INTRODUCTION

Salivary gland carcinomas (SGCs) account for approximately 6% of all head and neck cancers in the United States. They include more than 30 types of benign or malignant neoplasms with variable morphological and physiological traits (1). These traits can be extremely diverse between tumor types and even within individual tumors, thus making SGCs difficult to diagnose and manage. Standard treatment for localized SGCs is surgical excision with or without radiation therapy (1-3). If regional or distant metastases have occurred, therapeutic intervention becomes more limited and survival rates diminish to approximately 40% (4). Additionally, even with judicious intervention, the rate of recurrence for patients with SGCs is high (1). Therefore, identifying markers for early diagnosis and recognizing molecular mechanisms associated with tumor advancement are critical for improving SGC management.

The Wnt signaling pathway has been associated with epithelial-mesenchymal transition (EMT), cellular proliferation and differentiation, and with stem cell populations in multiple types of cancers (5,6). In the canonical pathway, Wnt signaling is dependent on β-catenin and with judicious intervention, the rate of recurrence for patients with SGCs is high (1). Therefore, identifying markers for early diagnosis and recognizing molecular mechanisms associated with tumor advancement are critical for improving SGC management.

The Wnt signaling pathway has been associated with epithelial-mesenchymal transition (EMT), cellular proliferation and differentiation, and with stem cell populations in multiple types of cancers (5,6). In the canonical pathway, Wnt signaling is dependent on β-catenin, a modulator of gene expression, through nuclear localization and removal of transcriptional repression from Groucho/T-cell factor (TCF)/Lymphoid enhancing factor (LEF) (7). Under normal circumstances, β-catenin is rapidly degraded through a destruction complex when not associated with cadherin-mediated adhesion (8). β-catenin uses the same binding interface to engage both cadherins and TCF, with greater affinity toward the cadherin-rein-catenin interaction. Thus, E-cadherin expression sets a baseline threshold for Wnt/β-catenin signaling through normal sequestration of β-catenin (9). However, loss of E-cadherin expression, increased Wnt signaling, or mutations associated with components of the destruction complex can lead to expression of cancer-related genes associated with tumor progression, metastasis, and invasive phenotypes (10,11). β-catenin expression has also been reported in several types of SGCs including benign and malignant phenotypes (12-18), however, findings were not conclusive across all studies. In two reports, predominant cytoplasmic β-catenin localization with reduced membranous expression was associated with decreased survival, invasiveness, aggressive behavior, and/or a lack of differentiation among malignant tumors (12,17). While in another study, aberrant nuclear localization was suggested to be a contributing factor to the aggressive behavior of epithelial-myoepithelial carcinomas (15). However, neither cytoplasmic nor nuclear localization alone denote malignancy, based on expression also occurring in benign salivary gland tumors (12). Based on this inconsistency among studies, there may be a morphological and behavioral effect from Wnt and β-catenin that differs among salivary gland neoplasms.

The aim of the present study was to evaluate expression of β-catenin in an in vitro model of salivary epidermoid carcinoma using the HTB-41 cell line. We also aim to investigate the effects of inhibiting the Wnt/β-catenin signaling pathway on cellular functions that affect cancer progression, including cell proliferation and migration.

MATERIALS AND METHODS

Human cell lines

A normal salivary gland cell line, HSG, was received as a generous gift (Dr. M. Hoffman, NIH, Bethesda, MA). A submaxillary salivary cancer cell line, HTB-41, was acquired from the American Type Culture Collection (ATCC, Manassas, VA). HSG and HTB-41 cells were aseptically cultured in DMEM/F-12 and McCoy5A media respectively (Corning Cellgro, Manassas, VA), supplemented with 10% FBS and 1% penicillin-streptomycin-ampicillin, in 5% CO2 atmosphere at 37°C. Cancer HTB-41 cell line was also treated with 10 µM and 50 µM of a Wnt inhibitor, IWR-1 (Sigma-Aldrich, St. Louis, MO) for 48 hours, and designated as HTB-41/IWR. All cell culture experiments were carried out in triplicate.

Immunofluorescence

HSG, HTB-41, and HTB-41/IWR cells were aseptically grown on tissue culture grade glass coverslips in sterile 12-well plates. On reaching 80% confluence, cells were fixed in 3% paraformaldehyde, and processed for immunofluorescence and confocal imaging. Fixed cells were permeabilized in 0.2% Triton X-100 and blocked with 10% bovine serum albumin for 1 hour. Cells were incubated overnight at 4°C, in anti β-catenin, anti Axin, and anti p-GSK3β primary antibodies (1:100; Santa Cruz Biotechnology, Inc., Dallas, TX). Following thorough washes with 0.07M PBS, donkey anti-mouse secondary antibody (Dylight 488; 1:200, Jackson ImmunoResearch Laboratories Inc., West Grove, PA) was used to tag the specific proteins of interest. Cells were incubated with Rhodamine-Phalloidin to visualize the actin cytoskeleton (1:50; Cytoskeleton Inc., Denver, CO). Coverslips were mounted with mounting medium containing DAPI for nuclear counterstain using a Confocal microscope (Olympus Fluoview FV300, Leeds Precision Instruments, MN).

Western blotting

HSG, HTB-41, and HTB-41/IWR cells were aseptically cultured in 100 mm sterile culture dishes till they reached 90% confluence. Total proteins were extracted using M-PER mammalian protein extraction reagent (Thermo Scientific/Pierce, Rockford, IL). Total proteins were also extracted from
HSG+ cells (HSG+ = normal HSG cells cultured in cancer secretome). Protein estimation was carried out using the RC DC protein assay (Bio-Rad, Hercules, CA) and equal amounts of protein were resolved by 10% SDS-PAGE under reducing conditions. After electrophoresis, proteins were electrotransferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA), blocked with 5% non-fat milk in 1X PBS, and probed with anti-β-catenin, anti-Ecadherin, anti-Axin, and anti-p-GSK3β antibodies (1:500; Santa Cruz Biotechnology, Dallas, TX). HRP-conjugated goat anti-mouse IgG was used as secondary antibody (1:10,000; Sigma-Aldrich, St. Louis, MO) and Clarity western ECL (Bio-Rad, Hercules, CA) was used as the substrate for HRP detection.

To determine equal protein loading, each membrane was carefully washed, treated for 5 minutes with stripping buffer (Thermo Scientific/Pierce, Rockford, IL) to eliminate the previous reaction and washed with PBS. Membranes containing whole protein were processed as above with anti-tubulin primary antibody (1:10,000; Sigma-Aldrich, St. Louis, MO) and HRP-conjugated goat anti-mouse IgG secondary antibody (1:10,000; Sigma-Aldrich, St. Louis, MO). Chemiluminescent detection was carried out for HRP detection, as described above.

2-Dimensional cell migration assay
HSG, HTB-41, and HTB-41/IWR cells were seeded on sterile, culture-grade glass coverslips, and grown to confluence. 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, respective growth media was added to the central well of 6-well transwell plates. 500 µl of DMEM/F-12 serum-free media was added to the well of the transwell plate. 2 ml DMEM/F-12 serum-free media was added to the central well. Transwell plates were incubated at 37°C in a 5% CO2 environment for 24 hours at every 24 hours, using an inverted microscope (Nikon Eclipse E600; Nikon, Melville, NY), until the “scratch” was completely eliminated by migrating cells. The experiment was repeated in triplicate.

3-Dimensional cell migration assay
HSG, HTB-41, and HTB-41/IWR cells were incubated in 1 µM of CM-Dil (Life Technologies, Grand Island, NY) tracking dye as per manufacturer’s instructions. Cells were washed in 1X PBS and resuspended in respective serum-free media. Sterile, culture-grade glass cover slips were placed at the borders of normal cells, where it co-localizes with actin (Figures 1A’ and 1A”). Chemiluminescent detection was carried out for HRP detection, as described above.

RESULTS

Preferential nuclear localization of β-catenin in salivary gland cancer was abrogated following treatment with Wnt inhibitor. Immunohistochemistry studies against β-catenin was carried out using normal human salivary gland cells (HSG) and human cancer salivary gland cells (HTB-41) and is depicted in Figure 1.

B, B’, B”, merge: Representative confocal images depict actin cytoskeleton, intracellular localization of β-catenin, and nuclei in cancer HTB-41 cells. The cytoplasm of cancer cells is filled with β-catenin and translocation into the nucleus is pronounced.

C, C’, C”, merge: Representative confocal images depict actin cytoskeleton, intracellular localization of β-catenin, and nuclei in cancer HTB-41 cells following treatment with a Wnt inhibitor. Wnt inhibition abrogates the excessive intracellular accumulation of β-catenin and its nuclear translocation that is seen in cancer cells with an activated Wnt pathway. β-catenin is seen present in the cytoplasm, as well as outlining cell boundaries.

As expected, β-catenin is seen present in the cytoplasm and outlining the borders of normal cells, where it co-localizes with actin (Figures 1A’ and 1A”). In cancer cells, β-catenin is strongly expressed within the cytoplasm, with no significant localization on the cell membranes. In addition, robust translocation into the nucleus is also clearly seen in cancer cells (Figures 1B’ and 1B”). Following treatment of salivary gland cancer cells with 10 µM IWR-1, a Wnt inhibitor, β-catenin no longer translocated into the nucleus, rather, a cytoplasmic expression coupled with membrane localization, as expected in normal cells can be seen (Figure 1C’ and 1C”).

Western blot analyses to evaluate overall levels of β-catenin showed significantly higher levels of the protein in cancer salivary gland cells as compared to normal cells, which were then significantly decreased following treatment with the Wnt inhibitor (Figure 2).

Cancer cells exhibited altered expression of β-catenin destruction complex components.

Figure 2) Evaluation of β-catenin expression in HSG, HTB-41, and HTB-41/IWR cells in Western Blot detection at 72 hours. Western blot revealed that β-catenin levels were elevated in cancer cells compared to normal cells, and following Wnt inhibition returned to lower levels. Analyses of relative band intensities followed by a student’s t-test revealed that β-catenin was significantly elevated in cancer cells. Although levels decreased compared to cancer cells following treatment with the Wnt inhibitor, they were not significant. (*p < 0.03; data expressed as mean ± SD)

Axin and phosphorylated glycogen synthase kinase-3β (p-GSK3β) form part of the β-catenin destruction complex and are found located in the cytoplasm in normal salivary gland cells (Figures 3A’, 3A’’, 4A’, and 4A’”). In cancer cells, with the activation of the Wnt pathway, the β-catenin destruction complex is dismantled, resulting in decreased expression of Axin and p-GSK3β in the cytoplasm (Figures 3B’, 3B’’, 4B’, and 4B’”). Inhibition of Wnt using 10 µM IWR-1 results in normal expression of p-GSK3β and a minimal change in distribution of Axin in the cancer cells (Figures 3C’, 3C’”, 4C’, and 4C’”).

Figure 3) Axin, part of the β-catenin destruction complex, moves out of the cytoplasm in cancer cells. Representative confocal images of HSG, HTB-41, and HTB-41/IWR cells depict localization of Axin. (Scale bar=5 µM)
A, A', A", merge: Representative confocal images depict actin cytoskeleton, intracellular localization of Axin, and nuclei in normal HSG cells. Axin shows a punctate presentation within the cytoplasm of normal salivary gland cells.

B, B', B", merge: In cancer cells, although Axin continues to be seen in the cytoplasm, it is no longer present as discrete punctae, but rather is seen as being dispersed.

C, C', C", merge: Following treatment with the Wnt inhibitor, Axin seems to resume its punctate intracellular expression.

A, A', A", merge: Representative confocal images depict actin cytoskeleton, intracellular localization of Axin, and nuclei in normal HSG cells. p-GSK3β shows a strong presentation within the cytoplasm of normal salivary gland cells, where it is present as part of the β-catenin destruction complex.

B, B', B", merge: In cancer cells, there seems to be a significant decrease in p-GSK3β in the cytoplasm.

C, C', C", merge: Following treatment with the Wnt inhibitor, p-GSK3β seems to exhibit resurgence within the cytoplasm.

Western blot analyses to quantify levels of Axin and p-GSK3β revealed decreased levels in cancer states as compared to normal salivary gland cells, followed by a significant reversal to normal levels of p-GSK3β and no reversal in Axin levels following inhibition of the Wnt pathway (Figure 5).

DISCUSSION

Ample evidence supports the idea that Wnt/β-catenin signaling is associated with resistance to chemotherapy, acquisition of epithelial-mesenchymal transition (EMT) features and is being studied as a potential therapeutic target in various cancers (5). Within a cell, β-catenin is known to play a dual role – as a linker protein in cell-cell adhesion, and as a component of the Wnt signaling pathway. In association with the catenins, E-cadherin exhibits its normal cellular adhesive function and sequestration of β-catenin (19). When the canonical Wnt pathway is activated, β-catenin, which normally undergoes phosphorylation/ubiquitination via targeting by a destruction complex (adenomatous polyposis coli (APC) + casein kinase-β (CK1β) + pGSK3β + Axin [main] and v-associates protein/transcriptional co-activator with PDZ-binding motif (YAP/TAZ) + β-transducin repeat-containing protein (β-TrCP) [secondary]) is not proteasomally degraded. Instead, β-catenin begins to accumulate in the cytoplasm with eventual translocation into the nucleus, and subsequent displacement of the transducing-like enhancer of split (TLE)/Groucho repression complex from TCF/LEF (6). Thus, in its dual role, β-catenin can function through the Wnt signaling pathway or through the E-cadherin catenin complex.

A 3-dimensional migration assay showed that cancer salivary gland cells migrated at a significantly faster rate as compared to normal salivary gland cells. Following treatment with the Wnt inhibitor, cancer cell migration rates were significantly decreased (Figure 7).

IWR-1, cancer salivary gland cells showed reduced migration rates, with the gap/wound not completely closing even after the fourth day (Figure 6).
Wnt related mutations have been shown to influence the initial development and the progression of several types of cancers, including carcinomas of the gastrointestinal tract, lungs, ovaries, uterus, thyroid, and pancreas (20-22). There is also mounting evidence that this pathway also contributes to tumorigenesis in salivary gland cancers, and inhibition of Wnt has been postulated as a potential mechanism to limit cancer progression. Here increased β-catenin activation, in combination with cAMP response element (CREB)-binding and mixed-lineage leukemia (MLL) proteins, has been associated with promoter methylation of self-renewal genes in salivary gland cancer (13). Furthermore, regulatory changes of Wnt, β-catenin, and Wnt inhibitory factor-1 have been linked to the development and/or progression of some adenocarcinomas, and of the invasive adenoid cystic carcinoma (14,19-24). Similarly, mutations of CTNNB1 (β-catenin), Axin1, Axin2, and APC have been shown in adenoid cystic carcinoma and basal cell adenoma (25,26). While our results are consistent with data showing immunofluorescence localization of β-catenin at cell–cell interfaces and within the cytoplasm of epidermoid carcinoma to Sengupta et al., we also observed nuclear translocation (27). This observation is supported by Chen et al., where Western Blot analysis of nuclear fractions revealed the presence of β-catenin and is consistent with immunohistochemical data from other studies (28,29). In addition, nuclear β-catenin could be abrogated when cells were treated with the Wnt inhibitor IWR-1. Concurrently, total protein concentration was also shown to decrease following treatment. These results are similar to previous studies using L-Wnt3a cells, osteosarcoma cell lines, colorectal and pancreatic cancer cells, and mouse embryonic stem cell (30-34). Furthermore, we observed no significant change in abatement between use of 10 μM and 50 μM doses of IWR-1. It is expected that upon further analysis of protein lysates between control, cancer, and treated groups we would observe a reduction in nuclear localization of β-catenin with an expected increase in phosphorylated β-catenin when IWR-1 is present.

In the case of the components of the β-catenin destruction complex, namely Axin1 and p-GSK3β, their distributions were not overtly altered following Wnt inhibition. Levels of Axin1 were not significantly altered between treated and untreated HTB-41 groups, while levels of p-GSK3β were prominently increased following Wnt inhibition between these same groups. Our data indicate higher levels of Axin1 in normal salivary gland cells than in the cancer cells, both in the cytoplasm and as clear specks on the cell membrane. Although our immunofluorescence data show a more distinct localization of Axin1 in the cytoplasm of cancer cells and increased representation on the cell membrane following Wnt inhibition, levels did not significantly change in cancer cells when compared to control or treated groups. We speculate that this might be associated with the mechanism of action associated with IWR-1. As shown by Bao et al., Axin1 is stabilized in MDAMB-231 breast cancer cells following treatment with 10 μM of inhibitor (35). Here, Wnt signaling was associated with no alteration to tankyrase 1/2, molecules that bind to a conserved domain on Axin1 and promote degradation. Further support is shown in a study conducted by Mashima et al., where Axin1 protein expression in colorectal cancer cells was unchanged when subjected to 3 and 9 μM of IWR-1 (31). This study further suggests that the salivary cancer cell line in use in our study may not be tankyrase inhibitor resistant, as we did not see similar trends to their COLO-320/WR cell line, where there was a coordinated increase in expression of both Axin1 and Axin 2. We would expect that on probing of protein lysates, Axin2 would also be increased in our cell line when treated with IWR-1, though further testing is needed to verify. Finally, it is also likely that the change in protein expression levels between HSG and HTB-41 cells are due to variable expression between cell types, and no significance between cancer and cancer treated cells being associated with the reported rate-limiting role of Axin1 on the destruction complex (36-38).

Expression levels of p-GSK3β significantly increased following treatment with IWR-1, with a mean intensity higher than that of the normal salivary gland cells used. This was an unexpected result as Cross, et al. reported GSK3-β phosphorylation at Ser9 caused inactivation, and Fei, et al. showed subsequent β-catenin stabilization, nuclear accumulation, and enhanced Wnt signaling (39,40). IWR-1 has also been shown to significantly decrease levels or inhibit activity of p-GSK3β in non-small cell lung cancer and mouse postnatal endothelial cells (41,42). However, other studies using retinal epithelial cells and skin fibroblasts found little expression difference between IWR-1 treated and control groups (43,44). These discrepancies between studies would suggest the role of IWR-1 on modulation of p-GSK3β might be cell type specific as shown in the previous information, or other signaling pathways might be influencing phosphorylation of GSK3β. In Mishra, et al. expression levels of GSK3β and β varied among different types of oral cancers, with p-GSK3β also varying among tissue samples (45). Alternatively, phosphorylation of GSK3β and β may also occur independently of Wnt signaling through insulin, platelet derived growth factor, fibroblast growth factor, epidermal growth factor, or retinol (46,47). It is worth noting that GSK-3 activity in the destruction complex has been shown to be impervious to Ser9 phosphorylated inactivation when bound to Axin (50). Therefore, it seems that only unbound GSK3β phosphorylated, leaving the very small cellular fraction that does associate with the Axin destruction complex active.

Finally, in addition to its effect on the destruction complex components and β-catenin in particular, IWR-1 use resulted in a significant decrease in cancer cell migration. Our data indicates both 2-dimensional cell migration rates and 3-dimensional cell invasion rates were significantly decreased in cancer cells following inhibition. These findings are not atypical, as other studies using IWR-1 have shown similar outcomes on cell migration, proliferation, and invasiveness in multiple cell types (35,44,51,52).

**CONCLUSION**

In summary, we have shown that inhibition of the Wnt pathway through use of IWR-1, can suppress nuclear localization of β-catenin, and cellular migration and invasiveness of HTB-41. This work helps to establish a link between the Wnt pathway and progression of epidermoid salivary gland carcinoma in vitro. Furthermore, it helps to establish the potential usage of Wnt inhibitors in treatment of oral cancers. However, further studies are required to determine the full involvement of the Wnt pathway in progression of HTB-41 and other oral cancers.

**CONFLICTS OF INTEREST**

The authors do not declare any conflict of interest for this work.

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