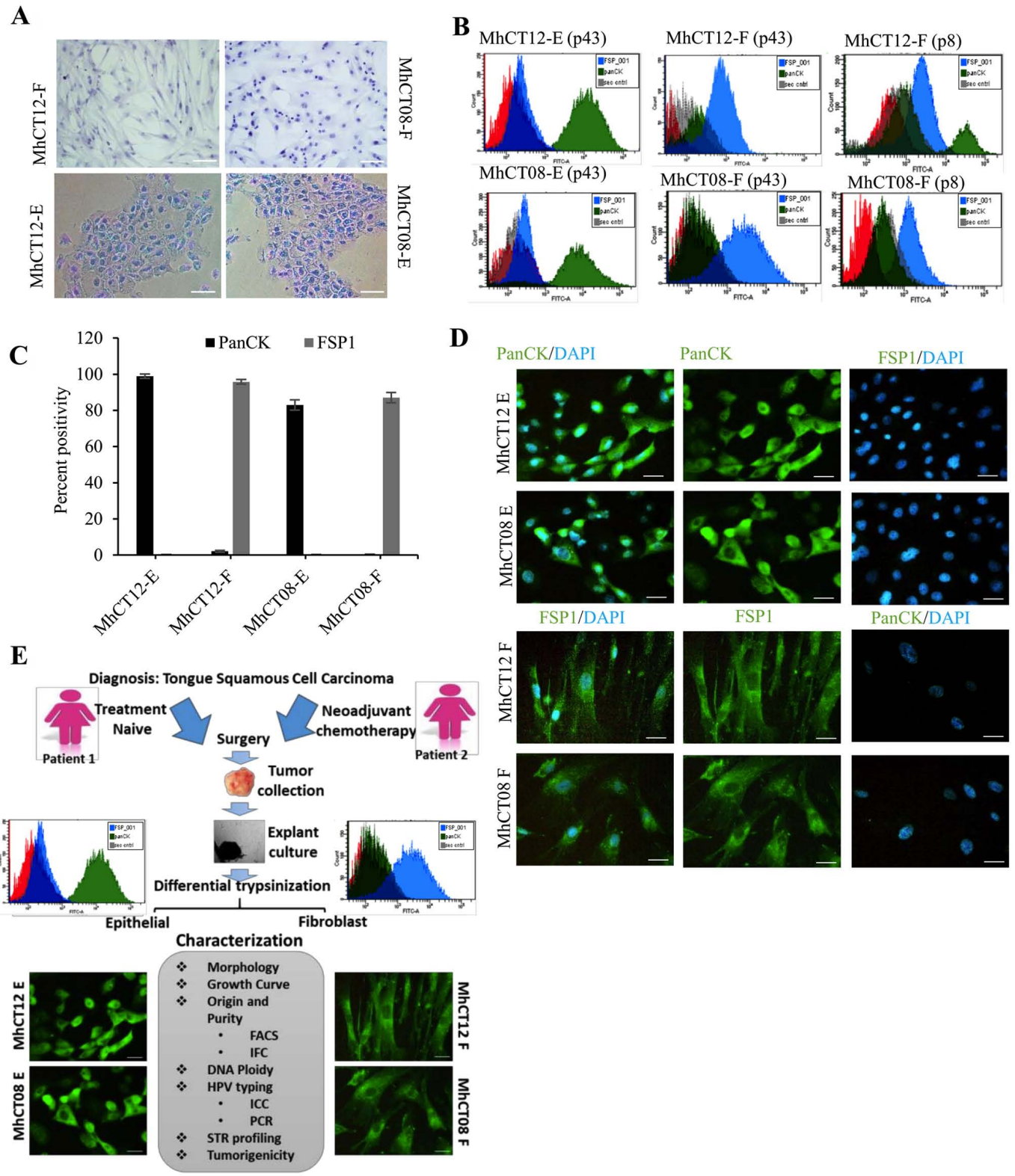

Figure 1. Purity of established cell lines. (A) Microscopic image of haematoxylin-stained cells. Scale bar=100 μm. (B) Representative flow overlay histogram for FSP-1 and PanCK staining in MhCT12-E,F and MhCT08-E,F at passage 8 and passage 43. (C) Quantification of percentage of positive population for PanCK and FSP-1 staining in the established cell lines. (D) Representative fluorescent images for FSP-1 and PanCK staining in MhCT12-E,F and MhCT08-E,F. Scale bar=50 μm. (E) Graphical abstract describing the work flow of the study. FSP-1, fibroblast specific protein; PanCK, Pan-cytokeratin.

(P<0.05) (Fig. 3D and F). Treatment with CAF-conditioned medium significantly increased the wound healing potential of their respective epithelial counterparts demonstrating the CAF-like nature of the established fibroblasts.

Invasion assay. Treatment with conditioned media from the autologous fibroblast pair significantly increased the invasive potential of both MhCT08-E and MhCT12-E cell types (P<0.05; Fig. 3G-J).

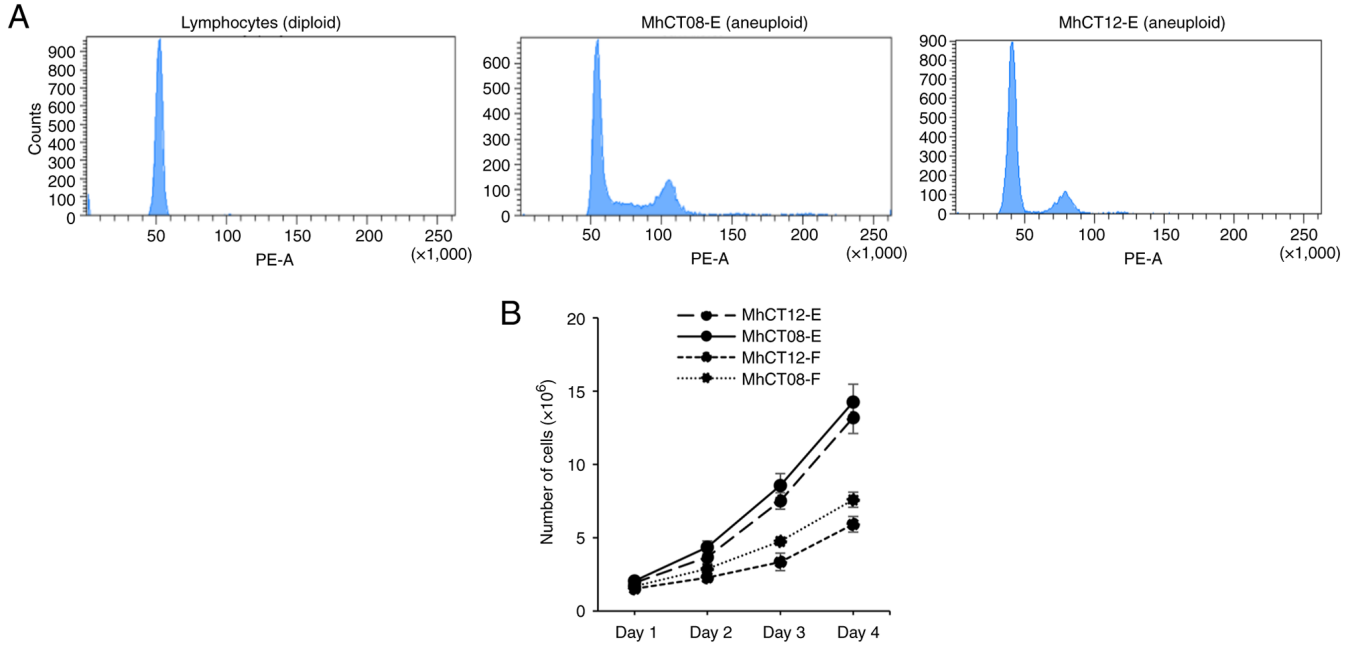

Figure 2. Characterization of established cell lines. (A) Ploidy determination by flow cytometric analysis. Propidium iodide-stained diploid human lymphocyte and aneuploid MhCT12 and MhCT08 cultures. (B) Doubling time of established cell lines.

Sphere formation assay. Treatment with conditioned media from the autologous fibroblast pair significantly increased the sphere formation potential of both MhCT08-E (n=60, P<0.01) and MhCT12-E (n=35, P<0.05) cell types, as compared with no treatment conditions of MhCT08-E (n=36) and MhCT12-E (n=24) as revealed in Fig. 4. Treatment with MhCT08-F CAF conditioned medium however influenced the epithelial cells to form bigger spheres ($5.4x10^7$ μm^3, P<0.01) as compared with the treatment with MhCT12-F CAF-conditioned medium ($1.2x10^7$ μm^3, P<0.01).

Discussion

Patient-derived primary cultures are an invaluable resource towards understanding the process of carcinogenesis. Given the heterogeneity of the tumors, cell cultures that represent multiple cell types of the tumor further enhance the significance of the *in vitro* models. In the present study, the establishment and characterization of two novel autologous epithelial and CAF cultures from two 65-year old Indian females without any risk habits, diagnosed with squamous cell carcinoma of the tongue, were described.

Traditional risk factors for the development of oral cancer include chewing, smoking, and alcohol consumption. The established cultures reported from previous studies were from different stages of tumor and included patients with/without habits. In the present study, both patients were without any reported risk habits. Overall in India, OSCC cases without any known risk habits are comparatively very less (49-51). Therefore, comparing these cell lines with the other established cultures, from patients with risk habits, may provide insights into the etiology of the disease progression in both cases. Furthermore, considering the rise in cancer in women without any known risk habits (51,52), increases the significance of this model towards understanding the carcinogenic process of this cohort of patients.

Most of the oral carcinoma cell lines established are either from the western population, or from the patients with habits. Such established platforms may provide insights into cancer progression in patients with habits, but lack in the complexity of disease progression in non-habitual patients diagnosed with oral cancer. There are very few studies of establishment of oral cancer cell lines from Indian subcontinent, even though oral cancer is among the top five cancer-related deaths in India. The aim of the present study was therefore to establish such a platform from Indian subcontinent in patients without habits. Establishment of this novel and unique platform will be helpful to understand the underlying molecular mechanisms in non-habitual oral cancer patients.

Multiple methods have been employed in the development of primary cultures. Numerous studies have described establishing cell lines by explant culture method (18,19,22,23,25). Certain studies have also reported directly digesting the tumor tissue and seeding the cell suspension (20,24). Mulherkar *et al* (26) described the establishment of NT-8e, an oral squamous cell carcinoma cell line using a mouse xenograft model. The current study used the explant culture method. A recent study demonstrated the use of different mediums to enhance the differential growth of epithelial and fibroblast populations (53). The cultures reported in the present study were established in RPMI-1640 complete medium to eliminate any phenotypic or genotypic changes arising due to patient-derived xenograft generations. The cultures did not require any feeder cells to grow and stabilized spontaneously, without the intervention of viral vectors. Further, cell-based separation and establishing homogeneous cultures are an essential requirement in primary culture generation. CD90/CD44-based separation has been successful in separating mesenchymal (CD90 positive) and epithelial (CD90 negative) populations from tumor tissue (21). Kaur and Ralhan (22) described an innovative method of treating the cultures with anti-fibroblast antibodies along

Table II. STR profiles of the established cell lines.

	Sample name	MhCT08-E		MhCT12-F		MhCT08-F		MhCT12-E	
SI no	Marker	Allele #1	Allele #2	Allele #1	Allele #2	Allele #1	Allele #2	Allele #1	Allele #2
1	TH01	6	8	3	8	6	8	6	8
2	D21S11	28	30	31	32.2	28	30	31	32.2
3	D5S818	11	11	11	12	11	11	12	12
4	D13S317	11	11	8	11	11	12	8	11
5	D7S820	7	8	11	11	7	8	11	11
6	D16S539	10	11	9	13	10	11	9	13
7	CSF1PO	11	12	10	11	11	12	10	10
8	AMEL	X	X	X	X	X	X	X	X
9	vWA	15	16	14	17	15	16	14	17
10	TPOX	12	12	8	11	8	12	8	11

Table III. Proof of novelty of established cell lines by STR (*<50% match cut-off from ATCC and CLASTR public databases). The numbers indicate the 'relatedness' of the cell lines to each other. According to ATCC, a STR profile match of ≥80% between cell lines indicate their 'relatedness' to each other. Cell lines with between a 55 to 80% match require further analysis for authentication of relatedness as per ATCC guidelines (48). ATCC, American Type Culture Collection; CLASTR, Cellosarus STR Similarity Search Tool.

Samples	MhCT08-E	MhCT08-F	MhCT12-E	MhCT12-F	Cal 27, HSC3
MhCT08-E	-	90	25	35	-*
MhCT08-F	90	-	30	40	-*
MhCT12-E	25	30	-	90	-*
MhCT12-F	35	40	90	-	-*

E, Epithelial; F, Fibroblast.

with complement rabbit serum treatment to remove fibroblast populations, however this led to the destruction of the valuable CAFs. The present study exploited the differential trypsinization method to establish the novel autologous pairs described.

Cancer-associated stem cells (CSCs), characterized by self-renewing oncogenic cells, form a small subpopulation of tumor stroma (54). While the explant culture method utilized in the study yielded distinct epithelial and fibroblast populations, isolation of CSCs was not undertaken. To overcome this limitation, further research should be attempted, where, the explant may be supplemented with additional growth factors. The isolated cells can then be sorted and enriched based on various stem cell markers (CD44 and CD24) via flow cytometry (55), followed by their culture in defined growth medium supporting the pluripotency of stem cells. However, since no such growth factors were added, current explant culture method is limited in isolation and growth of cancer-associated stem cells.

To the best of our knowledge, no previous studies have attempted to establish fibroblast and epithelial cultures from the same patient. The current study, at present, remains exclusive in establishing both the epithelial and fibroblast pairs from the same tumor sample. Most of the studies reported previously have used either different growth mediums, growth supplements, or feeder layers to establish epithelial cultures from tumors. However, the cultures established in the present study were not only novel in themselves, but also, did not require any additional supplements for their growth maintenance. This unique platform will provide answers to various research questions pertinent to tumor-stroma cross talk.

Characterization of the cells to establish purity and functional properties have been the mainstay of assigning an identity to the primary cultures. PanCK and FSP-1 staining have been reported to identify epithelial and fibroblast populations exclusively (56-58). In the present study, epithelial and fibroblast cells were stained with PanCK and FSP-1, respectively, with high specificity. However, MhCT08 exhibited lesser percentage positivity for FSP1 as compared with MhCT12 owing to the fact that it originated from a more aggressive tumor, and probably has fibroblast markers other than FSP-1. DNA ploidy determination can correlate with the cancer grade, aggressiveness and metastatic potential of a tumor and can provide insightful information about cancer diagnosis and prognosis (59). The DNA index calculation implied the aneuploid nature of the established cell lines, which may prove chromosomal instability, thus promoting a heterogeneous tumor evolution (44). However, since the ploidy level from

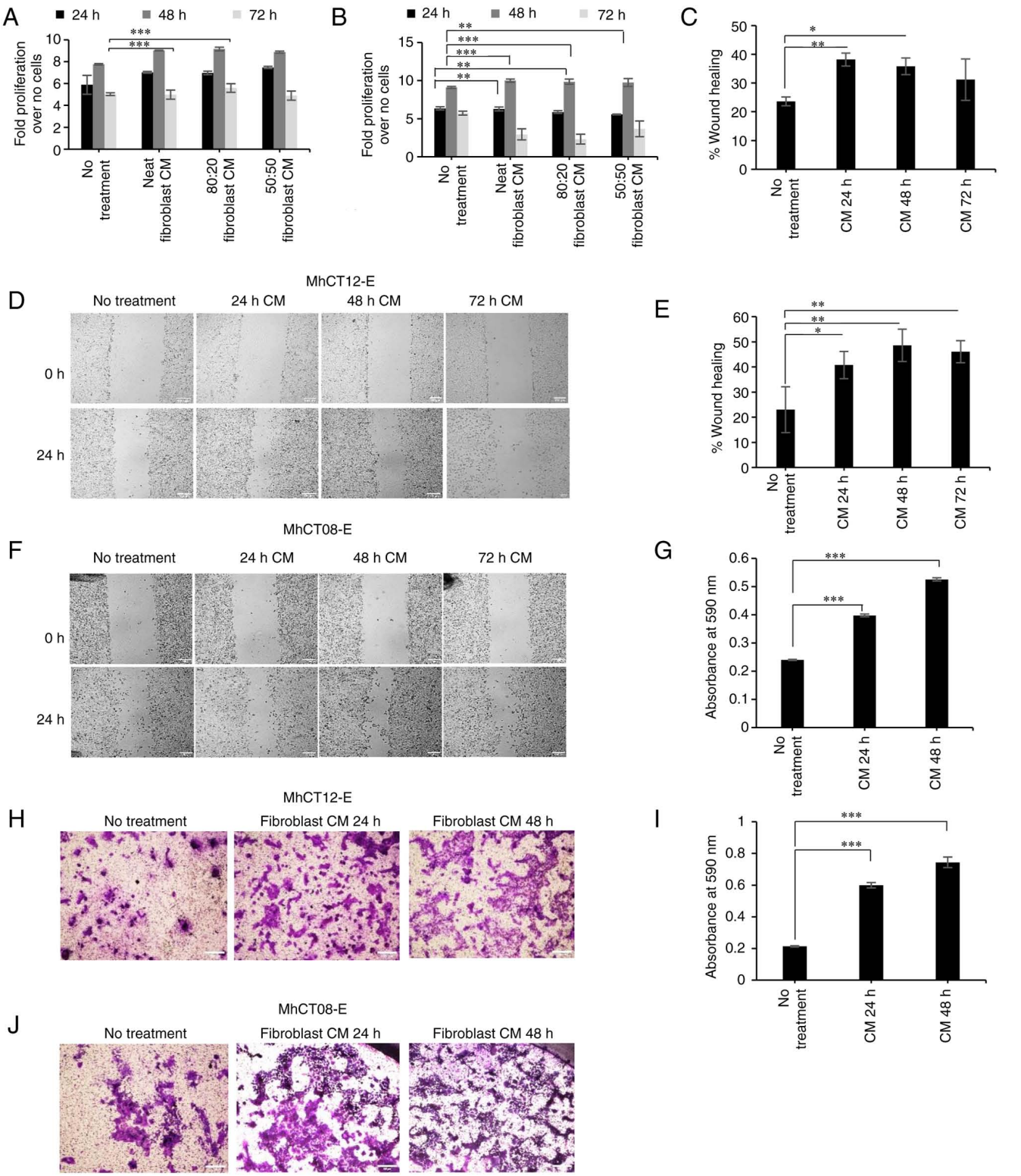

Figure 3. Tumorigenic properties of established cell lines. (A and B) Proliferation of (A) MhCT12-E and MhCT08-E (B) under the effect of CAF-conditioned medium at different time points. (C-F) Percentage of wound closure represented (C and E) graphically and (D and F) pictorially for MhCT12-E and MhCT08-E cell lines, respectively, under the effect of CAF-conditioned medium collected at indicated time points. Scale bar=100 μm. (G-J) Invasive potential as evaluated by crystal violet staining represented (G and I) graphically and (H and J) pictorially for MhCT12-E and MhCT08-E cell lines, respectively, under the effect of CAF-conditioned medium collected at indicated time points. Scale bar=100 μm. *P<0.05, **P<0.005 and ***P<0.001. CAF, cancer-associated fibroblasts. CM, conditioned medium. Neat, Complete conditioned medium; 80:20, 80% conditioned medium + 20% complete fresh medium; 50:50, 50% conditioned medium + 50% complete fresh medium.

karyotype of the original tumor has not been determined, the study is limited in drawing any hypothesis on the role of chromosomal multi-ploidy in spontaneous establishment of cell line from primary culture. Nevertheless, our analysis indicated abnormal DNA content in both cell lines, which may be responsible for their immortalization.

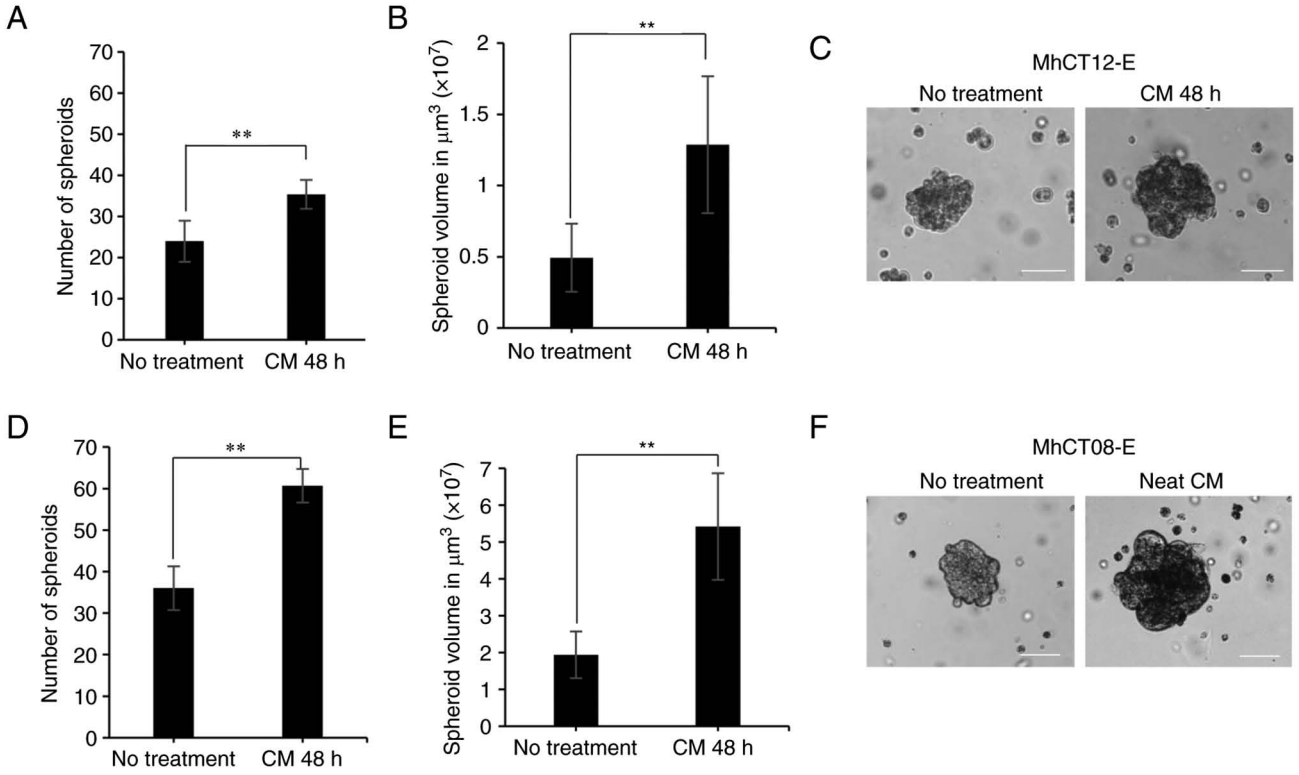

Figure 4. Sphere formation assay. (A-F) Sphere formation potential of (A-C) MhCT12-E and (D-F) MhCT08-E cells under the effect of CAF-conditioned medium. Scale bar=100 μm. CM, Conditioned medium. **P<0.01. CAF, cancer-associated fibroblasts.

In vitro assays such as proliferation, invasion-migration and sphere formation provide knowledge about the cancer stem cell subpopulation within a heterogeneous cell population (60,61). In a co-culture model, these studies further provide information of the tumor-stromal cross talk. Both MhCT12-E and MhCT08-E cell lines intrinsically had sphere formation ability, which was significantly increased upon treatment with corresponding CAF-conditioned medium. Similarly, the intrinsic migration, invasion and proliferative potential of the epithelial lines increased upon addition of CAF-conditioned medium. This may be due to various signalling factors released by the CAFs, which promote tumorigenesis and establishes the autologous pair of cells as an effective model to study tumor-stroma crosstalk (62,63). The *in vivo* tumor formation potential of the cell lines cannot be commented on, as *in vivo* tumorigenecity assays have not been performed yet. Unavailability of this data is a limitation to the present study in revealing the *in vivo* tumorigenicity of the established cultures.

High risk of HPV 16 and 18 infections have been associated with oral cancer. More than 25% of all oral cancers are associated with HPV16 infection, while a lower percentage of ~1-3% is attributed to HPV18 infection (42). Factually, HPV-positive oral cancers are associated with a favourable prognosis when compared with the HPV-negative oral cancers (41). Both MhCT12-E, F and MhCT08-E, F cultures were shown to be HPV-positive by PCR and ICC with anti-HPV antibodies.

The autologous pair of CAFs and cancer epithelial cells established in the present study serve as model systems to mimic the tumor-stroma cross talk, using multiple co-culture modes including conditioned medium-based assays, Transwell cultures and 3D models. This will aid in understanding the molecular mechanism behind stroma-tumor interaction leading to progression, treatment resistance, and other major problems in various types of cancer. The described co-culture system mimics the actual crosstalk between tumor cells and its static microenvironment, the CAFs. Moreover, the CAFs themselves can be excellent model systems to understand tumor progression. In summary, the cell lines developed in the present study have persistent growth potential and stable cell morphology. They hold great potential to be used as a novel drug testing platform in co-culture environment in addition to providing a useful tool to perform basic and translational research in the area of tumor-stroma interaction in human tongue cancer arising from no risk habits.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

MD and AS conceived and designed the study. CG collected the patient samples and isolated fibroblast and epithelial cells. ND performed the experiments. MAK and VP provided the samples. MD and ND wrote the manuscript, analysed and interpreted the data and confirm the authenticity of all the raw data. AS reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved [approval no. NHH/MEC-CL-2015-405 (A)] by the ethics committee of Narayana Health City (Bangalore, India). Informed consent for the study was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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