EVALUATION OF BRACHYURY EXPRESSION IN SPINDLE CELL CARCINOMA

A Thesis

by

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ABSTRACT

Epithelial-mesenchymal transition (EMT) is a complex biologic process by which epithelial cells lose their cytomorphologic characteristics and assume the phenotype of mesenchymal cells. EMT is essential to normal human embryogenesis, and also occurs in wound healing, tissue repair and cancer progression. Acquisition of a mesenchymal phenotype via EMT enhances the migratory capacity of carcinoma cells, increasing their metastatic capability and worsening prognosis for patients. One emerging marker of EMT is brachyury, a T-box transcription factor protein. Recent studies have demonstrated upregulation of brachyury in several carcinoma types, including squamous cell carcinoma (SCC). Spindle cell carcinoma (SpCC) is a variant of SCC in which cancer cells have a predominantly spindled phenotype. Whether brachyury is expressed in SpCC and contributes to this phenotypic change is not clear.

We aimed to examine brachyury expression by immunohistochemistry in SpCC of the head and neck, hypothesizing that brachyury expression is increased in SpCC, as compared with other forms of SCC.

Immunohistochemical staining for brachyury was performed on 20 head and neck carcinoma cases per group in each of the following four groups: SpCC; moderately- to well-differentiated SCC; moderately- to poorly-differentiated SCC; and verrucous carcinoma (VC). Uninflamed fibroma was the negative control; and human chordoma, along with a 14.5-day-old mouse embryo, were positive controls. Evaluation of brachyury reactivity was completed by light microscopy.

Brachyury expression was not seen in any of the experimental groups.

Brachyury did not appear to be involved in the cytomorphologic changes present in SpCC, nor was it expressed in any head and neck SCC variants. Our results are contrary to those found in previous reports. This result suggests that an alternate mechanism contributes to the phenotype of SpCC.

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CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis committee consisting of Dr. Yi-Shing Lisa Cheng (advisor) and Dr. John M. Wright of the Department of Diagnostic Sciences; and Dr. Jian Q. Feng, Dr. Kathy K. H. Svoboda and Dr. Mikhail Umorin of the Department of Biomedical Sciences.

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NOMENCLATURE

EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
PR	Progesterone receptor
SpCC	Spindle cell carcinoma
SCC	Squamous cell carcinoma
OSCC	Oral squamous cell carcinoma
H&E	Hematoxylin & eosin
HPV	Human papillomavirus
OpSCC	Oropharyngeal squamous cell carcinoma
CRB3	Crumbs protein homolog 3
LGL2	Lethal giant larvae homolog 2
TGF-β	Transforming growth factor beta
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
MAPK	Mitogen-activated protein kinase
ERK	Extracellular signal-related kinase
SOX2	SRY-box transcription factor 2
KLF4	Kruppel-like factor 4
WDSCC	Well-differentiated squamous cell carcinoma
PDSCC	Poorly-differentiated squamous cell carcinoma
VC	Verrucous carcinoma

IHC	Immunohistochemistry
TBS	Tris-buffered saline
BSA	Bovine serum antigen
HRP	Horseradish peroxidase
DAB	3,3’ Diaminobenzidine
SI	Staining index
ANOVA	Analysis of variance

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CHAPTER I

INTRODUCTION

Specific Aims

Epithelial-to-mesenchymal transition (EMT) was described as a well-described biologic process where epithelial cells lost their epithelial cytomorphologic characteristics and took on the phenotype of mesenchymal cells (1). This highly-conserved and complex process was shown to be essential in normal human embryogenesis, and it also occurred in the settings of wound healing, tissue repair and notably, cancer progression (1, 2). Acquisition of a mesenchymal phenotype via EMT enhanced the migratory capacity of tumor cells in several human carcinomas, thereby increasing the metastatic capability of these neoplasms (3). Brachyury, a T-box transcription factor protein, has been established as a marker of EMT (4-6). Recent studies demonstrated upregulation of brachyury in carcinomas of various anatomic sites, indicating increased occurrence of EMT within these carcinomas (7-10). Expression of brachyury in triple-negative breast cancer (negative for ER, PR and Her-2 expression) correlated with increased metastatic potential and more advanced stage of disease (11). Overall, patients with higher brachyury expression in their primary and metastatic tumors had poorer prognoses (11).

Spindle cell carcinoma (SpCC) is a variant of squamous cell carcinoma (SCC) in which the cancer cells are predominantly spindled morphologically (12). This variant is seen rarely in the oral cavity (12, 13). However, SpCC grows rapidly, metastasizes early, and is usually diagnosed at a later stage (13). Brachyury expression has been

found in conventional oral squamous cell carcinoma (OSCC), but information regarding its expression in SpCC was deficient (8, 9, 14). Therefore, the purpose of this project was to determine whether brachyury expression was increased in SpCC, compared with conventional OSCC. We examined brachyury expression in SpCC by immunohistochemistry, and compared those results with conventional OSCC. Due to the cytomorphologic characteristics of SpCC that were evident upon routine stain with hematoxylin and eosin (H&E), we hypothesized that brachyury expression would be increased in SpCC, compared to conventional squamous cell carcinoma of the oral cavity, head and neck.

Background and Significance

Today, oral squamous cell carcinoma (OSCC) represents over 90% of malignancies diagnosed in the oral cavity (15). The American Cancer Society has projected that, for the year 2020, approximately 53,260 people will be diagnosed with oral cavity or oropharyngeal cancer in the United States; and approximately 10,750 people will die of the disease (16). The etiology of OSCC was demonstrated to be multifactorial in nature. Tobacco use (cigarette-smoking in particular); alcohol use; oral habits such as chewing betel nut; vitamin and mineral deficiencies; and occupational exposure to chemicals have been shown to increase one's risk of developing OSCC (17-19). In addition, human papillomavirus (HPV) infection was identified as a common etiopathogenic factor in oropharyngeal squamous cell carcinoma (OpSCC); but evidence of HPV infection was seen in just a few OSCC cases and does not seem to be a driver of OSCC (3-5%) (20). Worldwide, OSCC predominantly affected males with a

ratio of 5.5 males with the disease for every 2.5 cases in women, per 100,000. Most often was diagnosed in individuals in the 5^{th}-7^{th} decades of life (21). OSCC may affect any oral mucosal site. The most common affected sites were the tongue, floor of mouth and gingiva; these locations together accounted for over half of OSCC cases (22). Clinically, OSCC typically presented as a mixed red and white surface lesion with ulceration and indurated borders. OSCC has mimicked other disease processes in the oral cavity, and unfortunately this led to frequent diagnostic delay (23). OSCC was shown to be an aggressive disease, characterized by local invasion and lymph node metastasis (24). Key prognostic factors included primary tumor size, depth of invasion, presence of lymph node metastasis, and distant metastasis (25). The 5-year survival rate for OSCC was approximately 64% in 2018 (16).

Histologically, OSCC may be divided into conventional types and non-conventional types. The term “conventional” is applied to tumors that appear on the following spectrum. Well-differentiated squamous cell carcinoma (WDSCC) is comprised of tumor cells that maintain cell-to-cell cohesion, exhibiting minimal cytologic atypia and forming keratin pearls. Moderately-differentiated squamous cell carcinoma (MDSCC) is the category into which intermediate tumors with increased pleomorphism and hyperchromasia (with or without formation of keratin pearls) are placed. Poorly-differentiated squamous cell carcinoma (PDSCC) is formed by wildly pleomorphic sheets of tumor cells with atypical mitotic figures and no keratin pearl formation (22). Other specific variants of SCC that fall outside of this spectrum include spindle cell carcinoma (SpCC), in which cells have a sarcomatoid cytomorphology (12); and

verrucous carcinoma (VC), a very well-differentiated SCC with no cytologic atypia and a unique, bluntly invasive growth pattern (22).

Spindle cell carcinoma (SpCC), a rare variant of SCC, represented less than 1% of OSCC cases (12, 13, 26). This tumor has been known by many names in the past, most specifically “sarcomatoid carcinoma” 24). SpCC was strongly associated with cigarette smoking, alcohol use, and prior radiation therapy; however, HPV infection was identified very rarely (26, 27). Like conventional OSCC, SpCC affected males more frequently, and tended to occur in the 6^{th}-7^{th} decades of life (24). SpCC occurred anywhere within the upper aerodigestive tract, with a predilection for the larynx and the oral cavity (12, 13, 27). Oral cavity site predilections for SpCC included the alveolar mucosa, tongue, buccal mucosa and lower lip (28, 29). The clinical presentation of SpCC was typically a rapidly-growing polypoid mass with or without ulceration. SpCC may cause pain and paresthesia, just like OSCC (12). Cytomorphologically, SpCC was biphasic in nature, with an often-minimal squamous epithelial component and a predominantly spindled population of cells (13, 26). SpCC has been considered a more aggressive neoplasm, compared to conventional OSCC because it grew faster, metastasized earlier (via lymphatics), and was typically diagnosed at a later stage (24, 30). Bice *et al*. (13) in 2015 investigated survival rates of patients diagnosed with SpCC of the head and neck, compared with patients with conventional OSCC. They divided their patient groups according to anatomic site affected, and one such group was designated “oral cavity.” The investigators found that the three-year disease-specific survival rate for patients with SpCC of the oral cavity was 49.9%, compared to a survival rate of 66.7% for patients diagnosed with conventional OSCC. The five-year survival

rate of oral cavity SpCC was 38.9%, compared to the survival rate of 58.1% for patients with conventional OSCC (13).

Epithelial-to-mesenchymal transition was defined as a process that allowed a polarized epithelial cell to assume a mesenchymal phenotype via a series of highly complex, evolutionarily-conserved and strictly regulated biochemical changes (1, 31). The physiologic ability of epithelial cells to transition into cells of a mesenchymal phenotype pointed to the phenotypic plasticity of epithelial cells (1). Greenburg and Hay described this change from epithelial to mesenchymal phenotype in the primitive streak of chick embryos in 1982 (32). It is important to note that EMT is a reversible process, with mesenchymal-to-epithelial transition (MET) occurring in the body as well (2). EMT occurred in three different settings. Type 1 EMT was vital to normal embryogenesis; type 2 EMT was found to be important in wound healing, regeneration and tissue fibrosis; and type 3 EMT was established as a critical step in the development and metastasis of many human cancers (1, 4, 31).

Epithelial cells are present in single or multiple layers on the surfaces of organs in the body. They exhibit the fundamental property of cohesion to one another via desmosomal attachments. Desmosomes contain intercellular bridges, which epithelial cells use to communicate with each other (31). They have apical-basal polarity, and their basal surfaces are intimately associated with a basement membrane. Normal epithelial cells function together to create selectively-permeable barriers for the organs which they cover. When EMT is induced in the setting of embryogenesis or pathologically, downregulation of epithelial proteins and increased expression of

mesenchymal proteins cause these cells to lose their basic epithelial cytomorphologic characteristics (4).

Many cytomorphologic changes take place during the process of EMT. One change is that epithelial cells undergo loss of their apical-basal polarity and acquire an end-to-end polarity (2). Epithelial integrity is maintained by tight cell-to-cell junctions and desmosomes, and during EMT, those junctional proteins become degraded and removed (2). E-cadherin, a calcium-dependent epithelial junctional protein responsible for cell-to-cell adhesion, becomes degraded. Loss of E-cadherin expression is a hallmark of the EMT process, identifiable by immunohistochemical studies. Initiation of EMT also destabilizes desmosomes, which attach epithelial cells to one another. After these junctions are degraded, epithelial cells lose their orientation with regard to their normal apical-basal polarity due to disruption of polarity complex proteins, CRB3 and LGL2 (1).

During EMT, the cytoskeleton was also reorganized to change the cell shape to one more elongated (end-to-end polarity), with directional mobility (spindled). Specific genes were activated that induced the transitioning cells to gain protrusions that promote cell movement. In addition, cells affected by EMT often acquired the ability to degrade the proteins of the extracellular matrix, which further enabled their capacity for motility (1, 2, 31). A mesenchymal phenotype and increased mobility seemed to correlate with cells' ability to detach from their original sites and travel to surrounding tissues or distant sites, potentially increasing invasive behavior. EMT also increased cells' resistance to apoptosis and senescence (1, 4).

In the last few years, EMT has been extensively researched in the context of tumorigenesis. EMT has been identified in many different human carcinomas, including those of breast, lung, colon, pancreas, ovary, cervix, esophagus, prostate and stomach (33, 34). Upregulation of EMT increased tumor cell invasion and metastasis via the cellular changes described above (3, 7, 33, 35, 36). Several proteins were identified as biomarkers of the EMT process, and the most widely-studied included E-cadherin, β-catenin, and vimentin (37). Another marker of EMT under active investigation is brachyury, a T-box transcription factor that has an important role in normal human embryogenesis (4). First isolated in humans in 1990, brachyury was identified as a nuclear protein whose gene was located on chromosome 6q27 (38). T-box transcription factors have been demonstrated to be responsible for regulating cell fate and differentiation during early embryogenesis (2). Specifically, brachyury induced EMT during gastrulation, during which it was located in the epiblast primitive streak (39). Brachyury was essential for appropriate notochord formation, mesoderm development and organ formation (39). Brachyury expression was strictly regulated, and the structure and function were evolutionarily conserved (5).

Brachyury was demonstrated in several different signaling pathways in tumorigenesis, such as the TGF-β, FGF/FGFR, and Wnt/β-catenin signaling pathways (4). Brachyury and TGF-β functioned together in a positive feedback loop to maintain the acquired mesenchymal phenotype of those cells that have transitioned via EMT (40). Activation of the FGFR/MAPK/ERK/brachyury pathway via FGF2 inhibited apoptosis of cancer cells and propagated EMT and cellular growth, as seen in chordoma (41). Finally, brachyury played a significant role in signal propagation from β-

catenin to influence the levels of an important cancer stem cell marker, CD133, in the Wnt/β-catenin signaling pathway, as was seen in colorectal cancer cells (42).

Brachyury's role in tumor development was described by Palena, *et al.* (6) in 2007. Palena's group found that brachyury mRNA was present in a variety of human tumors, including those of the small intestine, stomach, kidney, bladder, breast, uterus, ovary and testis. Expression of brachyury was also noted in cell lines derived from tumors of the lung, colon and prostate (6). In addition, increased brachyury expression has been found in chordoma (43), hemangioblastoma (44) and hepatocellular carcinoma (10). In each of these studies, high levels of brachyury expression correlated with worse biologic behavior of tumors, including increased tumor grade and stage; presence of distant metastasis; and poor prognosis for patients.

Brachyury expression was evaluated in two studies involving OSCC. The first was carried out by Imajyo, *et al*. (8) in 2012. The group examined brachyury expression and its correlation with EMT (as seen by loss of E-cadherin and gain of vimentin expression) in tumor tissue samples from 152 patients diagnosed with primary OSCC. They found that expression of brachyury was correlated with EMT, and was also significantly associated with lymph node disease and distant metastasis (8). More recently in 2016, a publication by Yoshihama *et al*. (9) evaluated expression levels of brachyury (along with SOX2, Oct4, c-Myc and KLF4) in tissue samples from 108 OSCC patients. They found that brachyury expression (and elevated levels of SOX2 and KLF4) was associated with cancer metastasis and poor prognosis (9). Both studies stated that the 5-year overall survival rate of patients with brachyury-positive OSCC was significantly less than that of patients with brachyury-negative OSCC (8, 9).

While it has been reported that brachyury (and therefore, EMT) was present at increased levels in some OSCC cases, to our knowledge no one has investigated the difference between brachyury expression in spindle cell carcinoma, compared with conventional OSCC. Because a mesenchymal phenotype of the cancer cells was the characteristic histologic feature of SpCC, the EMT process was expected to be responsible for this cytomorphologic change in SpCC. However, the specific pathway(s) leading to the phenotypic change seen in SpCC from squamous epithelium was not fully understood. The purpose of this project was to investigate brachyury expression in SpCC cases of the oral cavity, head and neck; and to test the hypothesis that brachyury expression in SpCC was increased, compared with brachyury expression in other variants of OSCC.

CHAPTER II

SUMMARY OF THE INVETIGATION AND CONCLUSION

Materials and Methods

Approval by Texas A&M University College of Dentistry's institutional review board was obtained for this study. Formalin-fixed, paraffin-embedded human archival specimens and pertinent clinical information regarding those specimens were obtained from the database of the Department of Diagnostic Sciences at Texas A&M University College of Dentistry, and from the archival database of the oral pathology service at the University of Kentucky College of Dentistry, contributed by Dr. Molly Housley-Smith. Secure de-identification of all specimens was completed by Dr. Karan Dharia, who was otherwise not involved in the project. Four test groups were established, with a sample size of 20 cases per group. They included:

1. Spindle cell carcinoma of the oral cavity and head and neck (SpCC)
2. Moderately- to well-differentiated oral squamous cell carcinoma (WDSCC)
3. Moderately- to poorly-differentiated oral squamous cell carcinoma (PDSCC)
4. Verrucous carcinoma of the oral cavity (VC)

Two positive and two negative controls were used in this study. The two positive controls were human chordoma biopsy tissue, and whole sections from a 14.5-day-old mouse embryo. Human chordoma was used because it is known to stain positive for brachyury in nuclei of tumor cells. The 14.5-day-old mouse embryo was the positive control tissue recommended by the primary antibody manufacturer, Abcam (Cambridge, UK). The two negative controls were tissue from a 14.5-day-old mouse embryo without

primary antibody incubation, and sections of human uninflamed fibroma, which represented normal oral squamous epithelium: the normal counterpart of all types of SCC investigated in this study. Based on the current knowledge that brachyury expression in normal adult human tissue was limited to testis and thyroid (4, 6, 45), normal oral squamous epithelium were not expected to express brachyury and served as a negative control. Every control tissue type was immunohistochemically processed along with each batch of experimental cases. Each control group consisted of mouse and human tissue samples to assure research validity. Fibroma sections used in this study were obtained from the archives of the Texas A&M University College of Dentistry's Department of Diagnostic Sciences. The mouse embryo sections were obtained from the lab of Dr. Jian Q. Feng, in the Department of Biomedical Sciences at Texas A&M University College of Dentistry. Human chordoma tissue was obtained from the archives of the University of Texas Southwestern Medical Center's Department of Pathology, courtesy of Dr. Justin Bishop.

Formalin-fixed tissue sections, (4 µm), were prepared from the paraffin blocks for each control and experimental case. Tissue sections were de-paraffinized with xylene and rehydrated with decreasing concentrations of reagent alcohol followed by tris-buffered saline (TBS). Antigen retrieval was accomplished by steaming the slides in 10mM Tris/EDTA buffer (pH 6.0) for 20 minutes. Endogenous peroxidase activity was inhibited using 3% hydrogen peroxide buffer. The slides were washed with TBS and blocked in 3% bovine serum albumin (BSA) and 10% goat serum, followed by incubation with a rabbit anti-brachyury monoclonal antibody [EPR 18113] (Cat. Ab209665, Abcam, Cambridge, UK) at a concentration of 1:4000 diluted in TBS with 1%

BSA overnight at 4°C. The tissue sections were then washed with TBS and incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (Abcam, Cambridge, UK) 1:50 diluted in TBS with 1% BSA for 1 hour at room temperature. After washing with TBS, antibody detection and chromogen development was completed using the EXPOSE rabbit specific HRP/DAB detection IHC kit (Abcam, Cambridge, UK), following the manufacturer's instructions. The slides were counterstained with 0.5% hematoxylin; dehydrated with ascending concentrations of reagent alcohol followed by three washes of xylene; and mounted/cover slipped using Permount mounting medium (Fisher Scientific, Waltham, MA).

Expression of brachyury, with "positive" defined as brown-colored staining localized to the nucleoplasm, was evaluated by light microscopy. A staining index (SI), determined by the staining intensity score multiplied by the proportion score (10), was used for analysis. A SI value was assigned to each experimental case. The staining intensity scores were defined as 0 (no reactivity); 1 (weak reactivity); 2 (moderate reactivity); and 3 (strong reactivity). The proportion scores were defined as 0 (no tumor cells were brachyury positive); 1 (less than 25% of tumor cells were positive); 2 (25-50% of tumor cells were positive) and 3 (more than 50% of tumor cells were positive). The staining intensity score and the proportion score of each case was determined with the assistance of ImageJ, an imaging analysis software. Control tissue sections were processed with experimental tissue on each slide to facilitate appropriate calibration. Five representative high-power fields (original magnification x 40) per case were evaluated using ImageJ, and those resulting SI numbers were averaged to yield a representative SI for each case. In turn, an average SI for each group was calculated,

and Analysis of Variance (ANOVA) was used to determine statistically significant differences between the experimental groups.

Results

In the positive controls, chordoma sections demonstrated epithelioid tumor cells arranged in nests and short cords (Fig. 1A, arrows), and nuclear staining of brachyury was observed in chordoma tumor cells (Fig 1B, arrows). The mouse embryo demonstrated nuclear brachyury expression in the nucleus pulposus of the notochordal remnants (Fig. 1C, arrows). The mouse embryo section without primary antibody incubation was negative for brachyury (Fig. 1D, arrow). The uninflamed, non-ulcerated fibroma showed normal squamous epithelium without cellular atypia and normal fibroblasts in the connective tissue (Fig. 1E), and no brachyury reactivity was seen in the keratinocytes of the epithelium, nor the fibroblasts of the lamina propria (Fig. 1F). All controls were appropriately reactive and validated the results of this study.

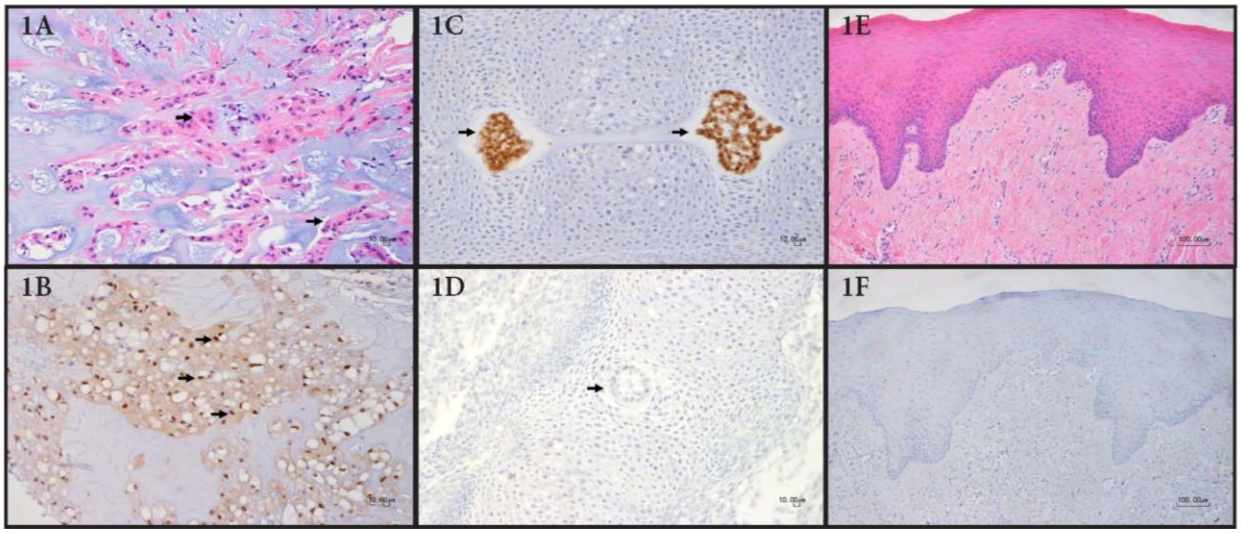

Figure 1. Control Tissue for Brachyury IHC.

1A. Human chordoma (positive control) with large epithelioid tumor cells (hematoxylin & eosin (H&E), scale bar = 10 µM).

1B. Nuclear brachyury was expressed (brown staining) in chordoma tumor cells (brachyury IHC, scale bar = 10 µM).

1C. Nuclear brachyury expression seen in nucleus pulposus of mouse embryo (second positive control) (brachyury IHC, scale bar = 10 µM).

1D. No brachyury expression seen in nucleus pulposus of mouse embryo without primary antibody (negative control) (scale bar = 10 µM).

1E. Uninflamed fibroma (second negative control) with squamous epithelium and connective tissue fibroblasts (H&E, scale bar = 100 µM).

1F. No brachyury expression seen in uninflamed fibroma (brachyury IHC, scale bar = 100 µM).

Hematoxylin & eosin (H&E)-stained slides were reviewed for each experimental case to confirm all diagnoses and assure adequate presence of tumor cells. The SpCC cases (N=20) demonstrated malignant epithelial tumors with prominent spindled morphology (Fig. 2A). Key histologic features included cytologic atypia and increased mitotic figures (Fig. 2B, arrows). No brachyury reactivity was observed in the SpCC cases examined (Fig. 2C, arrows). The 20 WDSCC cases showed invasive epithelial tumors with cohesive neoplastic cells forming characteristic keratin pearls (Fig. 3A, arrows). Tumor islands had cells with pleomorphism and hyperchromasia, with nuclear atypia (Fig. 3B, arrow). No brachyury expression was seen in the WDSCC cases examined (Fig. 3C, arrows). The 20 cases in the PDSCC group were comprised of non-keratinizing epithelial tumors with significant pleomorphism (Fig. 4A) Necrosis and atypical mitotic figures were noted with increased frequency (Fig. 4B, arrow). No brachyury expression was found in the PDSCC cases (Fig. 4C, arrows). The 20 cases in the VC group demonstrated hyperkeratotic epithelial neoplasms with keratin plugging and a broad, bluntly invasive front (Fig. 5A, arrows). Minimal cytologic atypia and a largely flat interface between tumor cells and connective tissue was seen (Fig. 5B, arrow). No brachyury expression was demonstrated in any of the examined VC cases (Fig. 5C, arrow).

With no staining present, the SI score for all test cases was 0. Due to negative results, ANOVA statistical analysis was not performed.

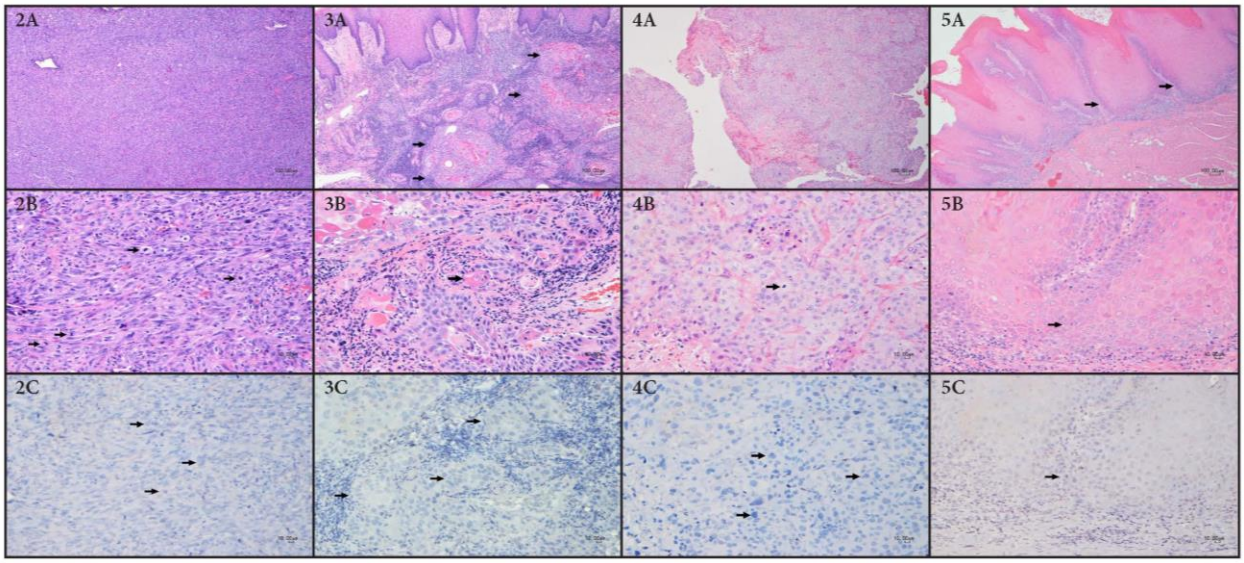

Figure 2. Representative Images of SpCC Cases (N = 20).

2A. SpCC showed sheets of spindled tumor cells (H&E, scale bar = 100 µM).
2B. Tumor cells were pleomorphic with increased mitotic figures (H&E, scale bar = 10 µM).
2C. No brachyury expression was seen in SpCC tumor cells (N = 20) (brachyury IHC, scale bar = 10 µM).

Figure 3. Representative Images of WDSCC Cases (N = 20).

3A. WDSCC cases showed invasive neoplasms forming keratin pearls (H&E, scale bar = 100 µM).
3B. Tumor cells were pleomorphic, hyperchromatic and cohesive (H&E, scale bar = 10 µM).
3C. No WDSCC tumor cells expressed brachyury (N = 20) (brachyury IHC, scale bar = 10 µM).

Figure 4. Representative Images of PDSCC Cases (N = 20).

4A. PDSCC cases showed invasive tumors growing in back-to-back islands and sheets with necrosis (H&E, scale bar = 100 µM).
4B. Nuclear pleomorphism and atypical mitotic figures were noted (H&E, scale bar = 10 µM).
4C. No PDSCC tumor cells expressed brachyury (N = 20) (brachyury IHC, scale bar = 10 µM).

Figure 5. Representative Images of VC Cases (N = 20).

5A. VC cases showed verrucoid tumors with a broad pushing front (H&E, scale bar = 100 µM).
5B. Minimal cytologic atypia was seen in tumor cells (H&E, scale bar = 10 µM).
5C. No VC tumor cells expressed brachyury (N = 20) (brachyury IHC, scale bar = 10 µM).

Discussion

Our study showed that brachyury expression was not seen in SpCC of the oral cavity, head and neck; nor was it expressed in any of the examined variants of OSCC. Our results differed from those reported by the two other studies that evaluated brachyury expression in OSCC (8, 9). The previous investigations also examined brachyury expression by IHC, and sought to determine whether it correlated with EMT, metastasis and poor prognosis in OSCC patients. In both studies, brachyury expression was noted in a majority of examined OSCC cases, and it was concluded that brachyury expression correlated with EMT and metastasis. Both groups suggested that brachyury was involved in EMT in OSCC. However, in Imajyo's study (8), no description of positive or negative control tissue for brachyury was presented. Instead, it was stated that brachyury positivity in lymphocytes was used to judge relative staining intensity in tumor cells. In Yoshihama's study (9), normal oral epithelium was used as the baseline tissue for brachyury staining intensity of OSCC tumor cells. The reason for using brachyury expression in lymphocytes and normal oral epithelial cells was not explained (8, 9). While minimal brachyury protein expression was found in normal human testis and thyroid tissues (45), expression of brachyury in normal lymphocytes or oral squamous epithelium has not been reported (46). In addition, both nuclear and cytoplasmic brachyury staining was observed in the OSCC cells in Imajyo's study (8), and the reason for cytoplasmic localization of this transcription factor was not addressed. We speculated that non-specific binding of the antibody used in those studies led to the positive result of brachyury expression in OSCC, lymphocytes and normal oral epithelium. Both investigations used polyclonal anti-brachyury antibodies produced by

Santa Cruz Biotechnology (Santa Cruz, CA). In contrast, the current investigation used monoclonal anti-brachyury antibodies produced by Abcam (Cambridge, UK). As previously reported, polyclonal antibodies (which can recognize and bind to multiple epitopes on the same antigen) had high binding affinity, but reduced specificity compared to monoclonal antibodies (which only recognized one epitope on the antigen) (46). This study was the first that demonstrated that brachyury was not expressed in conventional OSCC, VC, or SpCC (in which EMT was believed to occur) (47). More studies investigating brachyury expression in OSCC and SpCC at both mRNA and protein levels are needed to verify this preliminary result.

The results of our study were unexpected because brachyury was a known EMT marker involved in the TGF-β and Wnt/β-catenin signaling pathways, both of which were previously identified in OSCC (14, 48, 49). Brachyury was involved in the Wnt10 signaling pathway in colorectal carcinoma; and the TGF-β signaling pathway in lung and prostate carcinomas (4, 40). A review of EMT signaling pathways in OSCC, by Chang *et al.* (14), discussed the association of EMT with functional loss of E-cadherin, which was a key protein involved in maintenance of the epithelial characteristics of intercellular adhesion and cell polarity (14). Transcriptional control of E-cadherin was regulated by Snail, Slug, SIP1 and Twist (transcriptional repressors). These transcription repressor molecules were activated by a variety of EMT-inducing signaling pathways, including TGF-β (in which brachyury plays a role) (4). A group headed by Zidar (47) sought to compare oral SpCC (as an example of EMT) to conventional OSCC with and without nodal metastases, to test the hypothesis that EMT contributed to metastasis in OSCC. In addition to performing IHC for epithelial, mesenchymal, and stem cell markers, they

evaluated expression of Snail, Slug and Twist in human tumor specimens. They also analyzed the expression of several microRNAs, because increased expression had been associated with inducing EMT (50-52). The group found that Snail + Slug (one single IHC) and Twist were expressed in 93.3% and 86.7% of spindle cell carcinoma cases, respectively. Zidar's group concluded that, according to their criteria, EMT occurred in SpCC and served as a "positive control" when evaluating EMT in other variants of OSCC. Brachyury expression was not assayed in that study, so it would be beneficial to include brachyury alongside other EMT markers and microRNAs when studying development and progression of OSCC. The monoclonal antibody used in the current study was specific for the control tissues but did not label any cancer specimens. One known issue with IHC is that if the antibody does not bind, it does not necessarily mean that the protein is not present in the tissue; rather, the lack of reaction could be due to processing, antigen retrieval, or other protocol issues. Additionally, other methods not dependent on antibodies may have a different outcome. Clearly, further research could determine which specific signaling pathways contributed to EMT-like changes that were observed in SpCC so that potential targets may be identified for treatment of patients who develop this rare variant of SCC.

Conclusion

Brachyury expression was not found in SpCC of the head and neck, nor in VC or conventional OSCC. Brachyury did not appear to be involved in the cytomorphologic changes present in SpCC, and there may be a different mechanism contributing to the phenotype of SpCC.

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