ISSN: 2321-8819 (Online) 2348-7186 (Print) Impact Factor: 1.498 Vol. 5, Issue 9, September 2017

Small Integrin-Binding Ligand N-Linked Glycoproteins (Siblings): A Study on Human Salivary Gland Cancer

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Abstract: Salivary gland carcinomas constitute a rare but deadly group of head and neck cancers, but timely diagnosis is often delayed due to inherent variability in etiology, heterogeneity and histopathological characterization. SIBLINGs are a family of secreted glycophosphoproteins that include osteopontin (OPN), bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP), dentin matrix protein-1 (DMP-1), and matrix extracellular phosphoglycoprotein (MEPE). SIBLINGs were first discovered in bone and teeth, and were considered to be exclusively expressed in mineralized tissue. In addition to mineralized tissue, SIBLINGs have now been shown to have variable expression in normal, non-mineralized tissue and in cancers. However, there have been no studies evaluating SIBLING expression in human salivary gland cancers. Our study tested the hypothesis that SIBLINGs, specifically, BSP, DSPP and OPN, would be significantly overexpressed in human salivary gland cancer. We also hypothesized that the cancer secretome would influence SIBLING expression in normal salivary gland cells. Methods: Normal and cancerous human salivary gland tissue obtained from the NDRI were processed using routine immunohistochemistry techniques to evaluate expression of BSP, DSP, and OPN. In addition normal HSG cell line and cancer HTB-41 cell line were evaluated using immunofluorescence techniques to localize expression of BSP, DSP and OPN. Normal HSG, cancer HTB-41 and HSG* cells (normal HSG cells exposed to a cancer HTB-41 secretome) were propagated using routine cell culture techniques for 24, 48, and 72 hours. Western blotting techniques were utilized ii to quantify and compare SIBLING protein expression levels in HSG, HTB-41 and HSG* cells. Normal HSG, cancer HTB-41, and HSG* cells were processed via immunoflourescence in order to observe localization of SIBLINGs. Results: Immunohistochemistry and western blot showed increased expression of SIBLINGs in human salivary gland cancers. Furthermore, immunoflourescence revealed distinct localization of SIBLING proteins in HSG and HTB-41 cell lines. In terms of HSG*, it was found that cells exposed to cancer secretome exhibited similar SIBLING expression to HTB-41. Conclusion: Our studies confirm that SIBLING proteins are selectively expressed in human salivary gland cancer. Also, the cancer secretome is found to affect SIBLING expression in normal cells, similar to HTB-41 cancer cell lines.

Keywords: small integrin-binding ligand, n-linked glycoproteins (siblings), human salivary gland cancer

Introduction

Salivary gland cancers, while rare, are a group of exceedingly fatal cancers characterized by abnormal growth of salivary gland cells. Salivary gland cancer is a rare malignancy that can occur in the major or minor salivary glands (Di Palma and Guzzo, 1993; Gillespie et al., ; Guzzo et al.). Salivary cancers are a histologically diverse group of tumors with varying prognosis and treatment according to grade, histology, and stage. Treatment is primarily surgical, with postoperative radiotherapy for patients with a poor prognosis (Lang et al., 2005a; Mazeron et al., 2003; Medina, 1998). Treatment options are limited due to delayed diagnoses, complex histopathology and rarity (Lang et al., 2005b). Thus, salivary gland research is crucial to identify options for diagnostic and therapeutic options.

Malignant tumors are characterized by uncontrollable growth and replication, with possibility of metastasis. Many growth factors, cytokines, and glycoproteins play pivotal roles in the regulation of proliferation, survival, growth, adhesion, and migration of neoplastic or abnormal cells (Rangaswami et al., 2006a, , 2006b). One group of proteins thought to play a role in regulating or aiding in tumor growth and survival are the Small Integrin Binding N-Linked Glycoproteins (SIBLINGs). SIBLING proteins were first discovered in the mineralized matrices of bone and teeth. The SIBLING gene family, located on chromosome 4, is composed of 5 members: osteopontin (OPN), bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1), and matrix extracellular phosphoglycoprotein (MEPE) (Bellahcene et al., 2008). Moreover, this family of proteins is an emerging group that cancer cells use to facilitate tumor progression. SIBLINGs are soluble, secreted proteins that can additionally act as modulators of cell adhesion through their interaction with cell surface receptors, such as integrins (Bellahcene et

al., 2008; Ogbureke and Fisher, 2004, , 2007; Ogbureke et al.). Although initially thought to be associated only with mineralized compartments of bone and teeth, all five SIBLING family members have subsequently been found in specific soft tissues, including metabolically active ductal epithelial cells (e.g., sweat glands and kidneys) as well as in many different types of human cancer (Bellahcene et al., 2008). Studies, such as the one by Ogbureke and Fisher in 2005, have reported SIBLING expression, along with their respective matrix metalloprotinase partners, in normal human salivary glands (Ogbureke and Fisher, 2005). Researchers have also found SIBLINGs to be involved in tumor progression and metastasis by interacting with several integrins and CD44 to mediate cellular signaling (Bellahcene et al., 2008). However, limited studies have evaluated OPN, BSP, and DSPP expression exclusively in salivary gland cancer. With little known about the SIBLING family and their expression in cancerous states of salivary gland tissues, the current study will give insight into SIBLING expression in salivary gland cancer, specifically OPN, BSP, and DSP, a cleaved functional protein of DSPP previously shown to play a role in cancerous tissues (Figure 1). While DSPP is a key player in tooth development and mineralization in odontoblast, DSP is a specific marker for differentiation of odontoblast with little known about its functionality. Therefore, with little information within the literature, DSP is a pivotal protein to study in terms of cancer and its role. While OPN and BSP have been evaluated in cancers such as colon and breast, no studies have evaluated SIBLING expression in salivary gland cancer.

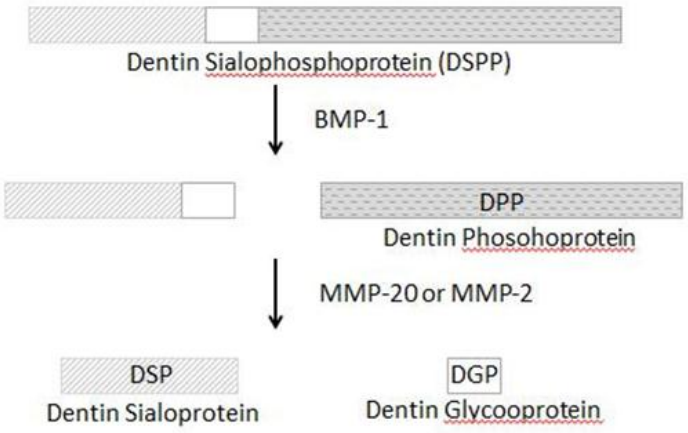


Fig. 1. Cleavage of dentin sialophosphoprotein into functional proteins DPP,

Previous studies have demonstrated OPN localization within the cytoplasm of both acinar and ductal cells of mouse salivary glands. It was also established that osteopontin mRNA was found to be highly expressed in salivary gland tissue (Oates et al., 1996). Although these findings have been represented, the function of OPN in salivary glands still remains unclear, as well as its localization and role in human salivary gland carcinoma. Ogbureke and Fisher (2004) have demonstrated co-expression of SIBLING proteins and their matrix-metalloproteinase partner (MMP) within salivary glands (Ogbureke and Fisher, 2004). Like OPN, BSP has been found to be expressed within acinar cells and the ductal system of salivary gland tissues (Ogbureke and Fisher, 2007). DSP, unlike BSP and OPN, has not been found to be expressed in acinar cells of the salivary gland. With this information characterizing salivary gland tissue and SBILING expression, localization of OPN, BSP, and DSP, within salivary gland cells, has not yet been characterized.

According to the National Cancer Institute at the NIH, the cancer microenvironment can be defined as, "the normal cells, molecules, and blood vessels that surround and feed a cancer cell". A cancer can alter its microenvironment, and the microenvironment can affect how cancer progresses http://www.cancer.gov/dictionary?cdrid=561725. The secretome is the collection of all macromolecules secreted by a cell, and is a vital aspect of cell–cell communication in eukaryotes. In cancer, tumor cells often have secretomes with altered composition compared to the normal tissue from which they are derived. These changes can contribute to the acquisition and maintenance of the recognized hallmarks of cancer. In addition, there is growing evidence that the tumor secretome has significant implications for malignant disease progression. Collectively, evidence from the current literature suggests that the tumor secretome, consisting of factors derived from

cancer stem cells, non-stem cells and the surrounding stroma, is critical in cancer progression, and may constitute a key therapeutic target in many cancers (Chang et al., ; Chenau et al., 2009; Lin et al., ; Paltridge et al., ; Pavlou and Diamandis, ; Ralhan, ; Xue et al., 2008).

Salivary gland cancer cells are capable of self-renewal and exhibit the ability to produce heterogeneous populations of cancer cells. Secreted molecules are key mediators in cell-cell interactions and influence the cross talk with surrounding tissues. Mimicking the tumor environment using salivary gland cancer secretome, or a secreted pool of proteins from a population of salivary gland cancer cells, is suggested as a method for identifying potential therapeutic targets. Strong evidence exists in support of crucial cellular functions such as proliferation, differentiation, communication, and migration, cellular functions that can be heavily regulated by the cell secretome.

Hypothesis

The current study tested the hypothesis that SIBLINGs, specifically OPN, BSP, and DSP, are significantly overexpressed in human salivary gland cancer, and that the salivary gland cancer secretome would influence BSP and DSP expression in normal salivary gland cells.

Specific Aims

Aim 1: To determine expression of OPN, BSP, and DSP in normal and cancerous human salivary gland tissues and cell lines. Using immunohistochemistry, OPN, BSP and DSP expression was evaluated in normal and cancer human salivary gland tissue. Using immunofluorescence techniques, OPN, BSP and DSP were localized in normal HSG and cancer HTB-41 cell lines.

Aim 2: To determine expression levels of OPN, BSP and DSP in normal and cancerous human salivary gland cell lines. Using western blot assays expression levels of OPN, BSP and DSP were evaluated in human normal HSG and cancer HTB-41 cell lines.

Aim 3: To evaluate if the cancer secretome derived from human salivary gland cancer cells would influence expression of OPN, BSP and DSP in normal human salivary gland cells. Using immunofluorescence and western blotting techniques, the influence of cancer secretome on expression of OPN, BSP and DSP in normal human salivary gland cells was investigated.

Significance of the Problem

Cancer cells are known to exhibit increased proliferation that allows uncontrolled growth of the tumor at the primary site. In addition, cancer cells have the ability to migrate and invade the surrounding tissue, a critical property that allows cancer cells to enter the lymphatic system or blood stream, resulting in metastasis to distant organs. Consequently, cancer progression and metastasis is the most common reason for fatalities in cancer patients. Salivary gland (SG) cancers are a rare but occasionally fatal variety of head and neck cancers. It is estimated that one adult out of 100,000 will be diagnosed with SG cancer each year and survival rates depend on the type of tumor, stage of cancer at diagnosis and metastasis. Although SG cancer boasts 5-year survival rates of 91%, this is true only if the cancer is diagnosed before metastasis. Survival rates drop to 75% with lymph node involvement, and 39% with metastasis. Survival of patients diagnosed with SG cancers is thus comparable to breast cancer (72% and 22%). The rarity of SG cancers, coupled with an inadequate understanding of cancer progression, has had an adverse effect on designing strategic therapies. Our data reveal that a family of proteins called SIBLINGs is overexpressed in human SG cancers. Specifically OPN, BSP and DSP are upregulated in human salivary gland cancers. In addition, our study provides evidence that the secretome derived from human salivary gland cancer cells has a significant influence on growth of and expression of OPN, BSP and DSP in normal human salivary gland cancer cells. Further understanding of the role of these specific SIBLING proteins, as well as the role of the secretome in cancer progression will help us design viable methods for early diagnosis and effective interventions in patients.

Review of literature

Salivary glands are a major exocrine glandular system, which aid in maintaining homeostasis of the oral cavity, and function to secrete saliva to assist the digestive and immune systems. Human salivary glands can be divided into two groups known as major and minor salivary glands. While the major group of salivary glands includes the parotid, submandibular, and sublingual glands, the minor group is made up of numerous small salivary glands which line the upper aerodigestive tract (Humphrey and Williamson, 2001). Anatomical locations of salivary glands differ, with the parotid gland situated along the posterior surface of the mandible, the submandibular gland bound by the inferior edge of the mandible and digastric muscle, and the sublingual gland lying within the anterior floor of the mouth and lateral to the mandible (Figure 2)

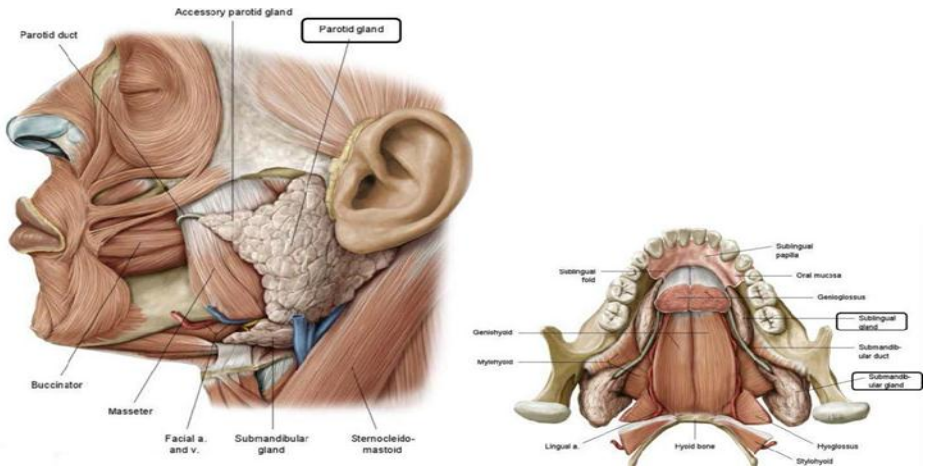


Fig. 2. Anatomical location of human major salivary glands, parotid, submandibular and sublingual glands.

Salivary glands are derived from epithelial cells and are made up of parenchyma and stroma. Histology of saliva glands includes acinar cells (mucous and serous), ducts (striated, excretory, and intercalated), and myoepithelial cells. Acini are responsible for secreting proteins necessary for producing saliva. Between the epithelial cells and basal lamina are the myoepithelial cells, responsible for contraction of muscle in order to force saliva through the salivary gland. The acini secrete salivary components into the intercalated ducts, which then empty into the striated ducts.

Parotid glands are the largest salivary glands in the human body, and are located around the mandibular ramus. The parotid gland secretes saliva through a duct, called Stensen's duct, into the oral cavity. Stensen's duct arises from the anterior of the parotid gland and penetrates through the buccinator muscle, allowing the duct to open into the oral cavity. In terms of histology, the parotid gland consists of striated and intercalated ducts, which are lined with columnar epithelium and cuboidal epithelial cells, respectively. Parotid gland function is defined by secretion of salivary alpha-amylase (sAA). By producing sAA, the parotid gland assists in decomposition of starches, such as amylose and amylopectin, and also prevents bacterial binding to oral surfaces.

Submandibular salivary glands are divided into lobes known as superficial and deep lobes, separated by the mylohoid muscle which forms the floor of the oral cavity. Secretions produced by the submandibular glands are emptied through Wharton's duct into the floor of the oral cavity. The sublingual glands lie anterior to the submandibular glands, inferior to the tongue and are also located in the floor of the mouth. The sublingual glands are fairly small glands. Parotid secretions are mainly serous, sublingual gland secretions are predominantly mucous, and submandibular gland exhibits a mixed sero-mucous secretion.

The main function of saliva, produced by the salivary glands, is to aid in digestion, taste, tooth maintenance, and immunity. Normally, the pH of saliva is between 6.0 and 7.2, meaning it is slightly acidic, which allows it to aid in digestion and food lubrication (Humphrey and Williamson, 2001). Lubrication of food particles during chewing allows for ease in swallowing and travel through the esophagus. Saliva contains a digestive enzyme, known as α-amylase, or ptyalin, whose function is to convert starch into maltose, maltotriose, and dextrins (Almeida et al., 2008). Furthermore, minor salivary glands located in the tongue secrete lingual lipase, which breaks down triglycerides (Humphrey and Williamson, 2001). Tooth maintenance is regulated with the help of saliva through the processes of demineralization and remineralization. Specifically, saliva influences the pH of plaque and acts as a buffer to inhibit formation of caries (Humphrey and Williamson, 2001). On another note, in terms of immunity, saliva is capable of producing secretory IgA, IgM, and IgG. IgA when active is capable of neutralizing viruses and inhibiting bacterial attachment to host tissue (McNabb and Tomasi, 1981). Another saliva component, lactoferrin, is produced by intercalated duct cells and can bind ferric iron. Binding iron is a crucial process for the human body in order to inhibit bacteria from consuming iron necessary for viability.

One of the major pathologies that affect salivary glands is salivary gland carcinoma. Salivary gland carcinoma is a rare form of cancer, with about 3000 new cases diagnosed annually. Due to variability in histological presentation between cancer types, treatment options are limited to surgical resections

and/or radiotherapy. Additionally, many salivary gland tumors exhibit features that make differentiation of malignant and benign tumors difficult to distinguish (Seethala, 2009). A major setback associated with salivary gland cancer is that tumors associated with salivary gland cancers have a high incidence of local recurrence despite aggressive treatment approaches. While there are many different types of salivary gland cancer, the most common are mucoepidermoid carcinoma, adenoid cystic carcinoma, acinic cell carcinoma, adenocarcinoma, and squamous cell carcinoma (Seifert and Donath, 1976a, , 1976b; Seifert et al., 1977; Seifert et al., 1976; Takata et al., 1987). Adenocarcinoma, a main focus for tissue processing in this study, is classified as an intrinsically high-grade, or high-risk cancer. Overall, salivary gland cancer effects salivation in the oral cavity due to changes in cell morphology in acini formation, ducts, and epithelial cells. Therefore, it is critical to identify potential protein markers that can be used as diagnostic or therapeutic targets to manage salivary gland cancers. The current study thus focused on evaluating OPN, BSP and DSP as potential markers of salivary gland cancers.

Previous studies have shown that SIBLING expression in cancers such as breast, colon, stomach, ovary, lung, thyroid, kidney, pancreas, and prostate, influence cell growth, cell adhesion, and cell migration during tumor progression. According to previous studies, SIBLINGs bind to cell surface integrins and sometimes CD44 in normal tissues, and function as signal transducers for cell adhesion, motility and survival through activating kinase cascades and transcription factors. The SIBLING family, located on chromosome 4, is defined as a soluble RGD motif containing integrin-binding ligands that distinguish them from large extracellular proteins. In cancer progression, adhesive properties of the SIBLINGs have been investigated in the context of bone targeting and recognition by metastasizing cancer cells. SIBLING molecules have also been implicated in enhancing the affinity of metastatic cancer cells for bone. Furthermore in cancer, SIBLINGs are bound by cancerous cells and affect cancer proliferation (Bellahcene et al., 1996a; Bellahcene et al., 2008; Bellahcene et al., 1997; Brown et al., 1994; Chaplet et al., 2003; Chaplet et al., 2006; Fisher et al., 2004; Furger et al., 2001; Hayashi et al., 2007; Jain et al., 2009; Ogbureke et al., ; Ogbureke et al., 2007; Ogbureke et al., ; Waltregny et al., 1998).

Osteopontin (OPN) is an extracellular matrix protein produced by osteoblasts and is capable of stimulating many cell activities through its multiple receptors. Additionally, osteopontin is found to be synthesized at the highest levels in bone and epithelial tissue (Denhardt and Guo, 1993). Specifically, an important structural characteristic of OPN its glycine-arginine-glycine-aspartate-serine (GRGDS or RGD) amino acid sequence (Hayashi et al., 2007; Scatena et al., 2007) (Hsieh et al., ; McKee et al.). OPN acts as a substrate for thrombin and tissue transglutaminase, thus, its function in promoting cell adhesion and migration is substantially enhanced when it is cleaved by thrombin (Furger et al., 2001). Therefore, the primary structure of OPN defines its role in calcification and mineralization control, cell signaling, and chemotaxis (Hsieh et al.). Furthermore, OPN engages with specific integrins to prevent apoptotic cell death through activation of the nuclear factor ĸB (NF-ĸB) (Furger et al., 2001; Tuck et al., 2001). Osteopontin (OPN) is an extracellular matrix protein produced by osteoblasts and is capable of stimulating many cell activities through its multiple receptors. OPN can promote adhesion of T-cells and possibly amplify a CD3-mediated proliferative response. Moreover, OPN is regulated by many transcription factors, cytokines, and growth factors. In pathological conditions, OPN expression in induced, allowing the SIBLING member to facilitate cancer progression, metastasis, and tumorigenesis (Hsieh et al.). A limited number of papers have documented OPN expression in normal salivary glands, pleomorphic adenomas, and in unspecified malignant tumors (Brown et al., 1992; Brown et al., 1994). Overall, OPN is involved in adhesion, chemoattraction, and immunomodulation within normal tissue conditions. However, in malignancy, OPN is found to be overexpressed in many forms of cancer, when induced by supplementary factors, and aids in promoting growth of the primary tumors (Chabas, 2005; Chen et al., 1993b; Chen et al., 2008; El-Tanani et al., 2006; Fisher et al., 2001; Furger et al., 2001; Hayashi et al., 2007; Jain et al., 2009; Kolb et al., 2005; Lee et al., 2007; Pritchett et al., ; Rangaswami et al., 2006b; Razzouk et al., 2002; Zhang et al., ; Zhang et al., 2000).

Bone sialoprotein (BSP), is a sialic acid rich, phosphorylated glycoprotein, which is secreted and part of the non-collagenous extracellular organic matrix in human bone. BSPis responsible for mediating cell attachment and regulating bone formation. It is thought that BSP may mediate the targeting and attachment of normal and metastasizing cells to the bone surface. In cancer, BSP has also been found to form mineral crystallization, thus causing cell damage (Bellahcene et al., 1998; Bellahcene et al., 1997; Bianco et al., 1991; Chen et al., 1993a; Chen et al., 2003; Chen et al., 1998; Chen et al., 1992; Chen et al., 1991; Fedarko et al., 2001; Fisher et al., 2004; Fisher et al., 2001; Malaval et al., 2008;

Ogbureke et al., ; Ogbureke and Fisher, 2004, , 2005, , 2007; Wang et al.) .

Dentin sialoprotein (DSP) is another SIBLING member which represents about 8% of the non-collagenous proteins in the dentin extracellular matrix of teeth and is a single mRNA transcript. DSPP is cleaved into dentin sialoprotein (DSP) and dentin phosphoprotein (DPP), also known as phosphophoryn. DSP, the amino-terminal part of DSPP, is a sialic acid-rich, glycosylated protein (Butler et al., 2002; Chen et al., 2008; Fisher, ; George et al., 1999; Ogbureke and Fisher, 2004, , 2005, , 2007; Suzuki et al., ; Trueb et al., 2007; Zhang et al.). Similar to OPN and DSP, BSP also plays critical roles in progression and metastasis of various epithelial cancers (Fisher et al., 2004; Jain et al., 2009).\Evaluation of SIBLINGs in salivary gland cancer has not been studied before. However, previous studies have evaluated OPN, BSP, DSP, and DMP1 in various organs and tissues such as the breast, kidney, colon, and others. Table 1 provides an overview of SIBLING expression in various normal and cancerous human tissues.

Organ/Tissue	OPN		BSP		DSPP	
	Normal*	Cancer[1]	Normal*	Cancer[1]	Normal*	Cancer[1]
Bone	High	High	High	High	Low	ND
Breast	Low	High	Low	High	ND	ND
Lung	No	High	Low	High	Low	High
Colon	Low	High	Low	High	Low	Low
Kidney	High	High	High	ND	High	ND
Pancreas	No	High	Low	High	ND	ND
Prostate	No	High	No	High	Low	High

Table 1. SIBLING expression levels in evaluated organs/tissues. Low expression of SIBLINGs usually exists in normal organs/tissues with exception to bone and kidneys. Moreover, SIBLING expression is found to be high in many tumorous organs/tissues. **Defines expression of specific*

The presence of high abundance or overexpressed proteins hinders the detection of tumor-specific biomarkers, which are usually at very low concentrations in normal states. With this knowledge, biomarker studies have been concentrated on analysis of the "secretome." The secretome is meant to describe the proteins released by a cell into the extracellular space, thus playing a role in cellular processes such as cell signaling and motility.

Tumor progression is characterized by tumor cell interaction with its extracellular environment, thus creating favorable conditions for growth and metastasis. Many cell-cell interactions are mediated by proteins within the secretome. Therefore, the cancer secretome can be described as constituting proteins released from tumor cells. Proteins secreted from malignant cells prove to be an auspicious source of serological biomarkers, due to their ability to enter the blood circulation (Chang et al., ; Chenau et al., 2009; Lin et al., ; Paltridge et al., ; Pavlou and Diamandis, ; Xue et al., 2008).

The cancer secretome is a valuable source for biomarkers, not only in that secreted proteins are indicators of the physiological conditions of the cells at any time point, but also because they have the highest likelihood of entering the blood circulation. Therefore, it is important to evaluate HTB-41 secretome and the presence of SIBLING proteins. Additionally, assessment of the cancer secretome and its effects on normal cell growth is imperative to the understanding of biological protein influences within the cancer secretome.

Methods

Human Cell Lines: The current study used two human cell lines, (i) normal human salivary gland cells, HSG (received as a generous gift from Dr. Baum B, NIH, Bethesda, MA), and (ii) human salivary gland cancer cells, HTB-41 (ATCC, Manassas, VA). HSG cells were aseptically cultured in DMEM/F-12 media (Corning Cellgro, Manassas, VA), supplemented with 10% FBS and 1% penicillin-streptomycin-amphotericin. HTB-41 cells were aseptically cultured in McCoys5A media (Corning Cellgro, Manassas, VA), supplemented with 10% FBS and 1% penicillin-streptomycin-amphotericin. All cells were maintained in a 5% CO2 atmosphere at 37°C.

Cancer Secretome Collection: HSG and HTB-41 cells were aseptically grown in sterile 100mm

tissue culture dishes to 90% confluence in respective growth media. All growth media was replaced with serum-free DMEM/F12 media for 48 hours. Serum-free conditioned media was collected, centrifuged at 10,000 rpm for 7 minutes, supernatant retrieved and stored at -20°C for use in secretome studies. Normal HSG cells cultured in the cancer secretome from HTB-41 cells were designated as HSG* cells. For all secretome experiments, three groups of cells were used – normal human salivary gland cells/naïve group (HSG), human salivary gland cancer cells/positive control group (HTB-41) and normal human salivary gland cancer cells treated with cancer secretome/experimental group (HSG*). All cell culture experiments were carried outin triplicate.

Protocols

Immunohistochemistry (IHC): IHC was utilized to determine the expression of OPN, BSP and DSP in normal and cancerous human salivary gland tissue samples. Salivary gland sections were fixed in 4% paraformaldehyde and cryosectioned at 10-12 µm onto glass slides. Tissue sections were immunostained with specific antibodies against BSP and DSP. Briefly, tissue sections were rehydrated through decreasing concentrations of a graded ethanol series, endogenous peroxidase activity was quenched using 3% hydrogen peroxide (Bloxall, Vector Laboratories, Burlingame, CA) for 30 minutes, followed by a 0.07M PBS wash. Tissue sections were then blocked with blocking serum, and reacted against anti-BSP, anti-DSP or anti-OPN primary antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX/1:100) overnight at 4˚C. Following a 0.07M PBS wash, sections were then conjugated with biotinylated secondary antibodies for 1 hour. Sections were then reacted against an avidin-biotin complex (ABC kit, Vector Laboratories, Burlingame, CA). The ABC complex was then reacted against diaminobenzidine/DAB, a horseradish peroxidase enzyme for the specific antigen being tested. Sections were counterstained with Hematoxylin, dehydrated through a graded ethanol series, mounted on glass slides and sealed with glass coverslips. All immunohistochemistry images were acquired using a light microscope (Leica DM2500, Leica Microsystems, Buffalo Grove, IL). Presence of a water-insoluble brown precipitate confirmed localization of the protein of interest (Figure 3).

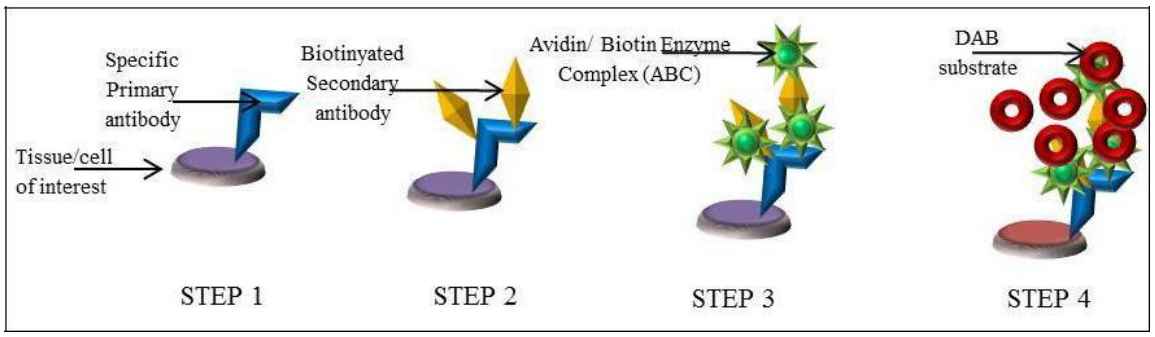


Fig. 3. Diagrammatic representation of the immunohistochemistry process

Protein extraction from HSG and HTB-41 cells: To extract protein from HSG and HTB-41 cell lines, cells were washed in 1X PBS before adding 1 ml of Mammalian Protein Extraction Reagent (M-PER). Cell plates were scraped and collected for 15 minute centrifugation at 4000 rpm. Supernatant was utilized for SDS-PAGE and Western Immunoblotting.

SDS-PAGE and Western Blotting: 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-BSP, anti-DSP and anti-OPN 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 min with stripping buffer (Thermo Scientific/Pierce, Rockford, IL) to eliminate the previous reaction and washed with PBS. Membranes containing whole protein and cytoplasmic fractions 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). Experiments were carried out in triplicate.

Immunofluorescence: HSG, HTB-41, and HSG* cells were processed for immunofluorescence in order to examine morphology. Cells were fixed

with 4% Paraformaldehyde, rinsed with 0.07M PBS, and permeabilized with 0.01% Triton X-100 for 15 minutes. Non-specific staining was blocked with donkey blocking solution for 45 minutes, then primary antibodies were diluted in blocking serum and added to the cells overnight at 4°C (1:500). The next day, cells were rinsed with 0.07M PBS three times, for 10 minutes each. Secondary antibodies were then diluted in 0.07M PBS and added to the cells for 2 hours at room temperature (1:1000). Counterstaining with Rhodamine-Phalloidin to visualize the actin cytoskeleton, and DAPI to stain nuclei were carried out. Cells were rinsed again with 0.07M PBS and mounted to glass slides with Prolong Gold mounting media.

Results

<u>BSP, DSP and OPN are enriched in human salivary gland cancer tissue (Aim 1).</u>

Immunohistochemistry on human salivary gland cancer tissue showed enhanced positive reaction against BSP, DSP and OPN antibodies as compared to normal human salivary gland tissue (Figure 4). As expected, the stroma of normal salivary gland tissue showed a diffuse brown staining for DSP and OPN (Figure 4B, 4C). In addition, epithelial cells lining the salivary gland ducts showed positive staining, indicating the presence of BSP, DSP and OPN (Figure 4A, 4B and 4C).

In contrast to normal tissue, BSP showed a stronger reaction in the stroma as well as inside the cellular profiles of human salivary gland cancer tissue (Figure 4A'). Cancer tissue also showed a robust staining against DSP in the stroma and more prominently within the cellular profiles of the ductal epithelia (Figure 4B'). In tissue stained against OPN, the extracellular and intracellular expression of OPN was evident (Figure 4C'). Tissue sections stained as negative controls did not show increased positive staining against BSP, DSP or OPN (Figures 4A", 4B" and 4C")

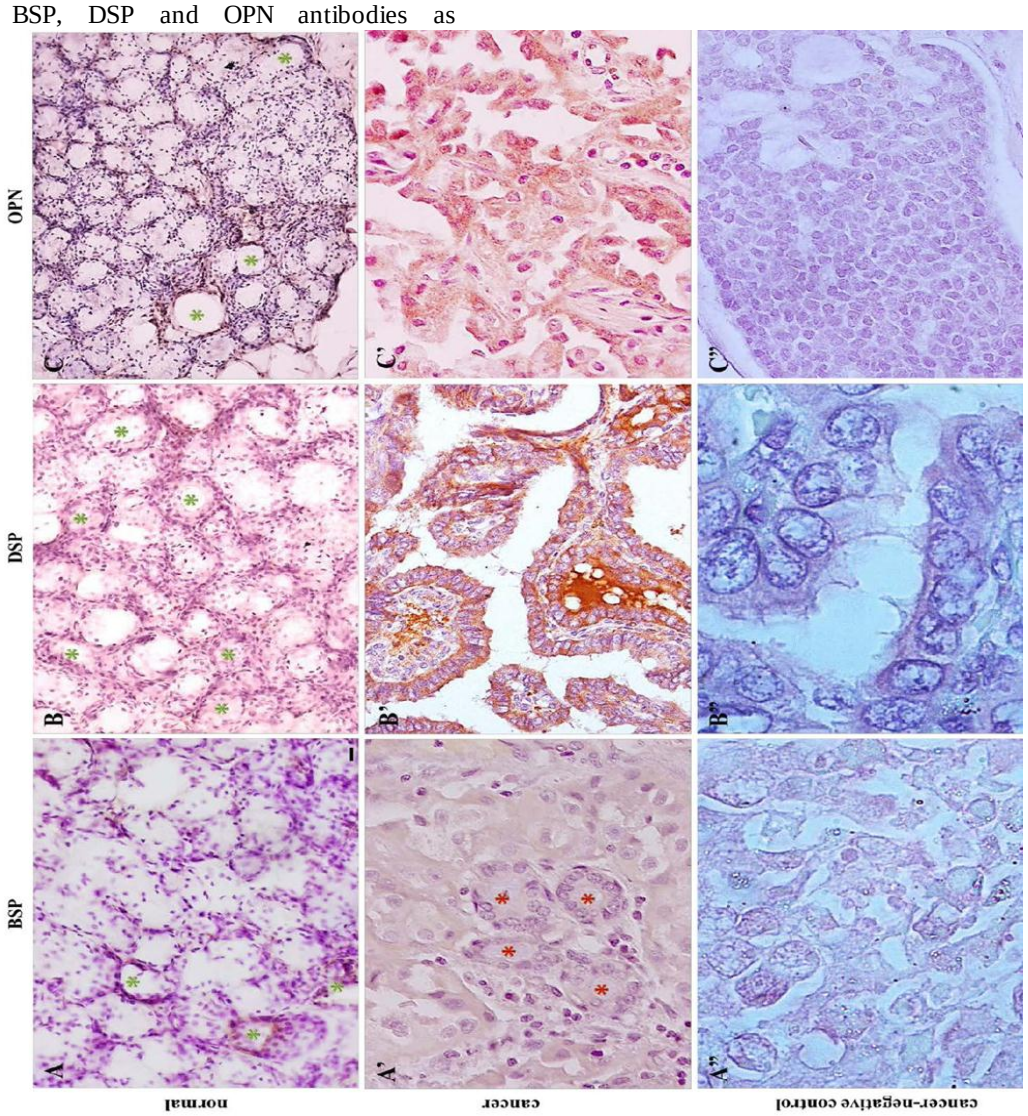


Fig. 4. BSP, DSP and OPN are strongly expressed in human salivary gland cancer samples

A, A', A": Immunohistochemistry images showing BSP expression in normal (A), cancer (A') and negative controls (A"). In normal tissue, nuclei stain dark purple and there is no positive DAB staining evident within the intracellular profiles of the acinar cells. The cytoplasm of the ductal epithelial cells (**green asterisks** indicate ducts) show positive brown staining indicating BSP expression. In cancer cells, there is cytoplasmic expression of BSP in both ductal epithelia **(red asterisks** indicate ducts) and acinar epithelia, as well as a diffuse positive DAB expression in the stroma/extracellular compartment.

B, B', B": Immunohistochemistry images showing DSP expression in normal (A), cancer (A') and negative controls (A"). In normal tissue, DSP expression is seen in the ductal epithelia (**green asterisks** indicate ducts), as well as in the stroma/extracellular compartment. The extracellular expression of DSP was more evident than that seen with BSP. In cancer tissue, a very robust DSP expression is seen associated with epithelia. Since the adenocarcinoma primarily originates from and affects ductal epithelia, all cellular profiles show the salivary gland ducts. Purple nuclear staining is evident with brown staining of the cytoplasm present around the nucleus.

C, C', C": Immunohistochemistry images showing OPN expression in normal (A), cancer (A') and negative controls (A"). In normal tissue, OPN expression is seen primarily as a brown precipitate in the cytoplasm of ductal cells (**green asterisks** indicate ducts). At the bottom left and right corners of panel C brown DAB staining of adipose tissue is evident, as expected. In cancer tissue, strong cytoplasmic staining of the ductal cells with a prominent purple nucleus is seen. Since the adenocarcinoma primarily originates from and affects ductal epithelia, all cellular profiles show the salivary gland ducts.

Negative controls of all cancer samples (A", B", C") did not show positive expression of BSP, DSP or OPN. In the absence of eosin staining, negative controls shows a diffuse blueish-purple hue of the cytoplasm and the extracellular compartment.

<u>BSP, DSP and OPN exhibit specific localization in human salivary gland cancer cells (Aim 1).</u>

Immunofluorescence studies against BSP, DSP and OPN antibodies were carried out in normal human salivary gland cells (HSG) and human cancer salivary gland cells (HTB-41), and is depicted in Figure 5. BSP, DSP and OPN were present in normal HSG cells, as expected. BSP specifically co-localized with the actin cytoskeleton, and is seen expressed along the actin cytoskeleton, as well as outlining the cell profiles together with actin (Figure 5; Panel A merge). In cancer HTB-41 cells, BSP continued to co-localize with actin, and exhibited a presence along the actin stress fibers (Figure 5; Panel B merge).

DSP expression in normal HSG cells was cytoplasmic (Figure 5C'). In addition, a discrete accumulation of DSP was seen localized as a cytoplasmic "cap" in relation to the nucleus (Figure 5C" and Panel C merge). In contrast, cancer HTB-41 cells showed a robust presentation of DSP in the cytoplasm, as a "perinuclear ring" (Figure 5D" and Panel D merge).

OPN in normal HSG cells exhibited a very strong cytoplasmic distribution (Figure 5E'), while in cancer HTB-41 cells, OPN was very distinctly seen to have a robust nuclear presentation (Figure 5F' and Panel F merge).

As evidenced by the actin distribution, the data also show that cancer salivary gland cells have a very distinct morphology as compared to normal salivary gland cells (Figures 5B, 5C, 5D). While normal cells exhibit a polyhedral shape, cancer cells exhibit a "stellate" configuration as a result of the elaborate filopodial extensions that project from the cells.

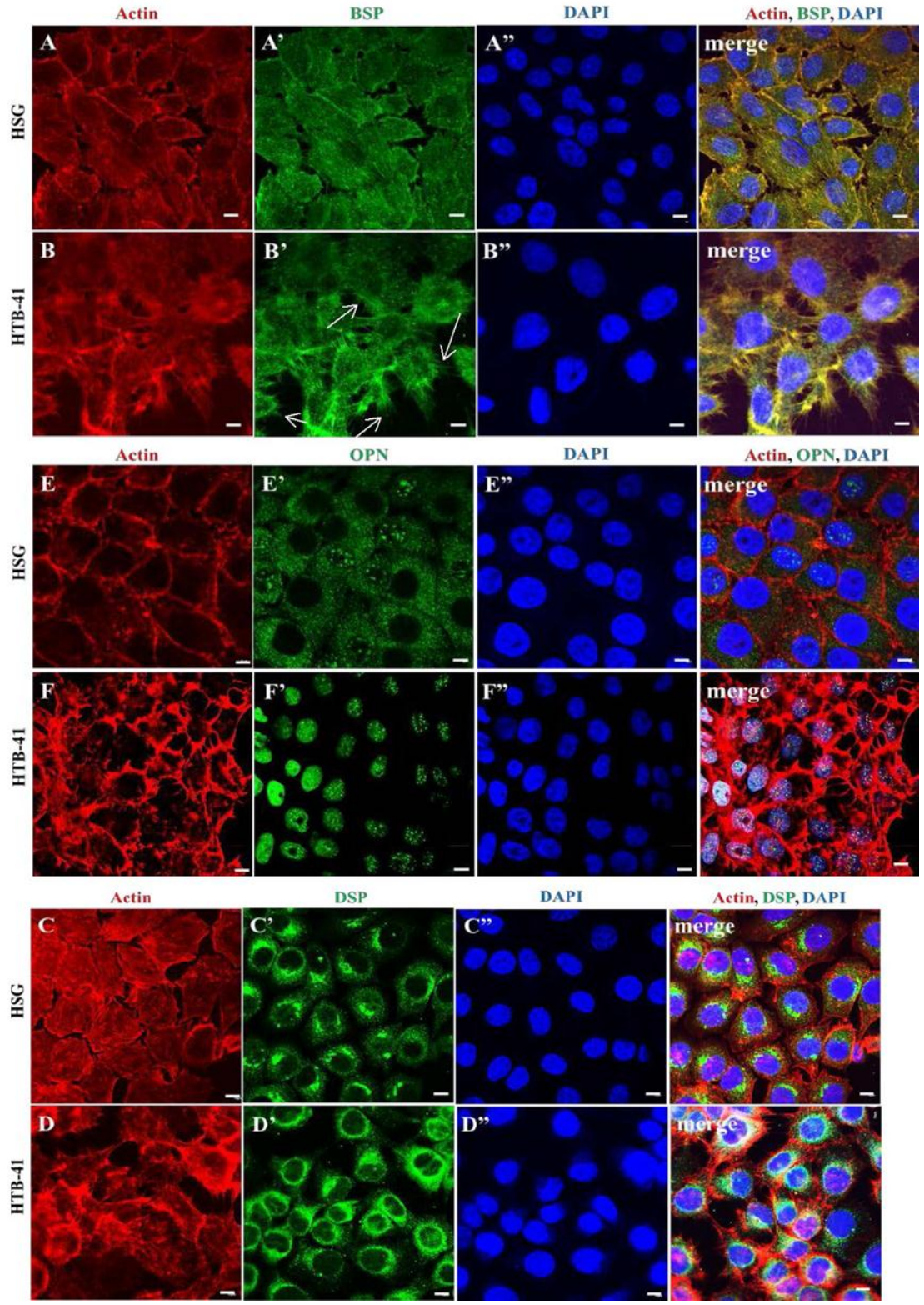


Fig. 5. (on opposite page): BSP, DSP and OPN exhibit specific intracellular localizations in normal HSG and cancer HTB-41 cells (Scale bar = 5µm)

A, A', A", merge: Representative confocal images depict actin cytoskeleton, intracellular localization of BSP and nuclei in normal HSG cells. The actin cytoskeleton and cortical actin clearly shows the normal, expected cell morphology of human salivary gland cells. BSP is localized to the cell membrane as well as the cytoplasm. The merged image shows BSP co-localizing with actin, both in the cell membrane, as well as in the cytoskeleton.

B, B', B", merge: Representative confocal images depict actin cytoskeleton, intracellular localization of BSP and nuclei in cancer HTB-41 cells. Cell morphology in the cancer cells are different from that seen in normal salivary gland cells, as evidenced by the extensive actin-lined membrane extensions or filopodia, giving them a "stellate" appearance (white arrows) . BSP continues to be associated with the cell membrane and extends along the filopodia. In the merged image, the strong co-localization between actin and BSP is clearly evident.

C, C', C", merge: Representative confocal images depict actin cytoskeleton, intracellular localization of DSP and nuclei in normal HSG cells. In normal HSG cells, DSP is distributed in the cytoplasm, and there is an accumulation of DSP towards one side of the cell. The merged image clearly indicates that in addition to the diffuse cytoplasmic distribution of DSP, there is a cytoplasmic accumulation that is localized as a "cap" in relation to the nucleus. Unlike BSP, DSP is not associated with actin.

D, D', D", merge: Representative confocal images depict actin cytoskeleton, intracellular localization of DSP and nuclei in cancer HTB-41 cells. In cancer cells, DSP continues to have a cytoplasmic presentation, but the staining is more intense than in normal salivary gland cells. Unlike normal cells, the cytoplasmic accumulation of DSP is distributed as an intense "perinuclear ring".

E, E', E", merge: Representative confocal images depict actin cytoskeleton, intracellular localization of OPN and nuclei in normal HSG cells. OPN is distributed all throughout the cytoplasm in normal HSG cells. Our data show that moderate nuclear presentation was seen in few cells.

F, F', F", merge: Representative confocal images depict actin cytoskeleton, intracellular localization of OPN and nuclei in cancer HTB-41 cells. In addition to a cytoplasmic distribution, OPN is seen to translocate into the nucleus.

<u>BSP, DSP and OPN levels are upregulated in human salivary gland cancer (Aim 2).</u>

Western blot analyses to evaluate levels of BSP, DSP and OPN in human salivary gland cancer cells (HTB-41) in comparison to normal human salivary gland cells (HSG) were carried out. Data were analyzed in triplicate, and show that expression levels of BSP, DSP and OPN are significantly elevated in cancer HTB-41 cells as compared to normal HSG cells (Figure 6).

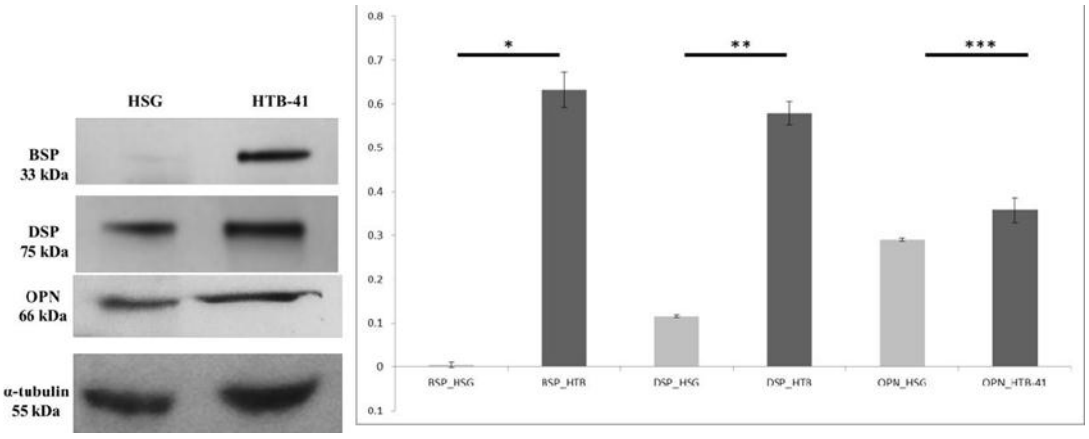


Fig. 6. BSP, DSP and OPN levels are significantly elevated in cancer HTB-41 cells.

<u>Cancer secretome enhances BSP, DSP and OPN expression in normal human salivary gland cells (Aim 3).</u>

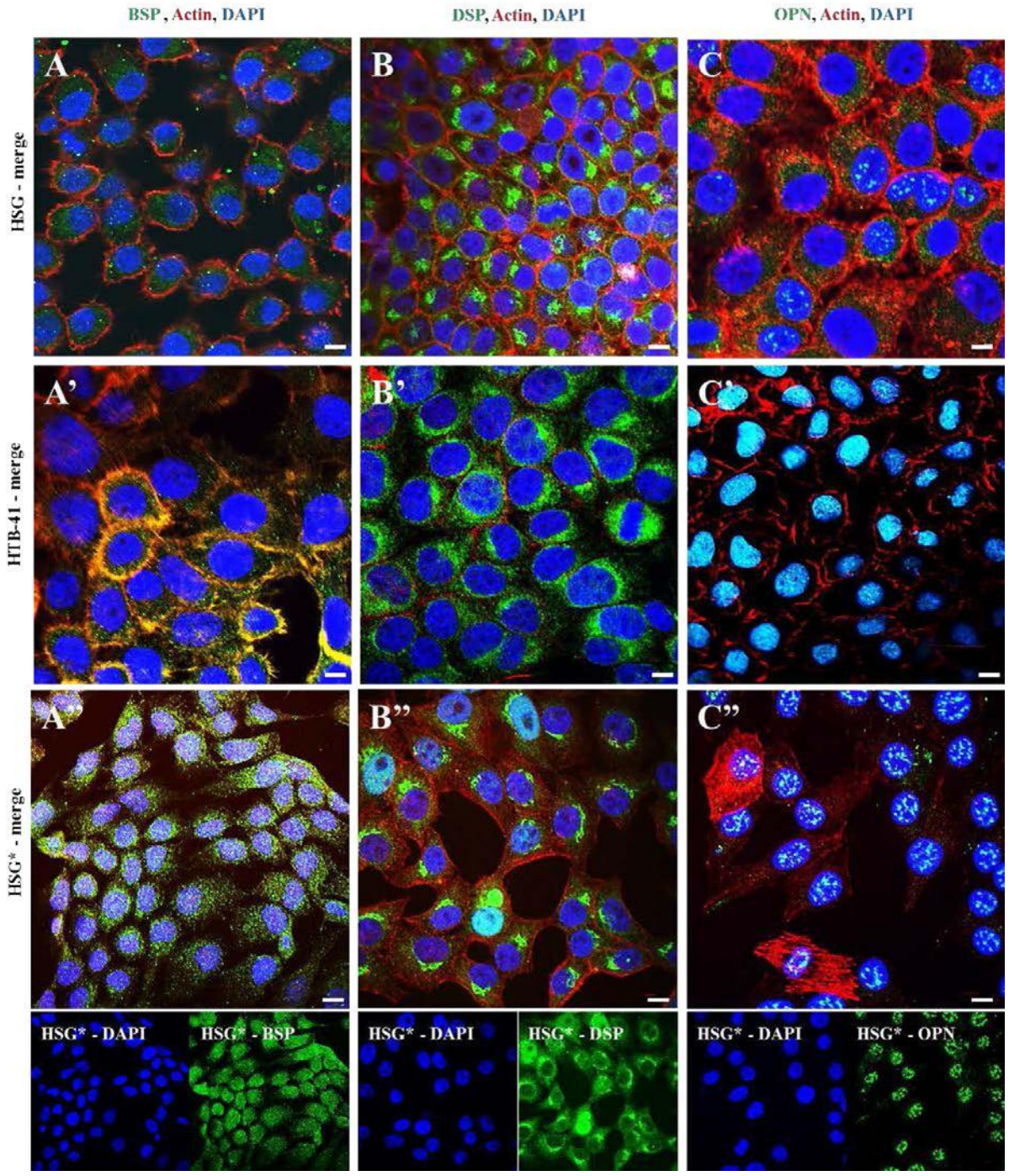
As described earlier, normal human salivary gland cells (HSG) were aseptically cultured in serum-free secretome collected from human salivary gland cancer cells (HTB-41) for 24, 48 and 72 hours, and designated as HSG* cells. Under the influence of the cancer secretome, BSP expression seemed to be increased in the cytoplasm and nuclear compartments of HSG* cells (Figure 7A, 7A' and 7A"). DSP expression in normal HSG cells is localized to the cytoplasm with an intense cytoplasmic accumulation, previously described as a "polarized cap" in relation to the nucleus. In contrast, HSG* cells show the perinuclear presentation seen in cancer HTB-41 cells (Figure 7B, 7B' and 7B").

Fig. 7 (opposite page). BSP, DSP and OPN expression is enhanced in normal HSG cells following exposure to a cancer secretome for 72 hours. Representative confocal images of HSG, HTB-41 and HSG* cells depict localization of BSP and DSP. (Scale bar = 5μm)

A, A', A": Merged confocal images depict the distribution of BSP in normal HSG, cancer HTB-41 and cancer secretome-induced HSG* cells. Under the influence of the cancer secretome, a nuclear presentation of BSP is seen in HSG* cells. The nuclear translocation of BSP is not evident in cancer HTB-41 cells and may be indicative of transformation of normal cells into a cancer phenotype by the influence of specific growth factors in the cancer secretome.

B, B', B": In HSG* cells under the influence of the cancer secretome, DSP expression is greatly enhanced.

C, C', C": OPN in HSG* cells shows strong nuclear translocation, as compared to that seen in normal.



In HSG* cells, OPN expression is visible as intense nuclear staining, as compared to normal HSG cells (Figure 7C, 7C', 7C"). The changes observed in BSP, DSP and OPN expression, indicate that under the influence of the cancer secretome, normal HSG

cells begin to more closely mirror the expression seen in cancer cells.

As described previously, in addition to the altered expression of BSP, DSP and OPN in HSG* cells, cell morphology was also distinctly different from normal. HSG* cells exhibited the more "stellate" cell morphology with associated membrane extensions that are typically seen in cancer cells (Figure 7A", 7B", 7C").

Discussion

SIBLINGs are a family of five proteins, that are encoded by identically oriented tandem genes located on chromosome 4 (BSP, DSP, DMP1, OPN and MEPE). They are small, soluble, RGD motif containing, integrin-binding ligands, and are unique from larger extracellular matrix proteins such as fibronectin and collagen. Four of the five members of this protein family (BSP, DSP, DMP1 and OPN) have been shown to be present in mineralized matrices of bone and dentin, while varying combinations of all five SIBLING proteins have been shown to be significantly upregulated in various epithelial cancers, which metastasize to bone (Bellahcene et al., 2008; Ogbureke and Fisher, 2004, , 2007; Suzuki et al., ; Trueb et al., 2007).

A previous study evaluating human gland tissue has shown that SIBLINGs are expressed in metabolically active, high energy-requiring sweat gland ducts as compared to metabolically inactive lacrimal ducts (Ogbureke and Fisher, 2007). Major salivary glands produce approximately 700 to 1200 ml of saliva daily. Primary saliva that is produced by the acinar cells is isotonic as compared to plasma. The striated cells of the salivary gland ducts modify the primary saliva by removing sodium and chloride ions from it, and secreting potassium and bicarbonate ions into it. The altered secretion is hypotonic and is referred to as secondary saliva. To support this high-energy requiring metabolic activity of the ducts, salivary glands are supplied by a very rich vascular supply, almost twenty times as much as the blood flow to skeletal muscle. Our studies of normal human salivary gland tissue show evidence of BSP, DSP and OPN expression and could indicate the high metabolic activity that typically characterizes the ductal cells of salivary glands. Evaluation of the normal salivary gland cell line, HSG, also confirms that BSP, DSP and OPN have very specific and unique intracellular localizations. In addition, protein quantification in the current study also shows that there is not a complete lack of BSP, DSP and OPN in normal salivary gland cells; rather, a baseline level of BSP, DSP and OPN is maintained, presumably to regulate the extraordinarily high metabolic requirements of the salivary gland ductal cells.

In contrast to normal human salivary gland tissue and cells, our data show that salivary gland cancer exhibits a more robust expression of BSP, DSP and OPN. *In vitro* studies of breast cancer cells have shown that BSP is capable of increasing cell proliferation (Sung et al., 1998). Human breast cancer cells transfected with BSP and injected into the mammary fat pad of nude mice stimulated migration, invasion and growth of primary and secondary tumors (Sharp et al., 2004). Elevated levels of BSP have also been found in cancers that have a high propensity to metastasize to bone, such as cancers of the breast (Bellahcene et al., 1996a; Bellahcene et al., 1996b; Bellahcene et al., 1994), prostate (Waltregny et al., 1998) and lung (Bellahcene et al., 1997). Our evaluation of human salivary gland cancer issue as well as the human salivary gland cancer cell line, HTB-41, indicate strong and very specific intracellular expression of BSP. In tissue samples, BSP was found to be localized to the extracellular matrix/stroma, as well as had an intracellular presentation of both acinar and ductal cells.

Confocal microscopy studies, as well as previous scanning electron microscopy studies have revealed that cancer HTB-41 cells exhibit a variant morphology and cytoarchitecture as compared to normal salivary gland cells (Figure 10). Specifically, salivary gland cells transform from a more rounded/polyhedral shape to a more "stellate" morphology with numerous membrane extensions extending in multiple directions. In addition, normal salivary gland cells grow as a cohesive "sheet" of cells, while cancer cells tend to grow in clumps/groups. The known biological activities of BSP, namely its role in cancer metastasis, coupled with co-localization with the actin cytoskeleton could indicate a potential role for BSP in promoting cell spreading, and initiating cell migration via filopodial extensions in human salivary gland cancers.

Dentin sialophosphoprotein (DSPP) and its cleaved products, namely, DPP/dentin phosphoprotein and DSP/dentin sialoprotein play important roles in biomineralization (Prasad et al.,; Sreenath et al., 2003; Verdelis et al., 2008; Xiao et al., 2001). In addition to its expression in mineralized tissue such as bone,

Interestingly, the immunofluorescence data closely mirrors the presentation seen in the tissue sections. In cancer HTB-41 cells, DSP expression is very clearly visualized as a "polar cap" located in the cytoplasm, but in close proximity to the nucleus. In addition, levels of DSP in cancer HTB-41 cells were significantly elevated as compared to normal

salivary gland cells. Although DSP has very specific roles in mineralized tissue, its roles in non-mineralized tissue have not been clearly elucidated yet. In non-mineralized tissues, it has been suggested that DSP is devoid of carbohydrate moieties (Prasad et al.), thus indicating a very different role for DSP in non-mineralized tissue as compared to mineralized tissue. Therefore, in salivary gland cancers, DSP expression could indicate important roles that include regulation of cell adhesion and cell migration. In addition, DSP has been shown to have a potential role in maintenance of blood vessel homeostasis and stem cell differentiation. In salivary gland cancers, DSP expression could potentially play a role in supporting angiogenesis and activating the stem cell niche in salivary gland ducts, thus promoting cancer progression and metastasis.

Osteopontin (OPN) is a multifunctional SIBLING protein which is highly expressed in bone, and also expressed in macrophages, endothelial cells and smooth muscle cells (Mazzali et al., 2002; O'Brien et al., 1994). In normal bone tissue, OPN is expressed by both osteoclasts and osteoblasts, and plays an important role in the regulation of bone remodeling. OPN expression is also prominent in tissues showing dystrophic or pathological calcifications such as arterial calcified plaques (Hirota et al., 1993; O'Brien et al., 1995; Srivatsa et al., 1997) and in the kidneys (Hess, 1994; Shiraga et al., 1992); in these tissues OPN acts as an inhibitor to prevent or limit calcifications. OPN is also a cell survival factor, and inhibits apoptosis (Ophascharoensuk et al., 1999). It is also an important molecule in wound healing and tissue regeneration. In addition to its diverse roles in normal cell biology, OPN is also important in tumor cell biology. In fact, OPN was first isolated from epithelial cells that had undergone malignant transformation (Senger et al., 1983; Senger et al., 1989). OPN has been shown to increase proliferation of cancer cells in a mouse model of prostate cancer (Khodavirdi et al., 2006). OPN also has critical roles in cell invasion that eventually can progress to cellular metastasis in cancer. Specifically, OPN has the ability to increase invasiveness of cancer cells that allow these cells to then invade into blood vessels (Khodavirdi et al., 2006). *In vitro* migration of several cancer cell lines, including skin, breast and blood have also been reported (Caers et al., 2006; Hayashi et al., 2007; Khan et al., 2005). The known biological activities of OPN thus help in the promotion of metastatic properties when expressed in cancer cells. Although OPN has been shown to be upregulated (Ogbureke et al., 2007) in oral cancers, a systematic evaluation of its cellular distribution and expression in salivary gland cancers is as yet lacking. Our data provide evidence of the expression of OPN in human salivary gland cancer tissue as well as in a human cancer HTB-41 cell line. In tissue samples, OPN shows a very robust intracellular staining that completely fills the cytoplasm. In the confocal images, a very clear cytoplasmic presentation of OPN is evident, along with a distinct nuclear translocation. Further analyses of nuclear versus cytoplasmic fractions are warranted to confirm elevated levels of OPN in the nuclear fraction as compared to the cytoplasmic fraction. OPN levels in cancer HTB-41 cells also showed a significant increase as compared to normal human salivary gland cells. Our data thus indicate that OPN, the first SIBLING protein that was found to be overexpressed in cancer (Senger et al., 1983), is in fact upregulated in human salivary gland cancers. Thus, very similar to BSP, OPN expression in cancer cells may not be restricted to cancer cells that metastasize to bone alone, but rather to cancer cells that have a general malignant phenotype. A previous study by Junaid et al, 2006 demonstrated that OPN translocates into the nucleus via an exportin-1-dependent mechanism (Junaid et al., 2007). Furthermore, this translocation concurs with the S phase and thus increases cellular OPN in the G2/M phase, while interacting with polo-like-kinase-1, a proto-oncogene often overexpressed in tumors (Junaid et al., 2007). The nuclear translocation of OPN into the nucleus could potentially indicate regulation of cancer cell migration and tissue invasion at a transcriptional level and warrants further studies.

The observations that confirmed upregulation of BSP, DSP and OPN in salivary gland cancer as opposed to normal human salivary gland cells, led to the hypothesis that the cancer microenvironment could potentially influence behavior of normal cells. Therefore, cancer secretome was collected and normal human salivary gland cells were cultured in the cancer secretome to understand the role of the cancer secretome in BSP, DSP and OPN expression.

Secreted factors are key mediators of cell-cell communication, and are responsible for regulating cell proliferation, cell migration, cell invasion, and evasion of apoptosis and several other hallmarks of cancer by cell signaling. The cancer secretome has multiple factors that can contribute towards various cellular functions, the goal of our study was to evaluate if the cancer secretome, with all its variant factors could potentially influence the expression of BSP, DSP and OPN. Future studies will evaluate the cancer secretome itself to identify key factors that may have a role in regulating SIBLING expression. Our study shows that under the influence of the human salivary gland cancer secretome, normal human salivary gland cancer cells begin to express BSP, DSP and OPN in a

similar phenotypic manner as seen in the cancer cells. Specifically, BSP and DSP levels are elevated significantly as compared to normal salivary gland cells, and intracellular localization of BSP, DSP and OPN are more similar to cancer HTB-41 cells than normal HSG cells. Our study supports the increasingly evident fact that cancer cells do not act in isolation during cancer progression and metastasis. Rather, primary tumor cells interact with non-transformed cells to create a microenvironment that is conducive to cancer cell survival, progression and metastasis. Although this close interaction between cancer cells and non-transformed cells can enhance cancer progression and increase malignancy, identifying key membrane and intracellular players such as the SIBLING proteins will allow us to develop strategic therapies targeting either specific factors in the cancer secretome or specific downstream molecules that are involved in the transmission of cell signals.

The current study thus provides valuable information regarding the expression of BSP, DSP and OPN in human salivary gland cancers; and for the first time, it specifically outlines intracellular localizations of these proteins. Although further studies are warranted, the data show a clear role of the cancer secretome in maintaining "cancer-ness", thus potentially promoting specific hallmarks of metastasis namely cell proliferation, cell migration, cell invasion and cell survival through downstream cell signaling pathways mediated via SIBLING proteins.

Human salivary glands offer a unique opportunity in future studies to identify other SIBLINGs or factors in the cancer secretome, which may be linked to activation and upregulation of SIBLING proteins. Furthermore, the future documentation of key membrane and intracellular players interacting with SIBLINGs in the cancer secretome may offer novel evidence leading to answers regarding therapeutic targeting. In regards to OPN, future studies may focus on its expression in nuclear and cytoplasmic fragments, as well as its mechanism of translocation into the nucleus. Studying the roles of SIBLING proteins will not merely give rise to forthcoming studies, but proposition additional data leading to therapeutic targeting.

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