# Unique Cohorts of Salivary Gland Cancer Cells as an *in-vitro* Model of Circulating Tumor Cells.

Callie Mincy [1, 2], Donald Reed [1], Kathryn Carter [1], Luke Revelt [1], Anita Joy [1, 2, 3*]

[1]Department of Growth, Development and Structure, Southern Illinois University School of Dental Medicine, Alton, IL

[2]Department of Biological Sciences, College of Arts and Sciences, Southern Illinois University Edwardsville, Edwardsville, IL

[3]Department of Diagnostic and Biomedical Sciences, School of Dentistry, University of Texas at Houston, Houston, TX

*Correspondence to: Dr. Anita Joy, Department of Diagnostic and Biomedical Sciences, UT Houston School of Dentistry, 7500 Cambridge Ave., Houston, TX 77479.

Telephone: 713-486-4530

Facsimile: 713-487-416

E-mail: Anita.JoyThomas@uth.tmc.edu

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**Abstract**

The characterization of circulating tumor cells (CTC) and circulating tumor microemboli (CTM) has emerged as both a challenge to the standard view of metastasis, and as a valuable means for understanding genotypic and phenotypic variability shown even within the same cancer type. However, in the case of salivary gland neoplasms limited data are available for the role that CTCs and CTMs play in metastasis and secondary tumor formation. In response to this, we propose that similarities between *in vitro* clusters of cultured salivary gland cancer cells may act as a surrogate model for *in vivo* CTCs and CTMs isolated from patients. Using techniques in immunofluorescence, immunoblotting, and 2-dimensional migration, we isolated and characterized a group of cohort cells from a commercially available cell line (HTB-41). Here cells exhibited a hybrid phenotype with simultaneous expression of both epithelial and mesenchymal markers (E-cadherin, vimentin, and α-SMA). Cohort cells also exhibited increased migration in comparison to parental cells, thus making these isolated cell clusters a potential *in vitro* model of CTCs and CTMs.

## Introduction

Salivary gland (SG) cancers comprise an exceedingly diverse group of tumors, often affecting both minor (labial, buccal, palatine, lingual) and major salivary glands (parotid, submandibular, sublingual). This diversity with respect to origin and pathology generates a multitude of pathophysiological variations, resulting in challenges to prompt diagnosis and subsequent obstacles in disease management [1, 2]. For localized SG cancers, the standard treatment is surgical excision or surgery in conjunction with adjuvant radiation therapy [3]. Diagnosis of the disease at later stages or advanced grades increases the likelihood of primary tumor metastasis [3-5]. This leads to the fundamental challenge of treating SG cancers due to the variable genotypic traits and phenotypic behaviors presented by each histologic subtype [6]. The presence of cells within a tumor that could predispose to enhanced metastasis have been identified. These cells are either seen as circulating tumor cells (CTCs) or when present as clusters in the peripheral blood of cancer patients, are referred to as circulating tumor microemboli (CTMs). CTCs originate when cells detach from the primary tumor or even from a metastatic site to invade the blood circulation, thus playing a critical role in cancer progression.

Growing evidence suggests that CTCs possess distinct phenotypic and molecular characteristics allowing for enhanced metastatic and migratory abilities [7]. The multicellular CTM clusters typically have been reported to be comprised of $2 \geq 100$ cells, with or without tumor-associated cells. These multicellular clusters correspondingly have high levels of heterogeneity and variable morphological appearances [7-10]. In addition to heterogeneity within the clusters, CTMs appear to also have increased metastatic potential and therapeutic resistance when compared to individual CTCs, and their presence greatly reduces progression-free survival time of patients [11-15]. Cells within CTMs show reduced apoptosis, increased resistance to anoikis, and

retention of cell-cell junctions. CTCs and CTMs also have the ability to "self-seed" or have increased association with stromal and circulating cells [16-18]. This problem is therefore a major area of cancer research to isolate and characterize both CTCs and CTMs, in order to identify molecular mechanisms associated with different stages of metastasis. Although both CTCs and CTMs have been observed and characterized in patient samples from several types of epithelial cancers (breast, colorectal, prostate, and lung), limited data are available for these unique cell groups in SG cancers [19-26]. In one study, CTCs and CTMs from ten metastatic adenoid cystic carcinoma (ACC) patients were isolated and characterized, while another study focused on CTCs from a single patient with salivary duct carcinoma [27, 28]. In addition to the limited SG cancer patient data available, there is also minimal data evaluating the phenotypic and genotypic traits of *in vitro* models of SG cancers to identify CTC markers [29, 30].

In the present study, we investigated a salivary gland mucoepidermoid carcinoma cell line (HTB-41) for isolation and characterization of cultured cell cohorts/clusters to mimic CTCs *in vitro*, with specific emphasis on quantification of cell-cell adhesion, epithelial-mesenchymal transition markers, and migratory ability.

## Materials and Methods

*Human Cell Lines*

A submaxillary salivary gland cancer cell line HTB-41 (A-253) was purchased from the American Type Culture Collection (Manassas, VA). HTB-41 cells were aseptically cultured in McCoy's 5A growth media (Corning Cellgro; Manassas, VA) and supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin/amphotericin. Cultures were maintained in 5% $CO_2$ atmosphere at 37° C. Culturing conditions for the cohort subculture lines (CTC mimics) were identical to the HTB-41 cell line.

*Cohort Cell Isolation (CTC mimics)*

Cohort cell lines were subcultured from the HTB-41 cancer cell line. A single HTB-41 100 mm plate culture was grown to 100% confluence and a paper grid was placed on the bottom of the 10 mL culture plate (Fig. 1). Cells were examined under a light microscope and after 120 hours of growth, areas of multi-layered growth were noted according to which grid block they were found in. Cells from within these selected grid blocks were then subcultured into individual 100 mm plates.

*Immunofluorescence*

HTB-41 cancer and cohort cells (CTC mimics) were aseptically grown on tissue culture grade glass coverslips in sterile 12-well plates. Cells were allowed to reach 60% confluence with subsequent fixation in 3% paraformaldehyde, and processed for immunofluorescence (IF) and confocal imaging. Fixed cells were permeabilized in 0.2% Triton X-100 and blocked with a 10% blocking solution for 1 hour. Cells were incubated overnight at 4° C in anti-: β-catenin, E-cadherin, vimentin, and α-SMA antibodies (1: 100; Santa Cruz Biotechnology; Santa Cruz, CA).

Following thorough washes using 1x PBS, donkey anti-mouse or anti-rabbit secondary antibodies (1: 200; Dylight 488; Jackson ImmunoResearch Laboratories; West Grove, PA) were used to tag the proteins of specific interest. Cells were incubated with Rhodamine Phalloidin (1:50; Cytoskeleton; Denver, CO) for 30 minutes to visualize the actin cytoskeleton. Coverslips were mounted with a medium containing 4',6-diamidino-2-phenylindole (DAPI) for nuclear counterstain (ProLong Gold; Life Technologies; Grand Island, NY). IF images were acquired using a confocal microscope (Olympus FluoView FV300; Leeds Precision Instruments; Minneapolis, MN). Experiments were repeated in triplicate.

*Western Blotting*

HTB-41 cancer and cohort cells (CTC mimics) were aseptically cultured in 100 mm culture plates until 90% confluence was reached. Total protein extraction for all cell lines was carried out using a mammalian protein extraction reagent (M-PER; Thermo-Scientific/Pierce; Rockford, IL). Quantification of total protein was carried out using a bicinchoninic acid assay (BCA; Thermo-Scientific/Pierce; Rockford, IL) with equal amounts of protein being resolved by 10% SDS-PAGE under reducing conditions. After electrophoresis, proteins were electrotransferred onto nitrocellulose membranes (Bio-Rad; Hercules, CA), and blocked with 5% non-fat milk in 1x PBS and probed with anti-: β-catenin, E-cadherin, vimentin, alpha smooth muscle actin (α-SMA), and tubulin antibodies (1: 10,000; Santa Cruz Biotechnology; Santa Cruz, CA). Either horseradish peroxidase (HRP) conjugated horse anti-mouse or goat anti-rabbit IgG were used as secondary antibodies (1: 20,000; Cell Signaling Technology; Danvers, MA). Chemiluminescent visualization was carried out using Clarity Western ECL (Bio-Rad, Hercules, CA) as the substrate for HRP detection.

*2-Dimensional Cell Migration Assay*

HTB-41 cancer and cohort cells (CTC mimics) were grown to 90% confluence on tissue culture grade glass coverslips in sterile 6-well plates. A uniform "scratch" was created in a straight line using a sterile micropipette tip on Day 0. Cells were gently washed with 1x PBS to remove any debris, with growth media being replaced for each respective cell line, and incubated at $37^{o}$ C in a 5% $CO_2$ environment. Starting at Day 0, images of the "scratch" were acquired from the same field every 24 hours, using an inverted microscope (Nikon Eclipse E600; Nikon; Melville, NY), until the "scratch" was completely eliminated by migrating cells. Experiments were repeated in triplicate.

## Results

*E-cadherin and β-catenin show increased localization at the cell membrane in cohort cells*

Immunofluorescence studies against E-cadherin and β-catenin were carried out in HTB-41 cancer and cohort cells (Figs. 2 and 3). In HTB-41 cancer cells, prominent distribution of E-cadherin within the cytoplasm was observed (Fig. 2A' and 2A"'). As expected, this distribution was diffuse across the entire cytoplasm, with limited expression at cell-cell junctions. In contrast, the cohort cells presented with increased redistribution of E-cadherin from the cytoplasm to the membrane (Fig. 2B"'). Robust intracellular localization of β-catenin was observed in cancer cells (Fig. 3 A' and 3A"'). Within some cells, nuclear translocation was seen, although this was not the prevalent presentation that was observed (data not shown). Within the cohort cells, β-catenin presented with a redistribution from the cytoplasm with more presentation along the cell membranes (Fig. 3B' and B"').

*E-cadherin and β-catenin in cohort cells are present at higher levels than the parental HTB-41 cell line*

Western blot and optical density analysis to evaluate overall levels of E-cadherin and β-catenin expression supported the IF data. E-cadherin expression in the HTB-41cohort cells had an optical density near 250,000, while expression in HTB-41 cells exhibited a lower expression (~50,000; Fig. 4). In contrast to E-cadherin, β-catenin expression was inverse for both cell lines. A higher optical density of ~150,000 was observed in HTB-41 cancer cells, with the cohort line showing a much lower value (~80,000; Fig. 4).

*Expression of mesenchymal proteins is decreased in cohort cells*

Immunofluorescence studies against α-SMA and vimentin were also completed in HTB-41 cancer and cohort cells (Fig. 5). As typically seen in cancer cells, strong α-SMA expression was evident in the cytoplasm and along the cell membranes of HTB-41 cancer cells (Fig. 5A', A'"), while decreased expression of α-SMA was seen in cohort cells as compared to cancer cells (Fig. 5B', B"'). Vimentin expression was observed to be robust in HTB-41 cells, while expression was very similar in the cohort (Fig. 6).

*Vimentin and α-SMA are expressed at a lower level in cohort cells*

Analysis by western blot and optical density showed that vimentin expression in the cohort cells had a lower optical density (300,000) when compared to the parental HTB-41 cancer cells (700,000). Similarly, optical density of α-SMA in cohort cells was also lower, but within the standard deviation for the HTB-41 cells (100,000 and 250,000 respectively) (Fig. 7).

*Cohort cells presented with an increased migratory ability when compared to parental cells*

A 2-dimensional gap/wound closure assay was carried out to evaluate the migration of cohort cells in comparison to parental HTB-41 (Fig. 8). Complete closure of the induced gap/wound was observed at Day 1 for the cohort line and Day 3 for the HTB-41 line. Essentially, cancer cohort cells migrated at an increased rate when compared to parental cells, closing a gap/wound of ~2.0 mm 48 hours faster.

## Discussion

The majority of cancer related mortalities are the result of metastasis [31]. While this conclusion has remained unchanged, the understanding of the initiation of secondary tumors has progressed. Rather than the ideology that only dissemination of individual cancer cells that are shed from a primary tumor causes formation of new lesions, multicellular cohorts are now perceived to also be involved in the process of metastasis [32]. This however, is not a new concept as it has been previously reported by Zeidman and Fidler that metastasis is correlated to number, size, viability, and dispersal route of CTCs [33,34]. These same associations with clinical outcome have recently been observed in colorectal, breast, lung, liver, pancreatic, gastric, and head and neck neoplasms [35-46]. However, as previously mentioned, limited data are available for CTC association with neoplasms originating from the salivary glands. Of the two reports found at the time of this writing, 10 patients with ACC were evaluated by Metcalf et al. for CTCs using markers for epithelial cell adhesion molecules, vimentin and cytokeratin [27]. Metcalf et al. were able to isolate CTMs and CTCs with epithelial, mesenchymal, and hybrid phenotypes. In the second study, Cappelleti et al. reported using CTC detection in a single patient with salivary duct carcinoma as a means for tailoring treatment and improving early detection [28]. Similarly, SG cancer *in vitro* data are also insufficient for evaluating CTC markers [29,30]. ACC was investigated in both cited studies for either expression of the epithelial marker cytokeratin-14 or for establishment of a tumor cell-cultured xenograft model. Thus, a paucity of studies that evaluate CTCs in SG cancer types is noted.

An abundance of evidence supports the idea that β-catenin is associated with cell-cell adhesion and transcriptional regulation [47,48]. Under normal circumstances β-catenin is rapidly degraded through the axin, adenomatous polyposis coli (APC), casein kinase-β (CK1β), glycogen synthase

kinase-3β (GSK3β) destruction complex when not bound to E-cadherin [49,50]. While β-catenin uses the same interface to engage both E-cadherin and the nuclear transcriptional molecule T-cell factor (TCF), it binds preferentially with cadherins, setting a baseline threshold for transcriptional activation [51]. However, changes in expression of E-cadherin, β-catenin, or the destruction complex caused by mutation or other signaling molecules can lead to tumor progression, metastasis, and more invasive cancer phenotypes [52-55]. This usually occurs through accumulation of β-catenin within the cytoplasm followed by subsequent nuclear translocation and displacement of the transducing-like enhancer of split (TLE)/ Groucho repression complex. Consequently, increased gene activation within the cancer cells occurs and causes the observable phenotype. Increased expression of β-catenin in CTCs has also been observed. In patients with metastatic colorectal cancer resistant to 5-fluorouracil, Satelli et al. observed overexpression of β-catenin with preferred nuclear localization in isolated CTCs [56]. Correspondingly, in their primary cancer cell spheroid model, they also observed increasing nuclear accumulation correlating to a more invasive phenotype. Similar findings were observed in early stage lung cancer patients where over-expression of β-catenin was detected in both patient tumors and isolated CTCs when compared to healthy controls [57]. In hepatocellular carcinoma, CTCs were predominantly epithelial when released from the primary tumor but started to undergo Smad2/ β-catenin directed epithelial to mesenchymal transition (EMT) during circulation [58]. Cytoplasmic and nuclear β-catenin accumulation have also been described as potential cancer stem cell biomarkers in CTCs [59]. Furthermore, positive cellular recognition of β-catenin in CTCs was also observed in metastatic castration-resistant prostate cancer, early stage breast cancer, pituitary adenomas, inflammatory breast carcinoma xenograft, and mouse colorectal cancer [60-65].

While it is not currently known what exact role β-catenin plays in SG cancer CTCs, this protein has been associated with both benign and malignant SG cancer phenotypes [66-78]. More specifically, predominant cytoplasmic or nuclear localization of β-catenin was reported to contribute to aggressive behavior, invasiveness, poor differentiation, and decreased patient survival [66, 71, 74, 78]. As shown in our immunofluorescence experiments, β-catenin was predominately observed in the cytoplasm for HTB-41 cancer cells while membrane redistribution occurred in cohort cells. Furthermore, cohort cells exhibited an overall reduction in β-catenin expression levels with localization that appeared to circumscribe the cell border, and had increased aggregation at cell-cell interfaces. These findings are consistent with β-catenin localization in other reported studies of SG cancers [79-81]. Moreover, changes in localization and expression may suggest a quasi-mesenchymal or migratory state [57].

In addition to β-catenin, we also characterized the expression and localization of other molecules (E-cadherin, vimentin, α-SMA) associated with EMT-like changes [82-84]. The classical representation of EMT is the loss of cell-cell junctions and polarity, with gain of migratory and invasive properties. Typically, this is accomplished through genetic and epigenetic transcriptional regulation which causes an overall change in expression of epithelial and mesenchymal markers [85]. In SG cancers, loss of E-cadherin has been associated with more undifferentiated, invasive, or metastatic epithelial tumors [86-90]. However, this trend is not consistent across all studies, as Furuse et al. showed loss of E-cadherin expression was not associated with greater invasive potential in the tumor types studied [78]. The most likely explanation for this inconsistency is the variable expression of E-cadherin across SG cancer types as reported in other studies [77-91]. Our data show E-cadherin expression is elevated and localized at cellular adhesion sites between cells when comparing the parental and cohort lines.

While these findings are in contrast to the classical EMT process, retained E-cadherin expression in mucoepidermoid carcinomas has been previously described [88, 91-93]. Furthermore, Armstrong et al. reported co-expression of epithelial proteins and mesenchymal proteins in prostate and breast cancer CTCs suggesting an intermediate state to either an epithelial or mesenchymal state [93]. Additionally, re-expression of E-cadherin in breast and prostate cancer cells has been associated with increase in metastatic cell survival [94]. Our data seem to indicate that the cancer cohort cells exist in a transitory stage between epithelial and mesenchymal-like characteristics that promote migratory characteristics in them.

In contrast, vimentin and α-SMA are cytoskeletal markers often associated with more mesenchymal phenotypes [82]. This general association is supported in SG cancers as well, where increased expression of both vimentin and α-SMA have been linked to EMT, disrupted cellular organization, tumor distribution, and reduced survival [95-98]. Interestingly, mucoepidermoid carcinomas (MECs) are typically reported to be negative for α-SMA expression, although there is some variability [96, 99-100]. This is at odds with our analysis as both the HTB-41 parental and cohort lines showed expression along the cell margins, although the level of expression was significantly decreased in the cohort cells. This observed lack of expression in prior studies may be accounted for by comparison of tissue samples rather than tumor transplants or cultured cells [101]. Conversely, we speculate that the potential expression of tumor protein 53 (TP53) in our cells could induce α-SMA expression as shown in myeloid leukemia and breast cancer cells [102-103]. This idea is further supported by Kang et al. where mutations of TP53 were found to be both common and correlated with an overall increase in gene mutations in MECs [104]. Similarly, vimentin has been associated with tumor growth in some MECs [100, 105]. Vimentin when present is usually co-localized with other cytoskeletal

structures within the cell [106]. Interestingly, our findings show vimentin expression as disorganized clumps within the cytoplasm as compared to the more punctate representation seen in parent HTB-41 cells. One explanation for this observance could be the nuclear localization of β-catenin that was observed in some of the parental cells. In the breast cancer cell line MCF10A, it was reported by Gilles et al. that nuclear redistribution of β-catenin can lead to upregulation of the vimentin promotor in invasive/migratory cells [107]. Similarly, in the metastatic MEC cell line YD15M, it was observed that upregulation of vimentin correlated with an increase in β-catenin nuclear localization, and knockdown of vimentin increased membrane extension with potential reorganization of the cytoskeleton [106, 108]. These same morphological traits were indirectly shown in HTB-41 cells by Chen et al. where it was observed that invasive subclones presented with a mesenchymal like morphology, having increased filopodia and lamellipodia, E-cadherin downregulation, and vimentin upregulation [109]. Thus, our cohort line presents as a more epithelial-derived line with clustered growth when compared to parental cells or sublines reported from the Chen et al. study.

The typically held view is that mesenchymal-like cells are more mobile. Our cohort cells migrated 48 hours faster than the parental cells closing a similar sized gap. Under conventional reports, increased expression of mesenchymal markers (i.e. vimentin and α-SMA) would lead to a more aggressive, migratory, and/or invasive cell [82-85]. However, many invasive carcinomas have retained E-cadherin expression suggesting that mobility is not precluded by its presence [110]. Control of cadherin expression alters adhesive activity at the cell surface thereby controlling epithelium formation, and allowing for the potential of collective migration [111, 112]. Through a positive feedback loop with Rac and actin assembly, E-cadherin is able to stabilize directional protrusion and movement between more motile and polar cells allowing for

clustered chemotaxis [113]. In a study of pancreatic cancer by Liu et al., tumor invasion was accelerated rather than inhibited by ectopic expression of E-cadherin [114]. Furthermore, reduced expression of E-cadherin can lead to a diminished capacity of SUM149, Mary-X, and 4T1 tumor cells to grow *in vivo*, through reduced adaptability to the microenvironment [115, 116]. Loss of E-cadherin may also cause changes in interactions between neoplastic and normal epithelial cells, thus making it an important biomolecule for metastasis of certain cancer types [117].

CTCs in clusters preserve strong cell-cell connections associated with the abundance of cells with epithelial and/or epithelial and mesenchymal like traits [8, 16, 58]. This composition indicating heterogeneity and/ or plasticity among cells within the collective is thought to contribute to a higher metastatic potential and therapeutic resistance relative to single CTCs. One prospective reason for this higher potential is the ability of clustered cells to undergo rapid and reversible unfolding into single-file chains through modulation of intercellular adhesions within capillary sized vessels [118]. Maintaining an epithelial- mesenchymal plasticity may also increase the interaction with endothelial and other cancer-associated cells (fibroblasts, platelets, immune cells, etc.) during early and late stages of metastasis [119]. This is specifically shown by Labernadie et al. where impairment of E-cadherin/N-cadherin between cancer cells and cancer-associated fibroblasts (CAF) lead to abrogation of guided migration and cancer cell invasion [120].

Similar to May et al., we believe that an *in vitro* model of clustered cells can serve as surrogate for comparison to *in vivo* CTCs and CTMs [121]. Based on our results we have isolated heterogeneous sublines of HTB-41 cancer cells that simultaneously express both epithelial and mesenchymal markers. These isolated cells present with a more epithelial-like phenotype and

morphology based on visual appearance and expression levels of β-catenin, E-cadherin, vimentin, and α-SMA. Conversely, these cells also migrate at a much faster rate than their more mesenchymal-like predecessor, most likely through clustered migration. Thus, we believe our HTB-41 cohort lines represent a group with epithelial-mesenchymal plasticity or a unique cluster of cells with a hybrid epithelial/mesenchymal phenotype. This hybrid phenotype is characterized by the coexistence of both epithelial and mesenchymal markers in cancer cells, allowing for adaptability to different microenvironments during translocation [84, 122-124]. While this hybrid phenotype may be less migratory/invasive when existing as solitary cells, an established cohort may be able to circumvent different inhibitory phases during metastasis, thereby allowing for maximum colonization at secondary tumor sites [125]. Nonetheless, further studies are required to verify if all of the cells within our cohort lines simultaneously express epithelial and mesenchymal markers or whether the cohorts are composed of individual mesenchymal and epithelial like cells. Furthermore, these cohort groups will also need to be analyzed to confirm if these epithelial/ mesenchymal cohorts lead to increased metastasis *in vivo* or confer any enhanced adaptability for survival.

## Conclusion

This study reports a group of epithelial/mesenchymal-like cells isolated from a mucoepidermoid salivary gland cancer cell line that may serve as a surrogate for salivary gland CTCs and CTC clusters *in vitro*. These isolated cells form aggregate groups with enhanced migration capabilities, and expression of molecules associated with epithelial and mesenchymal transitions (β-catenin, E-cadherin, vimentin, and α-SMA) similar to that seen within *in vivo* CTC clusters.

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**Competing Interests**

The authors declare that they have no competing interests.

**Availability of Data and Materials**

Supporting data can be provided on request.

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**Author Contributions**

CM performed the immunofluorescence and western blotting, and presented the data as part of a Master's thesis. CM helped with manuscript preparation. KC helped design the study, and supervised and helped with the immunofluorescence studies and all cell culture. KC also assisted with manuscript preparation. DR supervised and helped perform the immunoblotting experiments. DR assisted extensively with manuscript preparation. LR assisted with manuscript preparation and with immunofluorescence. AJ designed the study, supervised the study, analyzed

data, and drafted the manuscript. All authors have read and approved the final manuscript prior to submission.

## References


1. Barnes L, Eveson J, Reichart P, et al. World Health Organization classification of tumours: pathology and genetics of head and neck tumours. IARC Press: Lyon 2005.

2. Wang X, Luo Y, Li M, et al. Management of salivary gland carcinomas – a review. Oncotarget. 2017; 8: 3946-3956.

3. Chintakuntlawar A, Okuno S, Price K. Systemic therapy for recurrent or metastatic salivary gland malignancies. Cancers Head Neck. 2016; 1: 1-9.

4. Terhaard C, Lubsen, H, Van der Tweel, I, et al. Salivary gland carcinoma: independent prognostic factors for locoregional control, distant metastases, and overall survival: results of the Dutch head and neck oncology cooperative group. Head Neck. 2004; 26: 681-693.

5. Adams A, Warner K, Nor J. Salivary gland cancer stem cells. Oral Oncol. 2013; 49: 845-853.

6. Son E, Panwar A, Mosher C, et al. Cancers of the major salivary gland. J Oncol Pract. 2018; 14: 99-108.

7. Umer M, Vaidyanathan R, Nguyen N, et al. Circulating tumor microemboli: progress in molecular understanding and enrichment technologies. Biotechnol Adv. 2018; 36(4): 1367-1389.

8. Yu M, Bardia A, Wittner B, et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science. 2013; 339: 580-584.

9. Aceto N, Bardia A, Miyamoto D, et al. Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. Cell. 2014; 158(5): 1110-1122.

10. Cima I, Kong S, Sengupta D, et al. Tumor-derived circulating endothelial cell clusters in colorectal cancer. Sci Transl Med. 2016; 8(345):345ra89.

11. Au S, Edd J, Stoddard A, et al. Microfluidic isolation of circulating tumor cell clusters by size and asymmetry. Sci Rep. 2017; 7(2433): 1-10.

12. Bithi S, Vanapalli S. Microfluidic cell isolation technology for drug testing of single tumor cells and their clusters. Sci Rep. 2017; 7(41707): 1-12.

13. Liotta L, Saidel M, Kleinerman J. The significance of hematogenous tumor cell clumps in the metastatic process. Cancer Res. 1976; 36(3): 889-894.

14. Hou J, Krebs M, Lancashire L, et al. Clinical significance and molecular characteristics of circulating tumor cells and circulating tumor microemboli in patients with small-cell lung cancer. J Clin Oncol. 2012; 30(5): 525-532.

15. Dasgupta A, Lim A, Ghajar C. Circulating and disseminated tumor cells: harbingers or initiators of metastasis? Mol Oncol. 2017; 11: 40-61.

16. Giuliano M, Shaikh A, Lo H, et al. Perspective on circulating tumor cell clusters: why it takes a village to metastasize. Cancer Res. 2018; 78(4): 845-852.

17. Praharaj P, Bhutia S, Nagrath S, et al. Circulating tumor cell-derived organoids: Current challenges and promises in medical research and precision medicine. Biochim Biophys Acta Rev Cancer. 2018; 1869(2): 117-127.

18. Micalizzi D, Maheswaran S, Haber D. A conduit to metastasis: circulating tumor cell biology. Genes Dev. 2017; 31(18): 1827-1840.

19. Broncy L, Paterlini-Brechot P. Circulating tumor cells for the management of renal cell carcinoma. Diagnostics. 2018; 8(63): 1-15.

20. Kulemann B, Rosch S, Seifert S, et al. Pancreatic cancer: circulating tumor cells and primary tumors show heterogeneous KRAS mutations. Sci Rep. 2017; 7(4510): 1-11.

21. Boral D, Vishnoi M, Liu H, et al. Molecular characterization of breast cancer CTCs associated with brain metastasis. Nat Commun. 2017; 8(196): 1-10.

22. Wang L, Zhou S, Zhang W, et al. Circulating tumor cells as an independent prognostic factor in advanced colorectal cancer: retrospective study in 121 patients. Int J Colorectal Dis. 2019; 34(4): 589–597.

23. Pantel K, Hille C, Scher H. Circulating tumor cells in prostate cancer" from discover to clinical utility. Clin Chem. 2019; 65(1): 87-99.

24. De Souza L, Robertson B, Robertson G. Future of circulating tumor cells in the melanoma clinical and research laboratory setting. Cancer Lett. 2017; 392: 60-70.

25. Kapeleris J, Kulasinghe A, Warkiani M, et al. The prognostic role of circulating tumor cells (CTCs) in lung cancer. Front Oncol. 2018; 8(311): 1-11.

26. Yu J, Xiao W, Dong S, et al. Effect of surgical liver resection on circulating tumor cells in patients with hepatocellular carcinoma. BMC Cancer. 2018; 18(835): 1-9.

27. Metcalf R, Mohan S, Hilton S, et al. The application of liquid biopsies in metastatic salivary gland cancer to identify candidate therapeutic targets. Ann Oncol. 2017; 28(Suppl 7).

28. Cappelletti V, Miodini P, Reduzzi C, et al. Tailoring treatment of salivary duct carcinoma (SDC) by liquid biopsy: Arv7 expression in circulating tumor cells. Ann Oncol. 2018; 29(7): 1598-1600.

29. Gao X, Wu J, Cao M, et al. Cytokeratin-14 contributes to collective invasion of salivary adenoid cystic carcinoma. PLoS ONE. 2017; 12(2): 1-14.

30. Chen C, Choundhury S, Wangsa D, et al. A multiplex preclinical model for adenoid cystic carcinoma of the salivary gland identifies regorafenib as a potential therapeutic drug. Sci Rep. 2017; 7(11410): 1-15.

31. Chaffer C, Weinberg R. A perspective on cancer cell metastasis. Science. 2011; 331(6024): 1559-1564.

32. Au S, Edd J, Haber D, et al. Clusters of circulating tumor cells: a biophysical and technological perspective. Curr Opin Biomed Eng. 2017; 3: 13-19.

33. Zeidman I. Comparison of metastatic growth produced by tumor cell emboli in veins and lymphatics. Cancer Res. 1965; 25: 324-327.

34. Fidler I. The relationship of embolic homogeneity, number, size and viability to the incidence of experimental metastasis. Eur J Cancer. 1973; 9(3): 223-227.

35. Tsai W, Chen J, Shao H, et al. Circulating tumor cell count correlates with colorectal neoplasm progression and is a prognostic marker for distant metastasis in non-metastatic patients. Sci Rep. 2016; 6(24517): 1-8.

36. Campos M, Prior C, Warleta F, et al. Phenotypic and genetic characterization of circulating tumor cells by combining immunomagnetic selection and fiction techniques. J Histochem Cytochem. 2008; 56(7): 667-675.

37. Nakagawa T, Martinez S, Goto Y, et al. Detection of circulating tumor cells in early-stage breast cancer metastasis to axillary lymph nodes. Clin Cancer Res. 2007; 13(14): 4105-4110.

38. Bredemeier M, Edimiris P, Mach P, et al. Gene expression signatures in circulating tumor cells correlate with response to therapy in metastatic breast cancer. Clin Chem. 2017; 63(10): 1585-1593.

39. Agelaki S, Fiste O, Grapsa D, et al. Prognostic value of circulating tumor cells in non-small cell lung cancer. J Clin Oncol. 2017; 35:15 (Suppl): e20520-e20520

40. Zhang Z, Xiao Y, Zhao J, et al. Relationship between circulating tumour cell count and prognosis following chemotherapy in patients with advanced non-small cell lung cancer. Respirology. 2016; 21(3): 519-525.

41. Tong B, Xu Y, Zhao J, et al. Prognostic significance of circulating tumor cells in non-small cell lung cancer patients undergoing chemotherapy. Oncotarget. 2017; 8(49): 86615-86624.

42. Tien Y, Kuo H, Ho B, et al. A high circulating tumor cell count in portal vein predicts liver metastasis from periampullary or pancreatic cancer. Medicine. 2016; 95(16): e3407.

43. Chiba N, Abe Y, Ozawa Y, et al. The preliminary role of circulating tumor cells obtained from the hepatic or portal veins in patients with hepatobiliary-pancreatic cancer. Eur Surg. 2017; 49(1): 5-8.

44. Ishiguro Y. Prognostic significance of circulating tumor cells in patients with gastric cancer: Epithelial mesenchymal transition and perioperative kinetics. J Clin Oncol. 37(4): (Suppl 4):59.

45. Kulasinghe A, Kapeleris J, Kimberley R, et al. The prognostic significance of circulating tumor cells in head and neck and non-small cell lung cancer. Cancer Med. 2018; 7(12): 5910-5919.

46. Antonarakis E, Lu C, Luber B, et al. Clinical significance of androgen receptor splice variant-7 mRNA detection in circulating tumor cells of men with metastatic castration-resistant prostate cancer treated with first and second line abiraterone and enzalutamide. J Clin Oncol. 2017; 35(19): 2149-2156.

47. McCrea P, Gottardi C. Beyond β-catenin: prospects for a larger catenin network in the nucleus. Nat Rev Mol Cell Biol. 2016; 17:55-64.

48. Prakash S, Swaminathan U. β-catenin in health: a review. J Oral Maxillofac Pathol. 2015; 19(2): 230-238.

49. Chen G, Waterman M. Cancer: leaping the E-cadherin hurdle. EMBO J. 2015; 34(18): 2307-2309.

50. McCrea P, Maher M, Gottardi C. Nuclear signaling from cadherin adhesion complexes. Curr Top Dev Biol. 2015; 112:126-196.

51. Jeanes A, Gottardi C, Yap A. Cadherins and cancer: how does cadherin dysfunction promote tumor progression? Oncogene. 2008; 27(69): 20-29.

52. Pai S, Carneiro B, Mota J, et al. Wnt/beta-catenin pathway: modulating anticancer immune response. J Hematol Oncol. 2017; 10(101).

53. Krishnamurthy N, Kurzrock R. Targeting the Wnt/beta-catenin pathway in cancer: update on effectors and inhibitors. Cancer Treat Rev. 2018; 62: 50-60.

54. Luke J, Bao R, Sweis R, et al. Wnt/β-catenin pathway activation correlates with immune exclusion across human cancers. Clin Cancer Res. 2019; 25(10): 3074-3083.

55. Zhan T, Rindtorff N, Boutros M. Wnt signaling in cancer. Oncogene. 2016; 36:1461-1473.

56. Satelli A, Mitra A, Brownlee Z, et al. Epithelial-mesenchymal transitioned circulating tumor cells capture for detecting tumor progression. Clin Cancer Res. 2015; 21(4):899-906.

57. Zhang Z, Shiratsuch H, Lin J, et al. Expansion of CTCs from early stage lung cancer patients using a microfluidic co-culture model. Oncotarget. 2014; 5(23):12383-12397.

58. Sun Y, Guo W, Xu Y, et al. Circulating tumor cells from different vascular sites exhibit spatial heterogeneity in epithelial and mesenchymal composition and distinct clinical significance in hepatocellular carcinoma. Clin Cancer Res. 2018; 24(3):547-559.

59. Sun Y, Xu Y, Yan X, et al. Circulating stem cell-like epithelial cell adhesion molecule positive tumor cells indicate poor prognosis of hepatocellular carcinoma after curative resection. Hepatology. 2012; 57(4):1458-1468.

60. Bitting R, Boominathan R, Rao C, et al. Development of a method to isolate circulating tumor cells using mesenchymal-based capture. Methods. 2013; 64(2):1-20.

61. Goto W, Kashiwagi S, Asano Y, et al. Circulating tumor cell clusters associated gene plakoglobin is a significant prognostic predictor in patients with breast cancer. Biomark Res. 2017; 5(19):1-8.

62. Hamilton G, Hochmair M, Rath B, et al. Small cell lung cancer: circulating tumor cells of extended stage patients express a mesenchymal-epithelial transition phenotype. Cell Adhesion & Migration: 2016; 10(4): 360-367.

63. Hua G, Yanjiao H, Qian L, et al. Detection of circulating tumor cells in patients with pituitary tumors. BMC Cancer. 2018; 18(336):1-5.

64. Scholch S, Garcia S, Iwata N, et al. Circulating tumor cells exhibit stem cell characteristics in an orthotopic mouse model of colorectal cancer. Oncotarget. 2016; 7(19):27232-27242.

65. Tomlinson J, Alpaugh M, Barsky S. An intact overexpressed E-cadherin/a,b-catenin axis characterizes the lymphovascular emboli of inflammatory breast carcinoma. Cancer Res. 2001; 61:5231-5241.

66. Chandrashekar C, Angadi P, Krishnapillai R. β-catenin expression in benign and malignant salivary gland tumors. Int J Surg Pathol. 2011; 19(4):433-440.

67. Wend P, Fang L, Zhu Q, et al. Wnt/b-catenin signaling induces MLL to create epigenetic changes in salivary gland tumours. EMBO J. 2013; 32:1977-1989.

68. Wang R, Geng N, Zou Y, et al. Aberrant Wnt-1/beta-catenin signaling and WIF-1 deficiency are important events which promote tumor cell invasion and metastasis in salivary gland adenoid cystic carcinoma. Biomed Mater Eng. 2015; 26: S2145-2153.

69. Jo V, Sholl L, Krane J. Distinctive patterns of CTNNB1 (β-catenin) alterations in salivary gland basal cell adenoma and basal cell adenocarcinoma. Am J Surg Pathol. 2016; 40(8): 1143-1150.

70. Lee Y, Huang W, Hsieh M. CTNNB1 mutations in basal cell adenoma of the salivary gland. J Formos Med Assoc. 2018; 117(10):894-901.

71. Genelhu M, Gobbi H, Arantes D, et al. Immunolocalization of β-catenin in pleomorphic adenomas and carcinomas ex-pleomorphic adenomas of salivary glands. Appl Immunohistochem Mol Morph. 2007; 15(3):273-278.

72. Hakata Y, Fukui H, Sekikawa A, et al. Expression of b-catenin and REG Ia in relation to cell proliferative ability in salivary gland tumors. Exp Ther Med. 2010; 1(3):437-443.

73. Wilson T, Ma D, Tilak A, et al. Next-generation sequencing in salivary gland basal cell adenocarcinoma and basal cell adenoma. Head Neck Pathol. 2016; 10(4):494-500.

74. Lill C, Schneider S, Seemann R, et al. Correlation of b-catenin, but not PIN1 and cyclin D1, overexpression with disease-free and overall survival in patients with cancer of the parotid gland. Head Neck. 2015; 37(1):30-36.

75. Ishibashi K, Ishii K, Sugiyama G, et al. Regulation of β-catenin phosphorylation by PR55β in adenoid cystic carcinoma. Cancer Genomics Proteomics. 2018; 15(1):53-60.

76. Okuda Y, Nakano K, Suzuki K, et al. Wnt signaling as a possible promoting factor of cell differentiation in pleomorphic adenomas. Int J Med Sci. 2014; 11(9):971-978.

77. Tesdahl B, Wilson T, Hoffman H, et al. Epithelial-mesenchymal transition protein expression in basal cell adenomas and basal cell adenocarcinomas. Head Neck Pathol. 2016; 10(2):176-181.

78. Furuse C, Cury P, Altemani A, et al. β-catenin and E-cadherin expression in salivary gland tumors. Int J Surg Pathol. 2006; 14(3):212-217.

79. Sengupta P, Bouchie M, Kukuruzinska M. N-glycosylation gene DPAGT1 is a target of the Wnt/b-catenin signaling pathway. J Biol Chem. 2010; 285(41):31164-31173.

80. Chen L, Liu D, Chang J, et al. methylation status of insulin-like growth factor-binding protein 7 concurs with the malignance of oral tongue cancer. J Exp Clin Cancer Res. 2015; 34(20).

81. Prakash S, Swaminathan U, Nagamalini B, et al. Beta-catenin in disease. J Oral Maxillofac Pathol. 2016; 20(2):289-299.

82. Zeisberg M, Neilson E. Biomarkers for epithelial-mesenchymal transitions. J Clin Inv. 2009; 119(6):1429-1437.

83. Smith B, Bhowmick N. Role of EMT in metastasis and therapy resistance. J Clin Med. 2016: 5(17).

84. Pastushenko I, Blanpain C. EMT transition states during tumor progression and metastasis. Trends Cell Biol. 2019; 29(3):212-226.

85. Thierauf J, Veit J, Hess J. Epithelial to mesenchymal transition in the pathogenesis and therapy of head and neck cancer. Cancers: 2017; 9(76): 1-8.

86. Andreadis D, Epivatianos A, Mireas G, et al. Immunohistochemical detection of E-cadherin in certain types of salivary gland tumours. J Laryngol Otol. 2006; 120(4):298-304.

87. Yamada K, Kudeken W, Sumitomo S, et al. Immunohistochemical expression of E-cadherin in salivary glands and their tumors. Acta Histochem. 1996; 29(4):305-310.

88. Franchi A, Gallo O, Bocciolini C, et al. Reduced E-cadherin expression correlates with unfavorable prognosis in adenoid cystic carcinoma of salivary glands of the oral cavity. Anat Pathol. 1999; 111:43-50.

89. Namboodiripad P. A review: Immunological markers for malignant salivary gland tumors. J Oral Biol Craniofac Res. 2014; 4(2):127-134.

90. Prabhu S, Kaveri H, Rekha K. Benign; malignant salivary gland tumors: comparison of immunohistochemical expression of E-cadherin. Oral Oncol. 2009; 45:594-599.

91. Shieh Y, Chang L, Chiu K, et al. Cadherin and catenin expression in mucoepidermoid carcinoma: correlation with histopathological grade, clinical stage, and patient outcome. J Oral Pathol Med. 2003; 32(5):297-304.

92. Moghadam S, Pourmahdi M, Gholamian M, et al. Immunohistochemical analysis of E-cadherin expression in pleomorphic adenoma and mucoepidermoid carcinoma of salivary glands. J Dent Sch. 2013; 31(2):104-109.

93. Armstrong A, Marengo M, Oltean S, et al. Circulating tumor cells from patients with advanced prostate and breast cancer display both epithelial and mesenchymal markers. Mol Cancer Res. 2011; 9(8):997-1007.

94. Chao Y, Wu Q, Shepard C, et al. Hepatocyte induced re-expression of E-cadherin in breast and prostate cancer cells increases chemoresistance. Clin Exp Metastasis. 2012; 29(1):39-50.

95. Enescu A, Enescu A, Florou C, et al. E-cadherin and a-SMA expression in epithelial mesenchymal transition of salivary glands pleomorphic adenomas. Rom J Morphol Embryol. 2014; 55(4):1383-1387.

96. Ravi M, Boaz K, Natarajan S, et al. Expression of a-smooth muscle actin in benign and malignant salivary gland tumors: an immunohistochemical study. Ind J Pathol Microbiol. 2018; 61(4):479-484.

97. Weissferdt A, Kalhor N, Moran C. Pleuromediastinal epithelial-myoepithelial carcinomas: A clinicopathologic and immunohistochemical study of two cases. Am J Clin Pathol. 2016; 146:736-740.

98. Busch A, Bauer L, Wardelmann E, et al. Prognostic relevance of epithelial-mesenchymal transition and proliferation in surgically treated primary parotid gland cancer. J Clin Pathol. 2017; 70(5):403-409.

99. Omitola O, Iyogun C. Immunohistochemical study of salivary gland tumors in a tertiary institution in South-South Region of Nigeria. J Oral Maxillofac Pathol. 2018; 22(2):163-167.

100. Zhu S, Schuerch C, Hunt J. Review and updates of immunohistochemistry in selected salivary gland and head and neck tumors. Arch Pathol Labo Med. 2015; 139(1):55-66.

101. Yook J, Lee S, Chun Y, et al. The myoepithelial cell differentiation of mucoepidermoid carcinoma in a collagen gel-based coculture model. J Oral Pathol Med. 2004; 33(4):237-242.

102. Secchiero P, Rimondi E, Grazia di lasio M, et al. Activation of the p53 pathway induces α-smooth muscle actin expression in both myeloid leukemic cells and normal macrophages. J Cell Phys. 2011; 227(5):1829-1837.

103. Kim S, You D, Jeong Y, et al. TP53 upregulates a-smooth muscle actin expression in tamoxifen-resistant breast cancer cells. Oncol Rep. 2019; 41(2):1075-1082.

104. Kang H, Tan M, Bishop J, et al. Whole-exome sequencing of salivary gland mucoepidermoid carcinoma. Clin Cancer Res. 2017; 23(1):283-288.

105. Irani S, Jafari B. Expression of vimentin and CD44 in mucoepidermoid carcinoma: a role in tumor growth. Ind J Dent Res. 2018; 29(3):333-340.

106. Liu C, Lin H, Tang M, et al. Vimentin contributes to epithelial-mesenchymal transition cancer cell mechanics by mediating cytoskeletal organization and focal adhesion maturation. Oncotarget. 2015; 6(18):15966-15983.

107. Gilles C, Polette M, Mestdagt M, et al. Transactivation of vimentin by b-catenin in human breast cancer cells. Cancer Res. 2003; 63:2658-2664.

108. Lim W, Kim H, Kim Y, et al. Association between cancer stem cell-like properties and epithelial to mesenchymal transition in primary and secondary cancer cells. Int J Oncol. 2016; 49(3):991-1000.

109. Chen L, Hsu W, Tseng Y, et al. Involvement of DNMT 3B promotes epithelial-mesenchymal transition and gene expression profile of invasive head and neck squamous cell carcinomas cell lines. BMC Cancer. 2016; 16(431).

110. Gloushankova N, Rubtsova S, Zhitnyak I. Cadherin mediated cell-cell interactions in normal and cancer cells. Tissue Barriers. 2017; 5(3).

111. Hwang S, Zimmerman N, Agle K, et al. E-cadherin is critical for collective sheet migration and is regulated by chemokine CXCL12 protein during restitution. J Biol Chem. 2012; 287(26):22227-22240.

112. Cheung K, Gabrielson E, Werb Z, et al. Collective invasion in breast cancer requires a conserved basal epithelial program. Cell. 2013; 155(7):1639-1651.

113. Cai D, Chen S, Prasad M, et al. Mechanical feedback through E-cadherin promotes direction sensing during collective cell migration. Cell. 2014; 157:1146-1159.

114. Liu X, Huang H, Remmers N, et al. Loss of E-cadherin and epithelial to mesenchymal transition is not required for cell motility in tissues or for metastasis. Tissue Barriers. 2014: 2(4).

115. Chu K, Boley K, Moraes R, et al. The paradox of E-cadherin: Role in response to hypoxia in the tumor microenvironment and regulation of energy metabolism. Oncotarget. 2013; 4(3):446-462.

116. Elisha Y, Kalchenko V, Kuznetsov Y, et al. Dual role of E-cadherin in the regulation of invasive collective migration of mammary carcinoma cells. Sci Rep. 2018; 8(4986).

117. Rubtsova S, Zhitnyak I, Gloushankova N. A novel role of E-cadherin based adherens junctions in neoplastic cell dissemination. PLOS ONE. 2015; 10(7).

118. Au S, Storey B, Moore J, et al. Clusters of circulating tumor cells traverse capillary-sized vessels. PNAS. 2016; 113(18):4947-4952.

119. Quail D, Joyce J. Microenvironmental regulation of tumor progression and metastasis. Nat Med. 2013; 19:1423-1437.

120. Labernadie A, Kato T, Brugues A, et al. A mechanically active heterotypic E-cadherin/ N-cadherin adhesion enables fibroblasts to drive cancer cell invasion. Nat Cell Biol. 2017; 19:224-237.

121. May A, Crawford B, Nedelcu A. In vitro model systems to understand the biology and clinical significance of circulating tumor cell clusters. Front Oncol. 2018; 8(63).

122. Diepenbruck M, Christofori G. Epithelial-mesenchymal transition (EMT) and metastasis: yes, no, maybe? Curr Opin Cell Biol. 2016; 43:7-13.

123. Francart M, Lambert J, Vanwynsberghe A, et al. Epithelial-mesenchymal plasticity and circulating tumor cells: travel companions to metastases: Dev Dyn. 2018; 247:432-450.

124. Jolly M, Mani S, Levin H. Hybrid epithelial/ mesenchymal phenotype(s): the 'fittest' for metastasis? Biochim Biophys Acta Rev Cancer. 2018; 1870:151-157.

125. Jie X, Zhang X, Xu C. Epithelial to mesenchymal transition, circulating tumor cells and cancer metastasis: mechanisms and clinical applications. Oncotarget. 2017; 8(46):81558-81571.

**Figure 1**

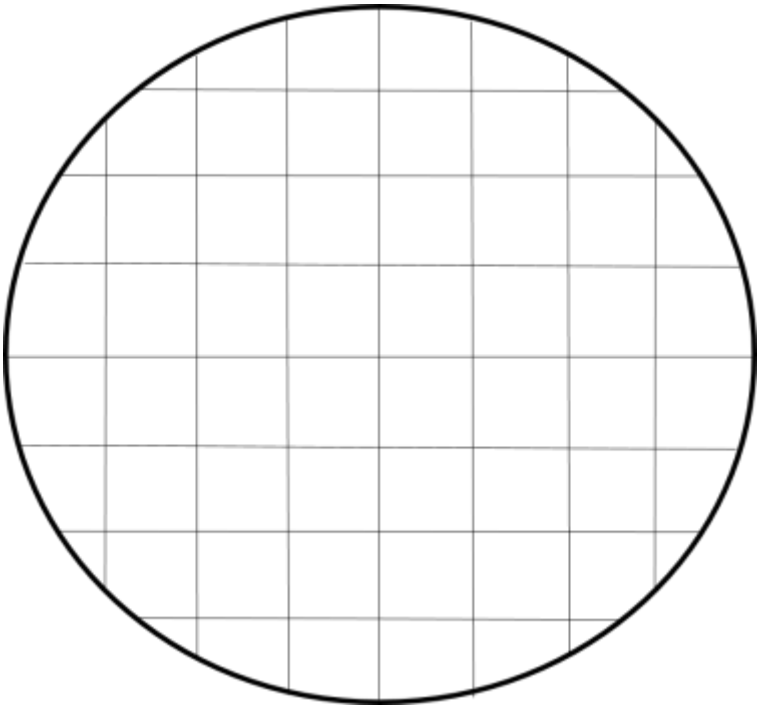

Figure 1. Diagrammatic representation of grid used for subculturing cohort cell populations.

**Figure 2**

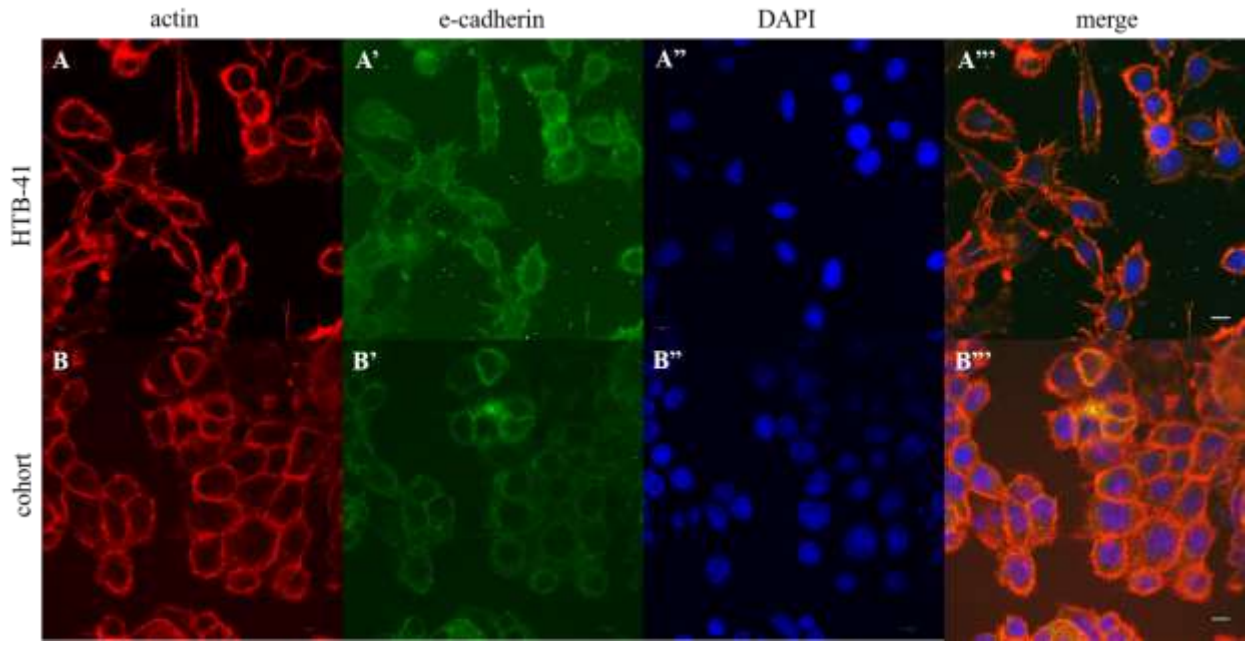


Figure 2: E-cadherin expression in cohort cells vs parental HTB-41. Salivary mucoepidermoid carcinoma HTB-41 and cohort cells were analyzed for expression and localization of E-cadherin using immunofluorescence. In HTB-41 cells there was noticeable dispersal of E-cadherin across the entire cytoplasm with more limited expression at cell-cell junctions. In cohort cells, E-cadherin was localized predominately to the cellular membrane with some cytoplasmic distribution. Scale bar = 5 µm.

**Figure 3**

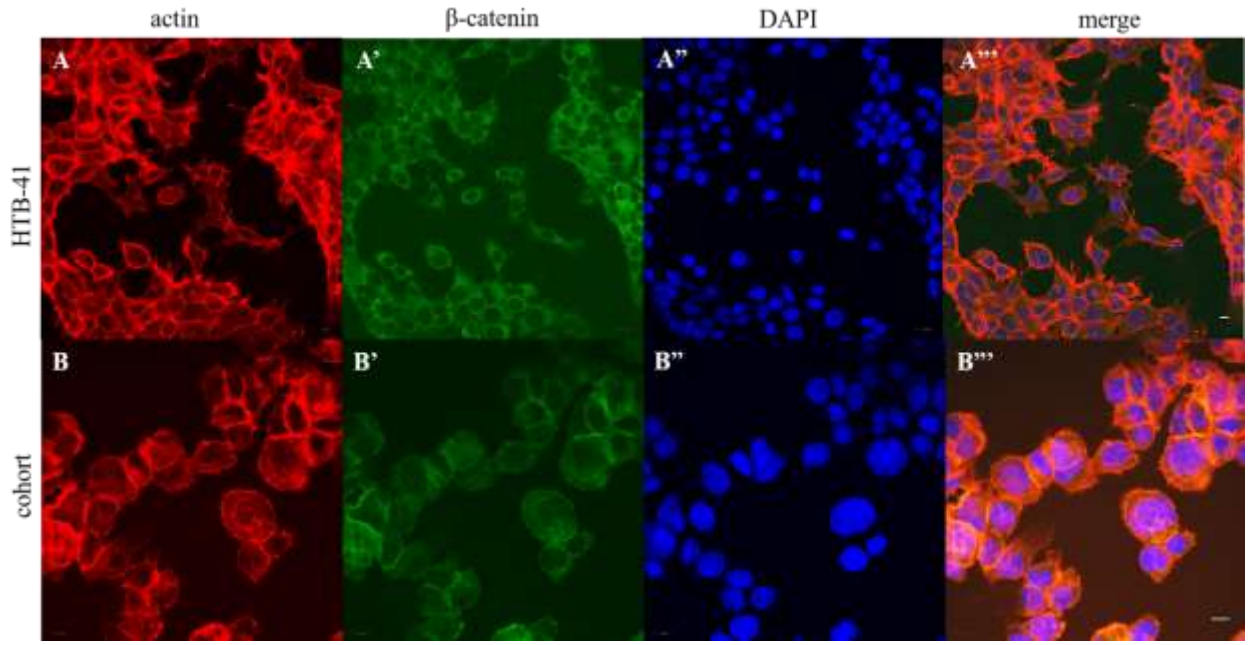


Figure 3: β-catenin expression in cohort cells vs parental HTB-41. Salivary mucoepidermoid carcinoma HTB-41 and cohort cells were analyzed for expression and localization of β-catenin using immunofluorescence. Scale bar = 5 µm. In HTB-41 cells β-catenin had a robust expression within the cytoplasm, whereas in cohort cells, a distinct membrane expression was seen. Scale bar = 5 µm.

**Figure 4**

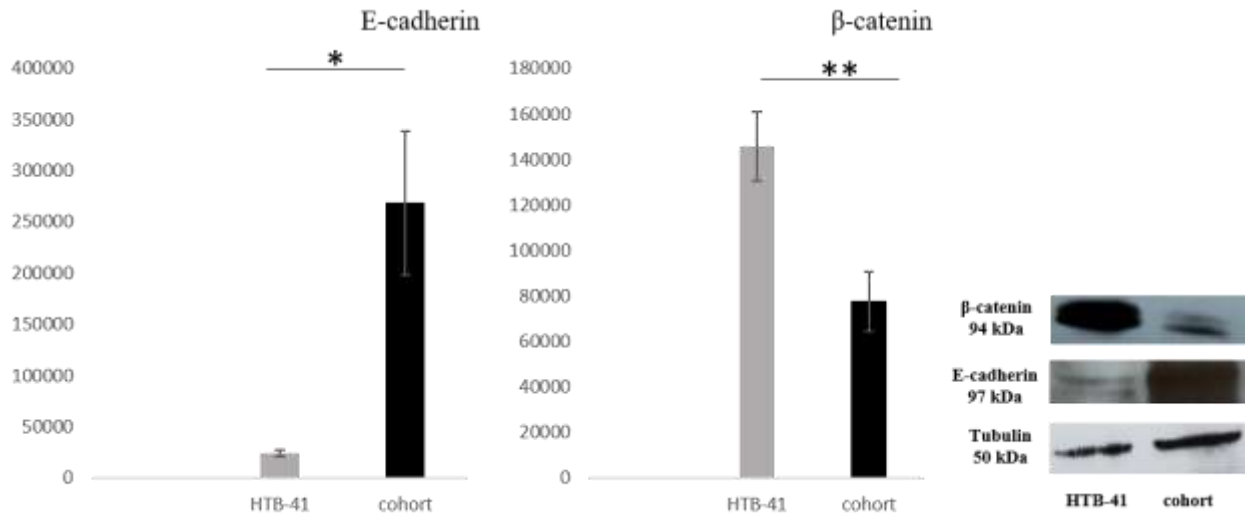


Figure 4. Evaluation of E-cadherin and β-catenin expression in HTB-41 and cohort cells in Western Blot detection. Western blot revealed that E-cadherin levels were elevated, while β-catenin levels decreased in cohort cells as compared to their parental HTB-41 cells. Analyses of relative band intensities followed by a student's t-test revealed that the change in expression levels were significant. (*$p \leq 0.001$ and **$p \leq 0.005$; data expressed as mean ±SD).

**Figure 5**

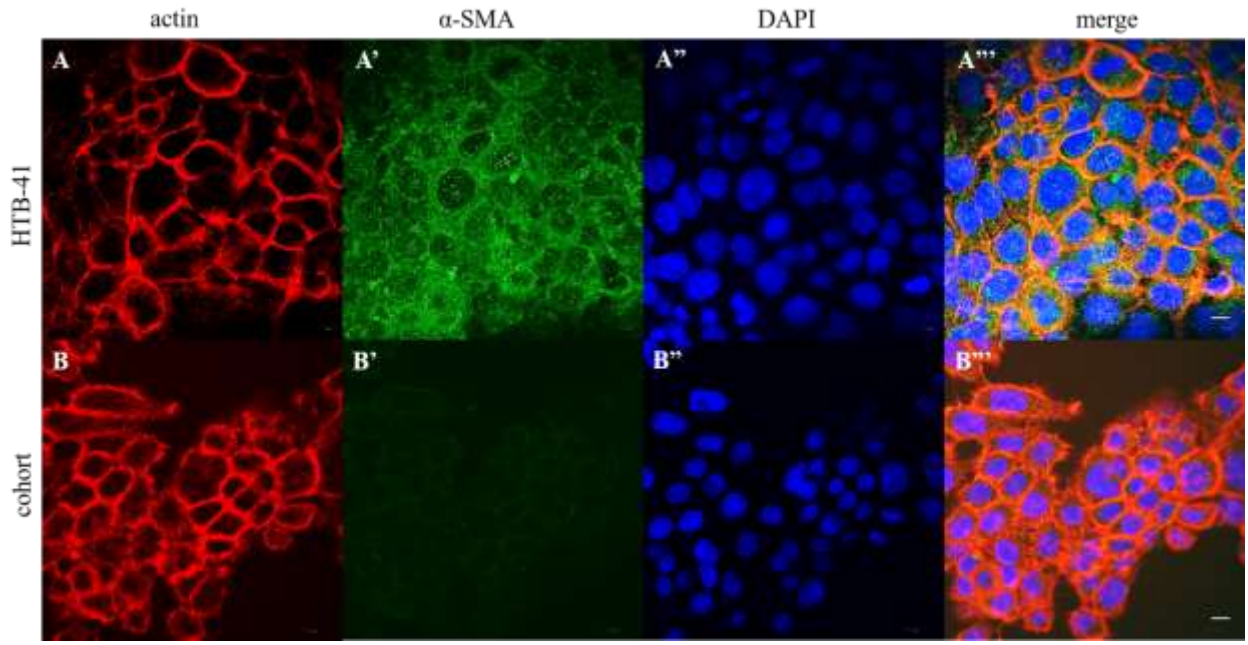


Figure 5. α-SMA expression in cohort cells vs parental HTB-41 cells. In HTB-41 cells, robust expression of α-SMA was observed, with localization to both the cytoplasm and membrane. In cohort cells α-SMA was observed more distinctly at cellular margins, co-localizing with actin. Scale bar = 5 μm.

**Figure 6**

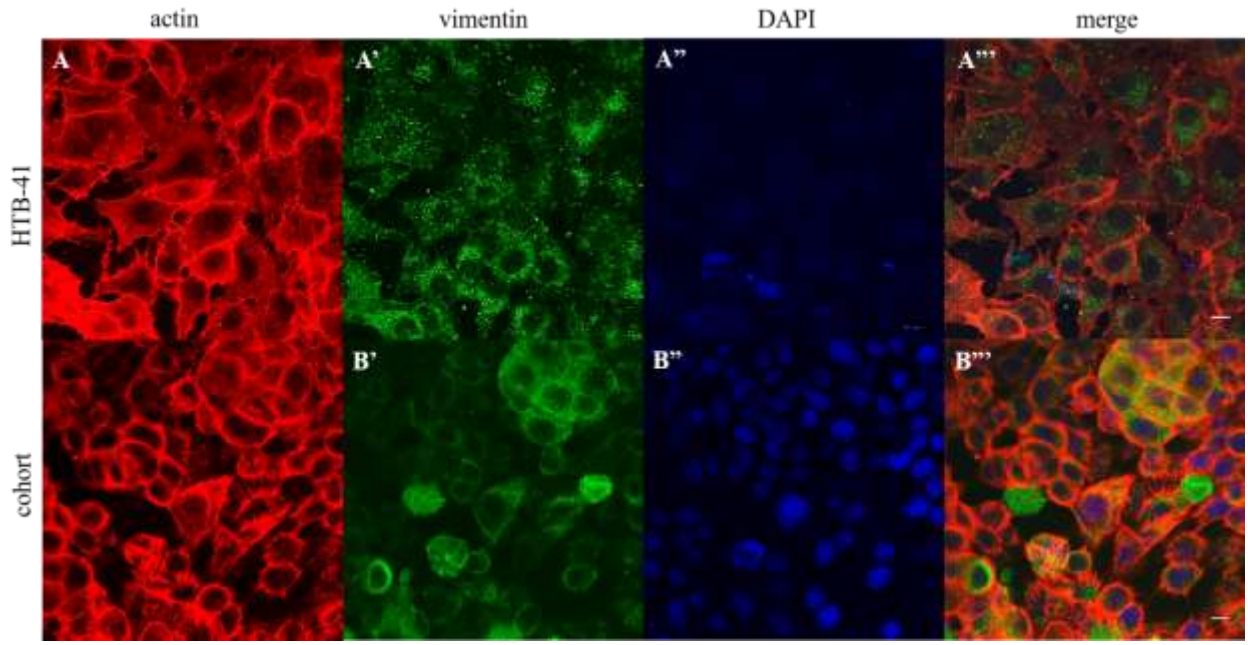


Figure 6. Vimentin expression in cohort cells vs parental HTB-41 cells. A cytoplasmic expression of vimentin with no presence in the membrane was visible in HTB-41 cells, while the cohort cells showed increased expression of vimentin as cytoplasmic clumps. Some expression in the membrane was also noticeable in these cells. Scale bar = 5 µm.

**Figure 7**

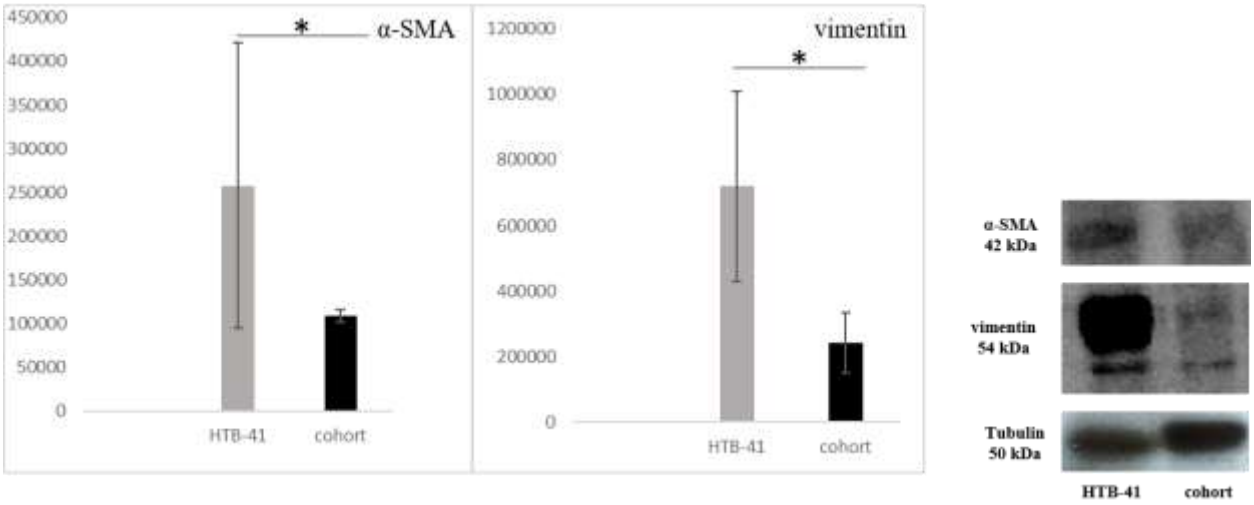


Figure 7. Evaluation of α-SMA and vimentin expression in HTB-41 and cohort cells in Western Blot detection. Western blot revealed that both α-SMA and vimentin levels were elevated in cohort cells as compared to their parental HTB-41 cells. Analyses of relative band intensities followed by a student's t-test revealed that the change in expression levels were significant. (*$p \leq 0.001$; data expressed as mean ±SD).

**Figure 8**

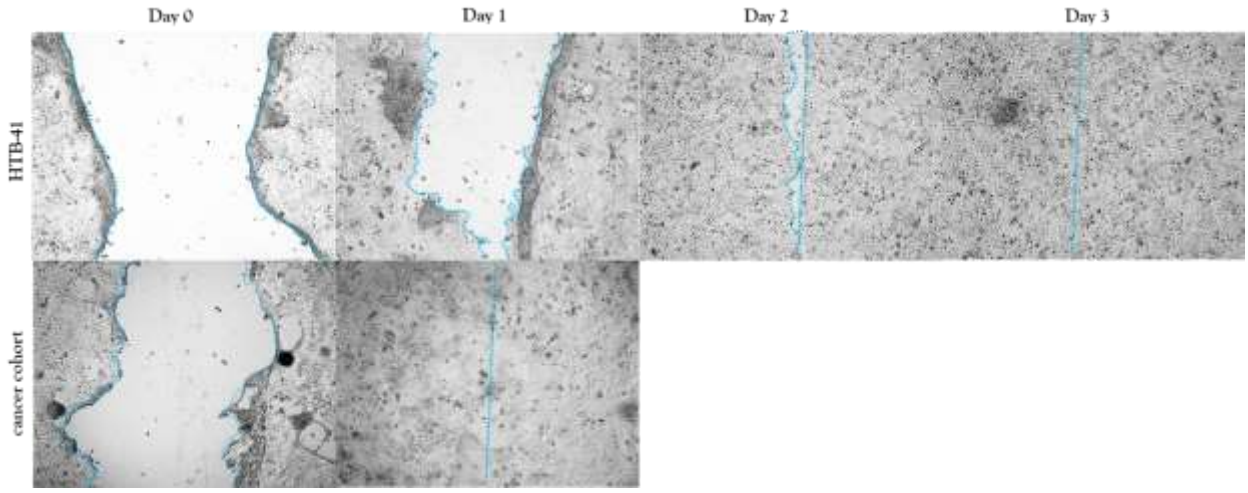


Figure 8. Increased migration is observed in HTB-41 cohort cells. HTB-41 parental and cohort cells were cultured until 90% confluence was reached. A uniform scratch or wound was created with a sterile micropipette tip on Day 0 (blue dotted line depicts the cell free region). Cells were imaged every 24 hours post Day 0 (4x magnification). Cohort cells migrated at a faster rate than parental HTB-41 cells, showing complete wound closure 24 hours earlier.