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SHORT COMMUNICATION

BSND is a Novel Immunohistochemical Marker for Oncocytic Salivary Gland Tumors

Kazuya Shinmura[1] · **Hisami Kato**[1] · **Yuichi Kawanishi**[2] · **Takaharu Kamo**[1] · **Yusuke Inoue**[1] · **Katsuhiro Yoshimura**[1] · **Kenta Sugiyama**[1] · **Kiyoshi Misawa**[3] · **Seiji Hosokawa**[3] · **Hiroyuki Mineta**[3] · **Haruhiko Sugimura**[1]

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Abstract BSND protein, which is involved in chloride transport, is expressed in normal kidney and the inner ear and is known as an immunohistochemical marker for chromophobe renal cell carcinoma (RCC) and renal oncocytoma; however, other organs and tumor types exhibiting BSND expression have not yet been reported. In this study, we investigated the expression of BSND using data from the Cancer Genome Atlas (TCGA) database and by performing immunohistochemical analyses. As a result, we found that BSND was also expressed in the striated duct cells of normal salivary glands. Next, BSND expression was examined immunohistochemically in 7 types of salivary gland tumors, and BSND positivity was found in Warthin's tumor (25 out of 25 cases; 100%) and oncocytoma (4/4; 100%), both of which are usually classified as oncocytic tumors, whereas BSND negativity was observed for pleomorphic adenoma (0/11), adenoid cystic carcinoma (0/7), acinic cell carcinoma (0/6), mucoepidermoid carcinoma (0/6), and salivary duct carcinoma (0/5). Finally, the expression of BSND mRNA in 30 types of tumors other than chromophobe RCC and salivary gland tumors was examined using data from the TCGA database, but none of these tumors exhibited BSND expression. These results suggest that BSND is expressed only in normal salivary glands and oncocytic salivary gland tumors such as Warthin's tumor and oncocytoma in addition to the two known organs and the two known renal tumor types mentioned above. The selective expression pattern of BSND suggests that BSND is an excellent novel immunohistochemical marker for oncocytic salivary gland tumors.

Keywords BSND · Immunohistochemical marker · Oncocytic salivary gland tumor · Oncocytoma · Warthin's tumor

Introduction

BSND is a protein encoding the β-subunit of ClC-K chloride channels, which play a pivotal role in chloride transport in the normal kidney and inner ear [1], and *BSND* germline mutations are known to cause Bartter syndrome type IV, which is a hereditary disease characterized by salt loss, metabolic alkalosis, hypokalemia, and sensorineural deafness [2]. Very recently, we reported that BSND is an immunohistochemical marker for differentiating between chromophobe renal cell carcinoma (RCC) and other subtypes of RCC (clear cell and papillary RCCs) and that it is also expressed in renal oncocytoma [3]. However, other organs and tumor types exhibiting BSND expression have not been reported to date.

Tumors in major salivary gland are relatively uncommon, and according to the WHO classification, 10 benign epithelial tumors (such as pleomorphic adenoma and Warthin's tumor) and 24 malignant epithelial tumors (such as adenoid cystic carcinoma, acinic cell carcinoma, and mucoepidermoid carcinoma) exist [4]. The pathological diagnosis of these tumors is

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✉ Kazuya Shinmura
kzshinmu@hama-med.ac.jp

[1] Department of Tumor Pathology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi Ward, Hamamatsu 431-3192, Japan

[2] Advanced Research Facilities and Services, Preeminent Medical Photonics Education and Research Center, Hamamatsu University School of Medicine, Hamamatsu, Japan

[3] Department of Otolaryngology/Head and Neck Surgery, Hamamatsu University School of Medicine, Hamamatsu, Japan

occasionally difficult because of the diverse histological features present in each tumor as well as the presence of a number of types and variants. Although hematoxylin-eosin (HE) staining is the golden standard method for diagnosis, immunohistochemistry can increase the accuracy and be helpful when a pathological diagnosis based on a routine examination of HE-stained sections is difficult. Unfortunately, the availability of salivary gland tumor type-specific markers is limited [5]. Thus, the identification of novel immunohistochemical markers for the diagnosis of salivary gland tumors is important. In this study, we searched for organs expressing BSND; since we found that BSND is expressed in salivary glands, we next investigated whether BSND could be used as an immunohistochemical marker of salivary gland tumors.

Materials and Methods

Collection of Publicly Available TCGA Data

Gene expression data for 31 tumor types (Supplementary Table S1) were collected from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/) in February 2016. Data composing a total of 692 non-tumorous tissues and a total of 9135 tumorous tissues were used in this study. The expression data were obtained as processed RNA-seq data in the form of RNA-seq by Expectation Maximization (RSEM). The RSEM expression value for the BSND gene was divided by that of the TBP gene, which is a control housekeeping gene, to compare the expression levels.

Paraffin-Embedded Tissues

Paraffin embedded blocks of salivary gland tumors consisting 25 Warthin's tumors, 11 pleomorphic adenomas, 7 adenoid cystic carcinomas, 6 acinic cell carcinomas, 6 mucoepidermoid carcinomas, 5 salivary duct carcinomas, and 4 oncocytomas as well as 30 non-tumorous human organs were obtained from Hamamatsu University Hospital (Japan). The use of these tissues was approved by the Institutional Review Board of Hamamatsu University School of Medicine.

Immunohistochemical Staining

Sections of paraffin blocks were used for immunohistochemical staining using an automatic immunohistochemical stainer, the HISTOSTAINER (Nichirei Bioscience, Tokyo, Japan). Briefly, the sections were incubated with a rabbit anti-BSND polyclonal antibody (1:1000; Sigma-Aldrich, St. Louis, MO, USA) for 30 min at room temperature (RT). After washing, the sections were incubated for 30 min at RT with an amino acid polymer conjugated with goat anti-rabbit IgG and horseradish peroxidase (Histofine Simple Stain MAX-PO Kit; Nichirei, Tokyo, Japan). The antigen-antibody complex was visualized using 3,3′-diaminobenzidine tetrahydrochloride, and the sections were counterstained with hematoxylin. If ≥10% of the tumor cells were immunostained in the cytoplasmic membrane or cytoplasm, the tumor was judged to be positive for BSND immunostaining.

Results

To search for tissues positive for BSND expression, we utilized RNA-seq mRNA expression data from the TCGA database. Among 15 organs (urinary bladder, breast, uterine cervix, bile duct, colorectum, esophagus, head and neck, kidney, liver, lung, pancreas, prostate, stomach, thyroid gland, and uterine corpus) that were examined, the 95th percentile mRNA BSND expression value divided by the expression value of TBP, a control housekeeping gene, was high (4.8) in kidney, but was low (8.9×10^{-4} to 3.4×10^{-1}) in the other 14 organs (Table 1). A high level of BSND expression in the kidney is compatible with our previous experiment, in which strong BSND expression was immunohistochemically detected in the thin limb and thick ascending limb of the loop of Henle, the distal convoluted tubule, and the collecting duct [3]. We next performed an immunohistochemical analysis to search for tissues that were positive for BSND protein expression. Among 30 organs (skin, tongue, tonsil, parotid gland, submandibular gland, esophagus, liver, gallbladder, pancreas, spleen, lung, trachea, adrenal gland, prostate, urinary bladder, testis, thyroid gland, breast, uterine cervix, uterine corpus, Fallopian tube, ovary, heart, aorta, lymph node, cerebrum, cerebellum, spinal cord, terminal filum, and vertebra), only the parotid gland and submandibular gland were positive for BSND protein (Fig. 1). In both salivary glands, striated duct cells, but not acinar cells, myoepithelial cells, or basal cells, were positive for BSND protein expression, and the protein was localized predominantly in the cytoplasmic membrane. These results suggested that BSND is expressed specifically in salivary glands, in addition to the kidney.

Based on our finding that BSND is expressed in salivary glands, we suspected that BSND might also be expressed in some types of salivary gland tumors. If so, BSND could be useful as an immunohistochemical marker for salivary gland tumors. Thus, we investigated the expression of BSND in various types of salivary gland tumors using an immunohistochemical analysis with anti-BSND antibody. The analysis of 64 salivary gland tumors in total revealed that BSND was immunohistochemically positive in Warthin's tumor (25/25; 100%) and oncocytoma (4/4; 100%), both of which are usually classified as oncocytic tumors, but negative in pleomorphic adenoma (0/11; 0%), adenoid cystic carcinoma (0/7; 0%), acinic cell carcinoma (0/6; 0%), mucoepidermoid carcinoma (0/6; 0%), and salivary duct carcinoma (0/5; 0%), all

Table 1 Ninety-fifth percentile of mRNA BSND expression values in various organs using data from the TCGA database

Organ	TCGA ID	No. of cases[a]	95th percentile expression value[b]
Urinary bladder	BLCA	19	0.002313855
Breast	BRCA	113	0.006162399
Uterine cervix	CESC	3	0.000888361
Bile duct	CHOL	9	0.003278632
Colorectum	COAD/READ	41	0.002462938
Esophagus	ESCA	11	0.002861848
Head and neck	HNSC	44	0.342610468
Kidney	KIRC/KICH/KIRP	129	4.787861972
Liver	LIHC	50	0.004669788
Lung	LUAD/LUSC	110	0.004451656
Pancreas	PAAD	4	0.005497353
Prostate	PRAD	52	0.059005434
Stomach	STAD	35	0.002676943
Thyroid gland	THCA	59	0.001211277
Uterine corpus	UCEC	13	0.014904746

[a] If the number of cases in a given organ was ≥3, the cases were included in this table

[b] The RSEM expression value of the BSND gene was divided by that of the TBP gene

of which are usually classified as non-oncocytic tumors (Table 2, Fig. 2). In the Warthin's tumors, BSND expression was found in the epithelial tissues but not in the lymphoid stroma (Fig 2a-c), while in oncocytoma, BSND expression was diffusely recognized in the tumorous portion (Fig. 2d-f). The sensitivity of the immunohistochemical detection of BSND expression for the diagnosis of oncocytic tumors was 100%, and the specificity was 100%. These results suggest that BSND is specifically expressed in oncocytic tumors, such as Warthin's tumor and oncocytoma, among salivary gland tumors.

To determine the expression status of BSND in tumors other than salivary gland tumors and renal tumors, we examined the RNA-seq mRNA expression data of 31 tumor types using the TCGA database. The 95th percentile of the mRNA BSND expression value was extremely low (<0.16) in 30 tumor types, when compared with the high value (14.7) observed in chromophobe RCC (Supplementary Table S2). These results suggest that BSND is a specific marker of a subset of renal tumors and salivary gland tumors.

Discussion

Our immunohistochemical analysis and data analysis using the TCGA database revealed for the first time that BSND was expressed in normal salivary glands and oncocytic salivary gland tumors such as Warthin's tumor and oncocytoma. In normal salivary glands, striated duct cells were positive for BSND expression, while in the tumor epithelial component of Warthin's tumor and all the components of oncocytoma were BSND-positive. Since normal striated duct cells, the epithelial component of Warthin's tumor, and oncocytoma cells are all known to have eosinophilic cytoplasm and abundant mitochondria [6], the phenotypes shared among these kinds of cells but not among other kinds of tumors might partly explain why BSND was positive only in Warthin's tumor and oncocytoma, among the salivary gland tumors that were examined. Regarding this point, since the physiological function of BSND protein is to play an important role in chloride transport [1], we speculated that the quantity of mitochondria in these cells was not directly related with the expression of BSND in those tissues. From the standpoint of a practical pathological diagnosis, mitochondrial antigen is known as an immunohistochemical marker for normal striated duct cells, Warthin's tumor, and oncocytoma [7], but mitochondrial antigen is also expressed in mucoepidermoid carcinoma, which is a non-oncocytic salivary gland tumor [8]. Therefore, as far as we know, BSND is the sole oncocytic salivary gland tumors-specific immunohistochemical marker. Because of the characteristic morphology of Warthin's tumor and oncocytoma, other lesions are not usually considered in differential diagnosis; however, diagnosis can be difficult under certain circumstances such as (1) the presence of secondary reactive changes in Warthin's tumors; (2) the presence of abundant lymphoid tissue in other epithelial lesions; or (3) the presence of squamous metaplasia in Warthin's tumors or clear cell change in oncocytomas [6]. Regarding this point, the

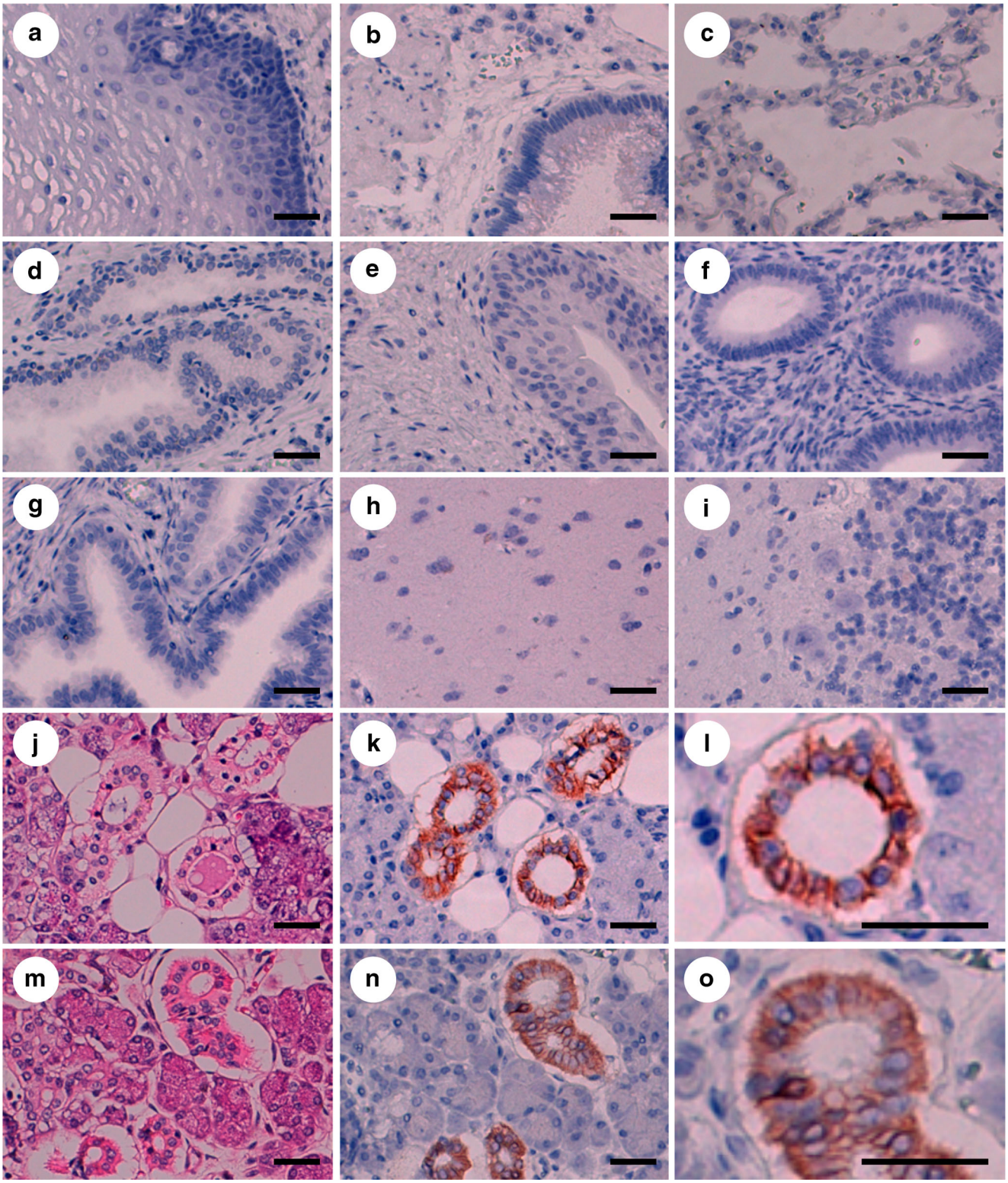

Fig. 1 Immunohistochemical results for BSND in various human organs. Negative immunohistochemical staining for BSND was observed in the esophagus (**a**), gallbladder (**b**), lung (**c**), prostate (**d**), urinary bladder (**e**), uterine corpus (**f**), Fallopian tube (**g**), cerebrum (**h**), and cerebellum (**i**), while positive immunohistochemical staining was observed in the parotid gland (**j**-**l**) and submandibular gland (**m**-**o**). (**l**) and (**o**) are higher magnifications of sections shown in (**k**) and (**n**), respectively. Hematoxylin and eosin stain; (**j**) and (**m**). Scale bar = 40 μm

percentage of the cells showing BSND positivity was low in squamous metaplastic lesions of Warthin's tumor cases, however the other epithelial lesions of the Warthin's tumor cases showed sufficient BSND expression (Supplementary

Table 2 Immunohistochemical results for BSND protein in a total of 64 salivary gland tumors

Tumor	No. of cases	Age (average ± SD)	Sex (M/F)	Positivity of BSND expression (%)
Warthin's tumor	25	64.3 ± 9.1	22/3	25/25 (100)
Pleomorphic adenoma	11	52.6 ± 10.5	4/7	0/11 (0)
Adenoid cystic carcinoma	7	58.4 ± 10.0	3/4	0/7 (0)
Acinic cell carcinoma	6	72.2 ± 13.8	3/3	0/6 (0)
Mucoepidermoid carcinoma	6	66.2 ± 12.6	3/3	0/6 (0)
Salivary duct carcinoma	5	71.8 ± 8.0	5/0	0/5 (0)
Oncocytoma	4	76.0 ± 6.2	1/3	4/4 (100)

SD standard deviation

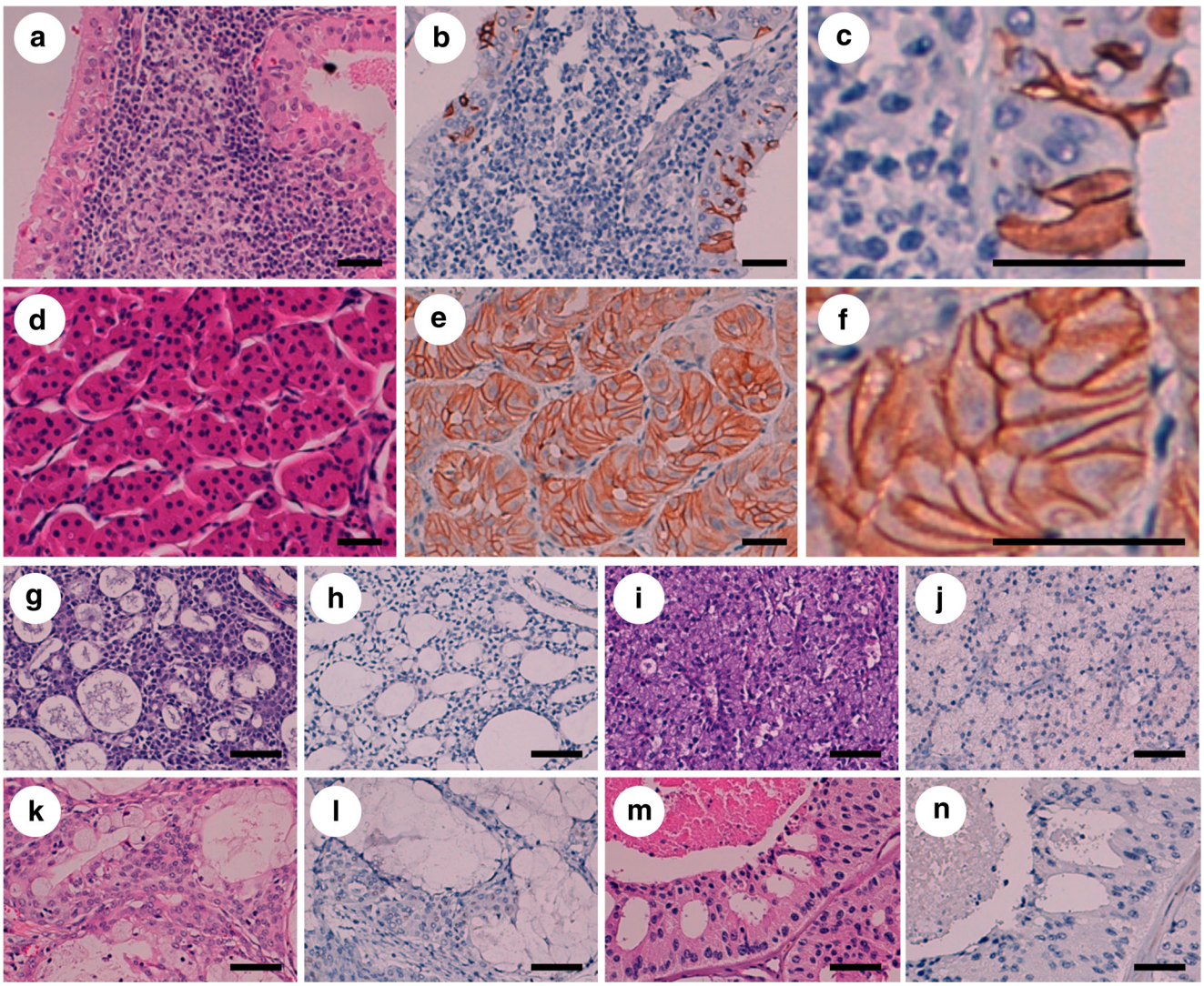

Fig. 2 Immunohistochemical results for BSND in salivary gland tumors. Positive immunohistochemical staining for BSND was observed in Warthin's tumor (**a**-**c**) and oncocytoma (**d**-**f**), while negative immunohistochemical staining was observed in adenoid cystic carcinoma (**g** and **h**), acinic cell carcinoma (**i** and **j**), mucoepidermoid carcinoma (**k** and **l**), and salivary duct carcinoma (**m** and **n**). (**c**) and (**f**) are higher magnifications of sections shown in (**b**) and (**e**), respectively. Hematoxylin and eosin stain: (**a**), (**d**), (**g**), (**i**), (**k**), and (**m**). Scale bar = 40 μm

Fig. S1). Thus, immunohistochemistry for BSND might be helpful for pathology studies.

Clinically, Warthin's tumor or oncocytoma are very slowly growing tumors with no symptoms. Thus, a definitive diagnosis of these tumors is important for the appropriate management of patients who do not require or are unable to undergo surgical excision. Among salivary gland tumors, only Warthin's tumor and oncocytoma manifest an accumulation of radioactivity during ^{99m}Tc-pertechnetate scintigraphy. The mechanism of ^{99m}Tc-pertechnetate accumulation in these tumors is considered to be due to the eosinophilic cytoplasm of the epithelial cells contained within these tumors [9]. We speculated that some similarities might exist between BSND and proteins involved in ^{99m}Tc-pertechnetate accumulation in eosinophilic cells. These results suggest that these proteins might be useful as therapeutic or diagnostic targets for the treatment of these tumors.

Our previous study [3] and present study revealed that BSND is expressed only in chromophobe RCCs, renal and salivary gland oncocytomas, and Warthin's tumors, but not in 5 other types of salivary gland tumors or 30 types of tumors derived from various organs. This means that the BSND marker is tumor-type specific in a manner similar to that of TTF-1, which is an excellent immunohistochemical marker used worldwide in pathological diagnosis. Thus, we believe that the application of BSND immunohistochemistry might be meaningful to the practical pathological diagnosis of kidney and salivary gland tumors.

The *BSND* gene is a responsible for Bartter syndrome type IV, and the renal abnormalities and sensorineural deafness seen in patients with a *BSND* germline mutation are considered to be attributable to a reduction in BSND activity arising from the effects of the *BSND* mutation in kidney and inner ear [1, 2]. Although other BSND-expressing human organs have not been previously reported, the present study found that the striated duct cells of salivary glands were also BSND-positive. Therefore, if the salivary glands of patients with Bartter syndrome type IV were to be investigated in a more detailed fashion, as yet unidentified abnormalities of the salivary glands might be found in such patients.

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