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A Loss of Profilin-1 in Late Stage Oral Squamous Cell Carcinoma

Guy R. Adami¹, Thomas N. O'Callaghan¹, Kolokythas Antonia², Robert. J. Cabay³, Yalu Zhou¹, and Joel L. Schwartz¹

¹Department of Oral Medicine and Diagnostics, Center for Molecular Biology of Oral Diseases, College of Dentistry, University of Illinois at Chicago, Chicago, IL

²Department of Oral and Maxillofacial Surgery, Center for Molecular Biology of Oral Diseases, College of Dentistry, University of Illinois at Chicago, Chicago, IL

³Dept. of Pathology, College of Medicine, University of Illinois at Chicago, Chicago, IL

Abstract

BACKGROUND—The genes for PFN1 and TMSB4 are both highly expressed in oral tissue and both encode actin monomer binding proteins thought to play a role in cell motility and possibly other crucial parts of tumor progression.

METHODS—Oral brush cytology of epithelium from Oral Squamous Cell Carcinoma (OSCC) was used to measure PFN1 and TMSB4 mRNA in OSCC while immunohistochemical analysis of tissue was used to check protein levels.

RESULTS—High but variable expression of mRNAs encoding these two proteins was observed suggesting they may contribute to tumor characteristics in a subset of OSCCs. Both proteins were highly expressed in normal appearing basal epithelium, in the cytoplasm and perinuclear area, while expression was minimal in upper epithelial layers. In OSCCs, expression of these proteins varied. In tumors classified as later stage, based on size and/or lymph node involvement, PFN1 levels were lower in tumor epithelium. A control gene, KRT13, showed expression in normal differentiated basal and suprabasal oral mucosa epithelial cells and as reported was lost in OSCC cells.

CONCLUSION—Loss of PFN1 in tumor cells has been associated with lymph node invasion and metastasis in other tumor types, strengthening the argument that the protein has the potential to be a tumor suppressor in late stage OSCC.

Keywords

Oral mucosa; Oral cancer; PFN1; TMSB4; Tumor progression

Introduction

PFN1 is a member of the profilin family of small proteins (12-15KD), which have the ability to bind actin, affect its polymerization, cytoskeletal growth that effects cell locomotion, cell shape and cell transport (1, 2). The protein is found extracellularly in saliva and in dental

crevicular fluid, among other places (3, 4), but it functions chiefly inside cells(1, 2)The ability of the protein to promote actin polymerization originally suggested the primary function was to regulate cell motility, but it is now clear that PFN1, like other profilins, can interact with additional proteins, including the phosphoinositides and polyproline-containing proteins, which may allow it to regulate multiple pathways(1, 2, 5). These interactions can affect cell signaling, membrane trafficking, and cell cycle arrest. PFN1 is also absolutely necessary for cytokinesis (6) and cell proliferation, however, enrichment of the protein in cell lines impairs cell motility and promotes apoptosis(7-10). These latter two observations are consistent with the protein being a tumor suppressor. Indeed, PFN1 is downregulated overall or at least in late-stage breast, pancreatic, laryngeal, and bladder cancers(7, 9, 11-13). In bladder cancer, for example, epithelial cancer expression of PFN1 decreases with worsening prognosis, while stromal levels increase(13). Thus loss of the protein seems to correlate with advanced tumors in at least some tumor types. Because of multiple protein interactions with PFN1 it is expected that many pathways are regulated, however, a clear mechanism for multiple functions in different cell types remains unclear. There is also evidence that changes in PFN1 levels and cell localization plays an important role in some tumor types(5). In short, the protein seems to have context-dependent effects on cell motility and on multiple pathways that can contribute to transformation though a tumor suppressor function.

TMSB4 is found at high levels in saliva in adults and even higher levels in neonatal saliva, which seems to indicate high expression in at least some tissues of the oral cavity. TMSB4 is the most abundant form of the multi-functional beta-thymosin proteins, that include TMB4, B10 and B15. The 43 aminoacid TMB4 protein is believed to be the main actin-sequestering protein, and there is evidence that it plays a role in regulating cell motility. In addition, the protein has roles in influencing angiogenesis, inflammation, cell survival, wound healing, and calcium deposition (14). There is evidence that TMSB4, a protein expressed at high levels in embryonic tissues, plays a role in expression of stem cell properties, and it is postulated that enrichment in some tumor types is linked to acquisition of embryonic cell properties or metastasis and tissue invasion For example, TMSB4, is linked to metastatic spread of various malignancies, such as colorectal and breast (15, 16) although enhanced expression most significantly occurs on the cancer border in stromal tissue and not in tumor cells themselves. In fact, in hepatocellular carcinoma TMSB4 has been noted to be poorly expressed in the majority of tumor tissue while easily detected in normal parenchyma. Additionally, the protein is totally absent in hepatocellular tumor cells undergoing stromal invasion (15). In general, its expression seems to counter differentiation and because TMSB4 is found at elevated levels in several tumor types it is thought to work as an oncogene but its effects are also likely related to an altered stromal microenvironment (17-21).

We used brush oral cytology to ascertain variability of the mRNAs encoding PFN1 and TMSB4 proteins between different OSCC tumors. If these proteins closely linked to tumor progression vary on the RNA level in OSCC epithelium, we reckoned they may also vary on the protein level. Given their links to tumor progression in other tumor types we reasoned protein variation may in turn correlate to OSCC stage. We note both these proteins can show robust expression in the stroma so usage of brush cytology allows rapid stroma free

epithelium isolation. These mRNAs both showed great variation in expression levels in a small group of tumors that were tested. We then tested if this variation also occurred with protein examined in intact tumor tissue sections in a larger tumor set, and if this correlated with tumor characteristics.

Materials and Methods

Clinical sampling

Paraffin-embedded tumor tissue was obtained from the Department of Pathology tumor bank at the University of Illinois at Chicago. Tumor tissue was from the March 2008 – 2012 period, with a preference for samples that included both normal tissue and tumor tissue. All patients were current or former smokers or betel users. Originally 42 samples were obtained but 2 were eliminated due to ambiguous diagnosis as OSCC, and three samples did not stain with at least one of the antibodies in normal or tumor tissues. OSCC samples came from: 6 buccal mucosa lesions; 14 tongue; 13 gingiva; 6 floor of mouth and 1 oropharynx lesion.

In addition, oral brush cytology samples were collected from 17 former and current tobacco and betel nut users, who presented with oral lesions, typically just prior to taking a diagnostic biopsy. Patients were seen in the Oral and Maxillofacial Surgery Clinic, the Multidisciplinary Head and Neck Cancer Clinic, and the Otolaryngology Clinic in the University of Illinois Medical Center. All diagnoses were verified by histopathologic examination of surgically obtained tumor tissue. All subjects provided consent to participate in accordance with guidelines of the Office for the Protection of Research Subjects of the University of Illinois at Chicago, and the Institutional Review Board of Biomedical and Biological Science. Care was taken to sample only epithelium in the case of ulcerative lesions. Samples were immediately placed in Trizol (Invitrogen, Carlsbad, CA, USA), mixed, and frozen. We used a cervical cytology brush with RNA purification as described in Schwartz et al. (22).

RT-PCR

RNA from brush cytology was converted to cDNA and quantitative RT-PCR was carried out using the iCycler iQ (Bio-Rad, Hercules, CA, USA) and SYBR Green fluorescence as described. 1 Values were normalized to the geometric mean of the controls, *GAPD*, *RPLP0*, and *RPL4*. Primers for these mRNAs, and those for *PFN1*, and *ANXA2* and *TMSB4* to detect the target mRNAs, were designed using Primer Express to give products of approximately 100 bases; sequences were previously published and/or included below. ¹
 PFN1 5'-TGTCACCAAGACTGACAAGAC-3' and 5'-
 GGTGGGAGGCCATTTTCATAA-3', PFN2 5'-CACTTCCTCCCATGACCTTAC-3' 5'-
 GTAGAGCTGGTGTGCAAAGA-3' and TMSB4 5'-
 TCCTCCGCAACCATGTCTGACAAA-3' TMSB4 5'-
 TTACGATTGCGCTGCTTGCTTCTC-3'

Cell lines

hTERT HOK cells were cultured in Keratinocyte-SFM medium supplemented with EGF, and Bovine Pituitary Extract as described by the manufacturer and supplemented with 400

micromolar for a final concentration of 800 nanomolar and 25 mg/mL of Gentamicin and 0.375 mg/mL of Amphotericin B all from Gibco, Invitrogen Carlsbad, CA, USA.

OSCC cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum (Sigma Chemical Co., St. Louis, MO) and 10,000 units of penicillin/ streptomycin and L-glutamine. The list of OSCC cell lines were: HSC-2 (mouth); HSC-3 (tongue), HSC-4 (tongue), Ca 9-22 (gingiva), SCC-9 (base of tongue), SCC-15 (base of tongue), and SCC-25 (base of tongue).

OSCC tissue and immunohistochemistry

Oral tissue was dehydrated and embedded in paraffin and 5-micron tissue sections that were stained using a standard immune-peroxidase technique after binding of primary antibodies for PFN1 and rabbit monoclonal (95847, Novus Biologicals, Littleton, CO, USA); TMSB4 (rabbit polyclonal, 14335, AbCam, Cambridge, MA, USA), and CYK13 (monoclonal mouse antibody, Novus Biologicals) specificity assessed with a control IgG antibody and counterstained with Mayer's hematoxylin. About half the samples were also tested with a different PFN1 antisera, (rabbit polyclonal, 19344 Novus Biologicals), to observe consistent tissue site expression specificity. Negative controls without primary antibody showed no visible staining. Positive controls for both TMSB4 and PFN1 antibodies were peripheral lymphocytes based on antibody supplier recommendation and/or reports on lymphocyte expression, <http://www.genecards.org/>

Evaluation of protein expression (IHC)

We used a relative index less than, equal to, or greater than the control with regard to percentage positive cells, and to a lesser degree staining intensity for examination of apparent normal oral mucosa in comparison to sites of dysplasia or invasive OSCC. In contrast to PFN1 and TMSB4 expressions which are preferentially expressed in the basal regions of oral mucosa, cytokeratin 13 is found not only in the basal region, but in the suprabasal regions of the oral mucosa, which provides a good counterbalance when we analyze for PFN1 and TMSB4 expressions (23). Histopathology of oral lesions and grade of OSCC were confirmed by two pathologists.

Statistical analysis

The *Fisher Exact Test* was used to determine the association of PFN1 and TMSB4 positivity and various clinical pathological parameters. Kirkman, T.W. (1996) Statistics to Use. <http://www.physics.csbsju.edu/stats/> (20 November 2015). The Wilcoxon Rank Sum Test was used to measure statistical significance of differences between tumor and normal tissue for PFN1 and TMSB4 levels based on antibody staining.

Results

TMSB4 and PFN1 mRNA expression in OSCC

PFN1 and TMSB4, proteins found at relatively high levels in saliva and oral tissue, have been shown in at least some cell types to regulate cell migration. We used RNA from brush cytology to measure the corresponding RNAs in epithelium from OSCCs. The mRNAs for

PFN1 and TMSB4 were observed to be expressed at high but variable levels in OSCC epithelium from different patients based on qRT-PCR after normalization to two housekeeping mRNAs (Fig. 1). This is in comparison to the mRNA encoding the cell adhesion protein ANXA1, which shows minimal variability in the epithelium of OSCCs from different tissue sites.

TMSB4 and PFN1 protein expression in normal oral tissue

As shown in figure 2, PFN1 was preferentially expressed in the inferior area of oral mucosa strata spinosum and strata basalis and localized to a perinuclear area, and in the cytoplasm of oral keratinocytes of both these regions. There was a sparse diffuse infiltration of lymphocyte and histiocyte-type cells interspersed between keratinocytes. The oral mucosa overlaid a submucosa of mesenchymal connective tissue with a sparse array of fibroblasts and endothelial-lined vascular spaces. In contrast, TMSB4 was expressed in the suprabasal and strata spinosum regions of the oral mucosa, but also in oral keratinocyte cytoplasm and perinuclear area. The submucosa description was given above. Lastly, KRT13 was highly expressed throughout the oral mucosa but particularly in the strata basalis but extending to the stratum spinosum in the cytoplasm of oral keratinocytes. H+E staining showed a parakeratinized epithelial tissue composed of squamous keratinocytes of several layers in thickness with slight diffuse hyperchromatism in the strata basalis.

OSCC and examination of correlation with tumor stage

Because of the variable mRNA expression in different OSCCs we sought to detect if the corresponding proteins levels varied. Immunohistochemical analysis was done and a comparison of protein levels was made between epithelium of the tumor and adjacent epithelial tissue with normal or near normal architecture and growth patterns in the same patient among patients described in table 1. Tumor tissue was identified by histopathology. Often interspersed between and surrounding OSCC was an inflammatory infiltration of lymphocytes, granulocyte populations (e.g., mast cell, eosinophils, polymorpho-nuclear leukocyte) which vary as to number and density for each sample. Histopathologically normal epithelium, used as an internal control in each sample, lacked these features.

As shown in figure 3, immunohistochemistry was used to measure PFN1, TMSB4 and KRT13 proteins in each of these regions and compared to regions that showed normal or near normal histology. Remarkably, as others have noted, in the majority of tumors tested, in this case 29 out of 32, tumor epithelium showed little or no detectable KRT13 protein (23). This selectivity helped expedite the localization of normal tissue for histopathology. For PFN1 and TMSB4, expression patterns were different from KRT13. The more normal-looking tissue adjacent to the tumor showed strong staining for both these proteins in the basal epithelium and in the suprabasal layers with greatly diminished expression in the upper levels of the stratum spinosum (Fig. 3). A quantitation of expression of these proteins was done on tumor tissue and staining for both proteins was quite variable. However, in each sample, expression was compared between tumor epithelium and normal adjacent epithelium in the same section. Each specimen was given a grade of staining by a single pathologist of less than, equal to, or greater than the control with regard to percentage positive cells, and to a lesser degree staining intensity. Overall, both PFN1 and TMSB4 were

more positive in tumors overall than normal epithelium based on staining intensity and percentage positive cells, $P < 0.001$, PFN1, and $P < 0.0194$, TMSB4. Interestingly, However, for late stage tumors PFN1 levels decreased and were more similar to normal tissue (Fig. 3). For TMSB4 there was a lack of significant difference for tumor stage (Fig. 3 and 4).

PFN1 variability is lost with cell culture

The results in figure 1 demonstrated that brush cytology samples from OSCC reveal high but variable mRNA levels for PFN1. When PFN1 mRNA levels were examined in head and neck SCC cell lines they in contrast demonstrated little variability. Also mRNA levels for PFN1 and PFN2 were on average no different than the one non-tumor control line tested (supplemental data).

Discussion

The analysis of protein expression in over 35 OSCC samples showed variability in PFN1 expression and TMSB4. In the case of PFN1 protein this variability was associated with tumor stage and the protein showed decreased expression in advanced tumors. This result was consistent with the large number of tumor types that similarly show decreases in PFN1 with tumor progression (7, 9, 11-13). However, the result was seemingly in contrast with that of Ma, et. al. who, based on cell line data (5 HNSCC lines and 1 normal cell line), showed minimal variation in PFN1 levels among the cell lines and speculated that there were no changes in the levels of this protein during tumor formation or progression (24). They concluded PFN1 was not likely to play a role in the HNSCC process and did not examine its level in tumor tissue (supplemental data). We found that there may be a lack of variable expression in PFN1 mRNA from OSCC cell lines though we found epithelial cells isolated directly from tumor without culturing showed over 20x fold variability (Fig. 1). Analysis of RNA from brush cytology allows one to assess *in vivo* expression of a near pure epithelial cell population without artificial gene expression changes that can occur in cell culture (25). Apparently OSCC maintained in culture are a poor model for monitoring PFN1 expression with this disease (supplemental figure). It is also notable that in their study of HNSCC, Ma et. al. focused on PFN2 and showed with immunohistochemistry (24) that the protein is reduced in late-stage HNSCC and because of this observation they proposed that for the PFN family, PFN2 plays the chief tumor suppressor role in OSCC. However, there are additional considerations: PFN1 and PFN2 are similar in sequence, and bind many of the same proteins, but typically PFN1 is at a higher concentration in non-neuronal cells. Despite their homology PFN1 and PFN2 can have opposing effects on cell migration in tumor cells (26, 27). In addition, because of the similarities in coding sequence for parts of PFN1 and PFN2, there is a potential for antibody cross-reactivity that requires careful selection of antibody. We used two different antibodies, one polyclonal to the carboxy half of PFN1 and the other specific to a small epitope predicted to be sufficiently different from PFN2 to reduce potential cross reactivity. This gives us confidence that our results reflect changes in PFN1 and not PFN2. Because we do not know the cross-reactivity of the antibody used by Ma et al. to recognize PFN1, at this point we would conclude from the two studies, that both PFN1 and PFN2 decrease in expression with advanced OSCC stage (Figs. 3 and 4) (24). To support our conclusion about PFN1 findings, Peng et al showed with another subtype of

HNSCC; an examination of 135 laryngeal tumor sections, that PFN1 reduction is associated with late tumor stage, $p = 0.031$, and a trend for enhanced lymph node invasion, $p = 0.057$ (28). We also note our result strengthens the premise of Peng's paper which was that actin binding proteins may play important roles in head and neck lymph node invasion. These findings agree with another report from HNSCC cell lines that induction of reduced PFN1 expression coincided with increased *in vitro* cell migration (29).

The majority of tumors we reviewed showed elevated expression of TMSB4 versus normal appearing tissue, consistent with its identification in other tumor types as an oncoprotein (Fig.1). We did not see any significant evidence for a change of expression with stage in tumor parenchyma, a finding consistent with other tumor types. As has been seen in other tumor types we also saw very high expression of TMSB4 in stromal cells. For this study, we focused on epithelium both in RNA and protein, and saw no evidence for, or against, a regulatory role for TMSB4 in OSCC aggressiveness (30).

Cytokeratins are well recognized to change in expression from high molecular weight to low molecular weight proteins in association with malignant keratinocyte transformation and loss of normal oral keratinocyte physiology. There are 54 cytokeratin genes that encode epithelial keratins, which are divided into acidic and basic types and are co-expressed during differentiation. KRT13 is more closely associated with normal oral mucosa and is progressively decreased in expression in OSCC as observed in our study (Fig. 4) (23). KRT13 has the potential to serve as a negative marker for oral malignancy. While we saw remarkable consistency in the loss of KRT13 in OSCC we note that this loss provided no information on tumor stage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

mRNA expression of PFN1, TMSB4, and ANXA1 from oral brush cytology samples of OSCC

RNA from brush cytology of OSCCs was subjected to qRT-PCR for the labeled mRNAs. Shown is the distribution of mRNA levels of PFN1, TMSB4, and ANXA1 in samples from OSCCs from different patients. All values are normalized to the expression of at least two housekeeping genes and the lowest value samples is given a value of one. ANXA1 serves as a negative control for variability.

Figure 2.

Photomicrograph of normal oral mucosa and expression of PFN1, TMSB4, and KRT13
Shown are micrographs of normal mucosa from tongue dorsum visualized by immunohistochemistry for the three proteins, PFN1, TMSB4, and KRT13, counterstained with hematoxylin. There is also an example of a control with hematoxylin and eosin staining.

Figure 3.

Photomicrographs of OSCC and expression of PFN1, TMSB4, and KRT13.

Shown are micrographs of sections of OSCCs, in this case all located at the lateral border of the tongue. Immunohistochemical visualization of the three proteins (PFN1, TMSB4, KRT13) is shown in representative tumor sections from the different tumor stages using the corresponding antibodies. The expression of PFN1 is lost and/or reduced in intensity with advancing stage of OSCC.

Figure 4.

Quantitation of protein levels in tumor sections according to tumor stage and presence of positive cervical lymph nodes.

A. Tumors were graded based on positivity of PFN1, and divided into two groups corresponding to PFN1 levels as described. Changes in protein level for lymph node negative versus positive tumors was statistically significant at $p < 0.016$, and for Stage I and II versus Stage III and IV tumors at $p < 0.018$ by the Fisher Exact Test.

B. As above except TMSB4 was measured and did not show a difference in the two groups.

Table 1

Clinical covariates for the subjects and OSCC PFN1 and TMSB4 levels

Covariates	PFN1 +/++	PFN1 +++	P-value ^a	TMSB4 +/++	TMSB4 +++	P-value ^a
Age, years, no.						
65	7	14	0.583	7	12	0.435
>65	5	9		6	7	
ND				1		
Gender, no.						
Male	10	13	0.195	11	10	0.122
Female	3	10		3	9	
Tobacco use, no.						
Yes	8	15	0.552	8	13	0.23
No	5	8		6	6	
Tobacco/Betel use, no.						
Yes	9	16	0.633	10	13	0.581
No	4	7		4	6	
Site, no.						
Tongue	3	4	0.318	5	2	0.336
Gingiva	6	3		2	3	
Buccal	2	6		2	5	
Floor of Mouth	2	1		0	1	
Other		1				
Tumor Grade/Diff.						
Well	5	10	0.482	5	7	0.574
Moderate	7	11		8	10	
Poor	1	1		1	1	
ND	0	1		0	0	

^aFishers exact test