Distribution of human PLUNC/BPI fold-containing (BPIF) proteins

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Abstract

Although gene expression studies have shown that human PLUNC (palate, lung and nasal epithelium clone) proteins are predominantly expressed in the upper airways, nose and mouth, and proteomic studies have indicated they are secreted into airway and nasal lining fluids and saliva, there is currently little information concerning the localization of human PLUNC proteins. Our studies have focused on the localization of three members of this protein family, namely SPLUNC1 (short PLUNC1), SPLUNC2 and LPLUNC1 (long PLUNC1). Western blotting has indicated that PLUNC proteins are highly glycosylated, whereas immunohistochemical analysis demonstrated distinct patterns of expression. For example, SPLUNC2 is expressed in serous cells of the major salivary glands and in minor mucosal glands, whereas SPLUNC1 is expressed in the mucous cells of these glands. LPLUNC1 is a product of a population of goblet cells in the airway epithelium and nasal passages and expressed in airway submucosal glands and minor glands of the oral and nasal cavities. SPLUNC1 is also found in the epithelium of the upper airways and nasal passages and in airway submucosal glands, but is not co-expressed with LPLUNC1. We suggest that this differential expression may be reflected in the function of individual PLUNC proteins.

As has been outlined elsewhere in this issue of Biochemical Society Transactions, the human PLUNC (palate, lung and nasal epithelium clone) family comprises nine genes located in a single locus on chromosome 20 [1]. The rapidly evolving nature of these genes has generated a significant diversification of PLUNC proteins within the eutherian lineage [1–4]. Although it is predicted that all PLUNC proteins are secreted [2,3], only a small number of human PLUNC proteins have been identified at the protein level, and an even smaller number of these proteins have been studied in any detail. Gene expression data suggest that PLUNC proteins exhibit very limited expression profiles. Owing to shared structural similarities, it was hypothesized that all family members would have similar functions [2,3]. This may not be the case, and it may also come to light that the function also differs across species. In the present review, the expression profiles of human SPLUNC1 (short PLUNC1), SPLUNC2 and LPLUNC1 (long PLUNC1) are discussed, as are their association with cancer, both as potential biomarkers and in tumorigenesis. As set out in detail elsewhere, our updated bioinformatic analysis of PLUNC family members has led us to propose a new phylogenetically based nomenclature whereby all family members are renamed using the root BPIF for ‘BPI (bactericidal/permeability-increasing protein) fold-containing’. In this systematic nomenclature, single-domain-containing family members have the designation BPIFA and two-domain-containing family members have the designation BPIFB [4]. For the sake of simplicity, in the present review, we will continue to refer to these proteins using previously used names.

SPLUNC1 (BPIFA1)

SPLUNC1 has been studied most extensively in comparison with the other family members, both in vitro, in primary airway cells [5–7] and in cell lines [8], and in vivo, in normal and diseased tissues [6,7,9].

The first description of mouse Splunc1 identified an extremely restricted expression profile limited to the palate, nasal cavity and upper respiratory tract [10]. We now know that, in humans, the protein is expressed in the epithelium of the upper airways, where it coats the surface of the epithelium and cilia, but significantly greater expression is seen in the submucosal cells and ducts of glands associated with the upper airways [6,9]. Cells of the normal peripheral lung do not produce SPLUNC1. The protein is also found in minor glands of the nose, sinus, posterior tongue and tonsil [9]. This suggests an association between SPLUNC1 production and mucous cells and this is further confirmed by the pattern of expression in major salivary glands where the protein is only produced in glands of a mixed or mainly mucous cell type (submandibular and sublingual), but not by the serous cells of the parotid gland [11]. However, despite being located in glandular mucous cells, SPLUNC1 expression is not seen...
in goblet cells in the airways; rather, the protein is located in non-ciliated non-goblet epithelial cells in these regions [9]. Recent studies in the chinchilla have indicated that SPLUNC1 is also a product of the middle ear, although the exact location, or type of cell, has not been elucidated [12]. A number of proteomic studies have confirmed the presence of a secreted form of SPLUNC1 in nasal lavage fluid, saliva, airway lining fluid and middle-ear effusions [13–16], thus confirming the immunohistochemical expression data.

SPLUNC1 has very limited expression in cell lines; however, protein expression and secretion, from some primary nasal and upper respiratory tract epithelial cells, appear to be very much dependent on the differentiation status of the cell population. In these cells, it is one of the most abundantly secreted protein products [6]. Array data have shown similar significant increases in expression associated with differentiation status [17]. If the cultures are allowed to dedifferentiate (by withdrawal of growth factors), then expression of SPLUNC1 is lost [7]. Modulation of expression of SPLUNC1 protein in nasal polyp epithelial cells in air/liquid interface cultures has also been reported [18].

Expression of SPLUNC1 has been determined in a number of inflammatory and/or infectious disorders. In end-stage CF (cystic fibrosis), expression was significantly elevated in diseased airways, but was not co-localized with MUC5AC, indicating that goblet cells were not the source of protein [7]. It was also noted that, unlike the small airways of normal lungs, where SPLUNC1 is not detected, the small airways of CF-affected lungs showed significant expression. However, SPLUNC1 is not detected in either normal or CF-affected alveolar epithelium. As the lungs studied were from end-stage disease, a number of larger airways were occluded with mucus plugs, which showed strong SPLUNC1 expression. Although the mucus plugs contained a significant number of inflammatory cells, SPLUNC1 was not associated with them, as co-localization with neutrophil elastase or CD68 could not be demonstrated [6]. This conflicts with a study by Bartlett et al. [19] in which specific granules of neutrophils were shown to contain and secrete SPLUNC1, possibly in response to inflammatory stimuli. The reason for these differences is unclear, but as our studies failed to show SPLUNC1 in peripheral blood-derived neutrophils [7], it is possible that expression is induced locally in extravasated cells.

Elevated SPLUNC1 gene expression has been identified in a large number of array-based studies [20], but has not been highlighted in many of these, perhaps due to lack of suitable antibody reagents. In one such array-based study aimed at identifying molecular biomarkers, gene expression levels were compared in patients with stable (slowly progressive) IPF (idiopathic pulmonary fibrosis) and those with progressive disease were compared [21]. SPLUNC1 mRNA levels were higher in the progressive group and this was confirmed by immunohistochemistry whereby increased SPLUNC1 was seen in bronchial columnar cells and noted in the mucus-filled cystic spaces. Interestingly, this paper confirmed the lack of SPLUNC1 in bronchial tissue of normal lungs.

Levels of secreted SPLUNC1 have been determined in nasal lavage fluid from subjects exposed to environmental or industrial pollutants, who are cigarette smokers or who suffer from allergies [22–24]. All of these have shown that irritation and inflammation of the nasal cavity decrease SPLUNC1 production. Exposure to chemical irritants reduced overall levels of SPLUNC1 and, although this was also seen in cigarette smokers, it was apparent that novel isoforms of the protein were also being expressed [23]. In patients with seasonal allergic rhinitis, protein levels appear normal, or similar to control subjects, until the allergy season, when they decrease and different isoforms are expressed with a significant reduction in sialylated forms [22]. SPLUNC1 levels in whole saliva from patients with oral lichen planus were lower than those found in normal controls, suggesting that SPLUNC1 could be a novel biomarker for oral lichen planus [25].

**LPLUNC1 (BPIFB1)**

Although LPLUNC1 has not been studied as extensively as SPLUNC1, it is clear that their expression profiles are very similar with regard to site of expression and secretion into nasal fluid, airway lining fluid and saliva [14,15,26]. We have shown that LPLUNC1 is found in the trachea, the epithelium and submucosal glands of larger airways and some smaller airways, but is absent from the alveolar regions of the lung [27]. It is also found in minor mucosal glands of the nasal cavity and more abundantly in minor, compared with major, salivary glands. Within the respiratory tract (including nasal and oral cavities), cells do not generally express both proteins. For example, LPLUNC1 is a product of a population of goblet cells in the nasal passage and airway epithelium, whereas SPLUNC1 is not detected in these cells [27]. Serous demilunes in airway submucosal glands stain positively for LPLUNC1 expression, but not SPLUNC1, while mucous cells within the same gland will express SPLUNC1, but not LPLUNC1. LPLUNC1 has been shown to be localized to the ducts of submandibular glands, but is not seen in the parotid gland [10]. LPLUNC1 from nasal secretions, airway lining fluid, sputum, saliva and differentiated TBE (tracheobronchial epithelial) cells in *vitro* presents as a number of glycosylated forms, a further similarity with SPLUNC1 [27–29]. The amount of LPLUNC1 secreted by the TBE cells increases as cells differentiate, and proteolytically cleaved products have not been reported in either TBE secretions or airway lining fluid, from normal healthy subjects [27].

Currently, there are very limited published data on differential expression of *LPLUNC1* in human disease. As with SPLUNC1, the gene appears as differentially expressed in many array datasets, but has not been studied in detail [20]. However, a significant association between a specific *LPLUNC1* SNP (single nucleotide polymorphism), found in the promoter region of the gene, and susceptibility to infection by the cholera-causing bacterium *Vibrio cholerae* has recently been identified [30]. Interestingly, the variant
is not found in a conserved region of the genome across mammals, and a further haplotype more significantly associated with disease than the variant itself was noted. The significance of these results is yet to be elucidated, but this genetic study was instigated after a previous study showed that LPLUNC1 gene expression was 7-fold higher in the duodenal mucosa of patients in the acute phase of cholera infection than those in the convalescent phase [31]. Clearly, further studies are needed to elucidate any role for LPLUNC1 in susceptibility to infection and/or development of disease.

SPLUNC2 (BPIFA2)

The expression pattern of SPLUNC2 is even more restricted than that of other family members, as it is only found in the oral cavity. No expression has been noted in the respiratory tract or nasal cavity. As highlighted previously, SPLUNC2 and related proteins, including rodent PSP (parotid secretory protein), show the greatest diversity across the PLUNC family [1], and this suggests that extrapolation of studies from one species to another may be difficult. Taking this into consideration, the pattern, or site of expression, across species is somewhat similar, as the rodent, bovine and human proteins are all found in both major and minor salivary glands [11,32–34]. In human salivary glands, serous cells produce SPLUNC2, and thus there is considerable expression in the parotid gland. In the submandibular and sublingual glands, it is again a population of serous cells which secrete SPLUNC2, in direct contrast with the secretion of SPLUNC1 from the mucous cells in these glands. The functional significance of this has yet to be elucidated, as both proteins are secreted into saliva. SPLUNC2 is also expressed in some minor mucosal glands of the oral cavity and, in some instances, it may be co-expressed with SPLUNC1 and LPLUNC1 [34].

A number of independent proteomic studies have identified SPLUNC2 in whole saliva [14,28,35], in minor gland saliva [36] and as a component of acquired pellicle [37]. It is also apparent that, as with other family members, SPLUNC2 is a heavily glycosylated protein [28,34]. SPLUNC2 is differentially secreted into saliva by the three major salivary glands [35], and it may be that each gland contributes proteins with distinct glycosylation.

SPLUNC2 levels in saliva from diseased individuals have not been studied extensively, but examples of differential production are emerging. For example, it appears that SPLUNC2 is reduced in the saliva of patients with periodontitis [38], and we have recently shown a significant difference in the expression of SPLUNC2 in HIV patients who were also infected with either CMV (cytomegalovirus) or with mycobacteria [39]. In particular, increased expression of SPLUNC2 was noted around the viral lesions within all major salivary glands. Again, the functional significance of this remains unclear, and future work is clearly needed to determine whether differences in tissue expression translate into altered salivary levels of the protein.

PLUNC proteins and cancer

The role of PLUNC proteins in the development and progression of cancer has been studied in nasopharyngeal carcinomas, and their potential use as diagnostic markers has been investigated in NSCLCs (non-small cell lung carcinomas) [9] and salivary gland tumours [11]. With the exception of our study in salivary gland tumours [11], all other studies have focused solely on the founder member of the family, SPLUNC1, and all but one of these has only investigated gene expression.

Iwao et al. [40] first described an association between PLUNC mRNA expression and cancer. Their novel lung-specific gene, LUNX (lung-specific X protein), which is in fact SPLUNC1, showed increased mRNA levels in 84% of the NSCLCs examined. This study also suggested that SPLUNC1 mRNA could provide a novel diagnostic tool for the detection of micrometastases in the lymph nodes. Benlloch et al. [41] assessed SPLUNC1 and a second marker, CEACAM5, based on their expression in primary NSCLCs, but not in benign lymph nodes [41]. Then 16% of pathologically negative lymph nodes were found to be positive for SPLUNC1, indicating that there is greater precision in detecting metastases if a molecular marker such as SPLUNC1 is used. They also found that the survival rate of patients was poorer if extra-thoracic lymph nodes were positive for either CEACAM5 or SPLUNC1.

Mitias et al. [42] examined the gene expression of a panel of markers in NSCLC tumour cells circulating in the peripheral blood and found that SPLUNC1 was the most sensitive marker in detecting these cells. A similar study by Cheng et al. [43] showed SPLUNC1 as the only marker in their panel able to discriminate between NSCLCs and other epithelial tumours and also to be a sensitive marker in both peripheral blood cells, correlating with pathological stage and pleural effusions. In a further study, SPLUNC1 apparently had a small tendency to be overexpressed in tumour cells compared with normal samples, but was suggested to have a greater potential as a biomarker for adenocarcinomas than for all NSCLCs [44]. We examined the expression of SPLUNC1 in a range of lung cancers and showed that the protein was expressed in adenocarcinoma, mucoclapidermoid carcinoma and bronchusalveolar carcinoma [8]. Our own studies do not show a similar difference in LPLUNC1 expression (L. Bingle and C.D. Bingle, unpublished work). The prognostic and diagnostic significance of this remains unclear.

SPLUNC1 has also been suggested to be a useful marker for hepatoid adenocarcinoma of the stomach following bioinformatic analysis of SAGE (serial analysis of gene expression) and microarray datasets from gastric cancer cases [45]. The protein was focally distributed in 7% of gastric adenocarcinomas and, more importantly, it was shown that 100% (eight of eight) of cases of hepatoid adenocarcinoma were strongly positive for SPLUNC1, suggesting that this may be a useful diagnostic tool for this disease [46].

We have studied the expression of a number of family members in a range of tumour types arising in salivary
The majority of tumours investigated did not show any PLUNC expression; however, in mucopideroid carcinomas, strong expression of LPLUNC1 was detected in mucous cells and mucus plugs, and both LPLUNC1 and SPLUNC2 were detected in intermediate cells. In one case of a papillary cystadenoacinar carcinoma, all PLUNC proteins analysed (LPLUNC1, SPLUNC1 and SPLUNC2) stained positively, but the intensity of staining varied; LPLUNC1 and SPLUNC1 being stronger than SPLUNC2. In this limited group of cases, there was no correlation in any of the positive samples with tumour grade, size, age, gender or site [11].

The search for biomarkers does not require any understanding of the biological function or regulatory mechanisms of candidates, neither are such investigations likely to provide this information. The effectiveness of SPLUNC1 as a biomarker for NSCLCs is not clear-cut, and it is entirely possible that, as is almost certainly the case in the salivary gland tumours, expression reflects the predominant cell type found within a particular tumour at the time of testing.

The other aspect of cancer biology with which PLUNC proteins have been shown to be associated is in the development of NPC (nasopharyngeal carcinoma). NPC shows a definite racial and geographic distribution, with the highest incidence being found in people from southern China. The first study to establish a link between PLUNC gene expression and NPC showed that both SPLUNC1 and LPLUNC1 were down-regulated [46]. A further genetic study identified eight SNPs in the PLUNC gene and could show that two of these, found in the promoter region, showed a significant association with susceptibility to disease [47]. EBV (Epstein–Barr virus) is an established risk factor for the development of NPC, and it has been demonstrated that SPLUNC1 can inhibit the potential oncogenicity of the virus [48]. More recently, the regulation of NPC development by PLUNC has not been investigated beyond the initial description of a down-regulation of gene expression.

**Funding**

This work is funded by The Wellcome Trust and the Sheffield Hospitals Charitable Trust.

**References**


Received 27 April 2011
doi:10.1042/BST0391023

Proteins with a BPI/LBP/PLUNC-Like Domain: Revisiting the Old and Characterizing the New 1027