—Original Article—

Immunohistopathological properties of pleomorphic adenoma in salivary gland

Toshikazu Hirano^{1,2)}, Izumi Kashiwado¹⁾, Isao Suzuki¹⁾, Takamasa Yoshihiro^{1,2)},
Kurata Yuge¹⁾ and Goro Asano²⁾

¹⁾Department of Otorhinolaryngology and ²⁾Department of Pathology,
Nippon Medical School

Summary

Fifty-eight salivary gland pleomorphic adenomas and 5 normal salivary glands were studied immunohistochemically with respect to intermediate filaments (keratin, desmin, and vimentin), actin and S-100 protein to observe the cellular differentiation of these tumor cells. Normal myoepithelial cells showed positive immunostaining for actin, vimentin and S-100 protein. Pleomorphic adenomas expressed keratin, vimentin and S-100 protein to various degrees, but only a few tumor cells of pleomorphic adenoma revealed actin. The results indicate that the tumor cells of pleomorphic adenoma show a bipolar differentiation capability of both epithelial and mesenchymal origins, although normal myoepithelial cells show only mesenchymal characteristics. The findings also support previous reports using light and electron microscopy, and also contribute to more precise diagnosis and a better understanding of the histogenesis of this tumor.

Key words: pleomorphic adenoma, immunohistochemistry, intermediate filaments, actin, cell differentiation

Introduction

Several salivary gland tumors have been known for their unique histological features. This is based on the facts that these tumors histogenitically possess both ectodermal and mesenchymal characteristics. In an attempt to explain these features, many studies have recently been performed using light microscope, electron microscope¹⁻⁶⁾, and immunohistochemical techniques⁷⁻¹¹⁾. According to these studies, myoepithelial cells play an important role in the histogenesis of some salivary gland tumors^{8,9,12,13)}, but some controversial points still remain to be clarified.

Recently, several studies have shown that the localization of intermediate filaments could be used to identify the histogenic origin of tumors¹⁴⁻¹⁶⁾, because tumors continue to express the major intermediate filament types that show the characteristics of the cell of origin. The analysis of the intermediate filaments might provide a better understanding of cellular differentiation and histogenesis of the tumors. Therefore, in the present study, normal salivary glands as well as pleomorphic adenomas were examined for the expression of their intermediate filaments (keratin, vimentin, and desmin), actin and S-100 protein, using an immunohistochemical technique. The

Correspondence to Toshikazu Hirano, Department of Otorhinolaryngology, Nippon Medical School First Hospital, 3-5-5, Iidabashi, Chiyoda-ku, Tokyo, 102 Japan

results are compared in this paper with the morphological findings and a discussion is presented of the unusual histological complexities and the histogenesis of pleomorphic adenoma.

Materials and Methods

(1) Tissue specimens

Primary tumor tissues from fifty-eight patients with pleomorphic adenoma of the salivary gland were obtained from the files of Nippon Medical School. In addition, tissues from five submandibular glands were used as controls. The tissues were fixed in 10% buffered formol-saline and embedded in paraffin. Sections of 2.5 μm thickness were stained with hematoxylin and eosin for routine morphological study.

(2) Immunohistochemistry

An avidin-biotinylated-peroxidase complex (ABC) method was used for the immunohistochemical study. Deparaffinized sections of 2.5 μm thickness were soaked in methanol containing 0.3% hydrogen peroxide for 30 minutes at room temperature in order to block endogenous peroxidase activity. After washing three times with PBS (phosphate-buffered saline; 0.15 M NaCl in 0.01 M phosphate buffer; pH 7.2), the sections were incubated for 10 minutes with either 5% normal goat serum (Dakopatts) for polyclonal antibodies, or 5% normal horse serum (Dakopatts) for monoclonal antibodies. After washing three times with PBS, they were reacted with each antibody at the following dilutions: actin 1/50 (polyclonal antibody) (Biomedical Technologies Inc.), vimentin 1/100 (monoclonal antibody) (Dakopatts), S-100 protein 1/250 (polyclonal antibody) (Dakopatts), keratin 1/300 (polyclonal antibody) (Dakopatts), desmin 1/100 (monoclonal antibody) (Dakopatts). Preincubation with 0.1% protease was performed for 10 minutes prior to immunohistochemical staining for desmin, at room temperature. After washing with PBS, the sections were reacted with biotin-labeled anti-rabbit gamma globulin (Dakopatts) for polyclonal antibodies and biotin-labeled anti-mouse globulin (Dakopatts) for monoclonal antibodies for 30 minutes. They were then washed with PBS again, with avidin-biotinylated peroxidase complex (ABC) reagent being applied to the sections for 30 minutes in a moist chamber at room temperature. After washing with PBS, they were finally soaked in DAB- H_2O_2 reagent (0.02% 3,3-diaminobenzidine tetrahydrochloride; 0.005% H_2O_2 ; 0.05 M TRIS buffer; pH 7.6), which produced a brown reaction product, and counterstained with Mayer's hematoxylin.

Individual sections were histologically classified according to degree of immunoreactivity on a scale of no stain to varying intensities of stainings (slight, moderate, and intensive). Immunostaining of control sections was performed by incubation of sections with non-immune rabbit or mouse serum, instead of the primary antibody.

Results

Pleomorphic adenoma is composed of several different types of histological structures. The authors have classified these tumor tissues light microscopically as being composed of duct lining cells, cells resembling myoepithelial cells, squamous epithelial cells, cartilage, and myxomatous components, solidly proliferative cells, and stroma. The results of the immunohistochemical studies are presented in **Table 1** and are summarized as follows.

Table 1 Immunohistochemical findings of normal salivary gland and pleomorphic adenoma

	actin	keratin	vimentin	S-100	desmin
normal salivary gland					
epithelium					
duct	—	+	—	—	—
acini	—	—	—	—	—
myoepithelial cell	+	—	+	+	—
blood vessel					
endothelium	—	—	+	—	—
smooth muscle	+	—	+	—	+
pleomorphic adenoma					
myoepithelial type cell	±	±	++	++	—
duct lining cell	—	+	—	—	—
squamous epithelium type cell	—	++	±	±	—
solidly proliferative cell	—	±	++	+	—
cartilage type cell	—	—	+	+	—
myxoma type cell	—	±	+	+	—

— : no, ± : slight, + : moderate, ++ : intensive staining

Fig. 1 Actin is localized in myoepithelial cells surrounding acini and excretory ducts, normal submandibular gland. ×200

Fig. 2 A portion of two cases of pleomorphic adenoma shows a positivity for actin in the outer cells of glandular structure, those similar to myoepithelial cells, pleomorphic adenoma. ×200

1. Actin

In normal salivary gland tissues, actin was localized immunohistochemically in the slender cytoplasm of the myoepithelial cells (**Fig. 1**), and in the smooth muscle cells of vessels.

In the pleomorphic adenoma, the tumor cells of almost all cases showed no localization of actin. Only a portion of two cases, however, revealed positive actin staining in the spindle cells surrounding the duct lining cells, i.e., those similar to myoepithelial cells (**Fig. 2**).

2. Keratin

In the normal salivary glands, keratin was observed only in the duct epithelium from the intercalated duct to the interlobular duct.

In the pleomorphic adenomas, intensive reaction to keratin occurred in the duct lining cells and those cells resembling squamous epithelial cells (**Fig. 3**). Slight to moderate reaction was

Fig. 3 Intensive positive reaction for keratin is observed in squamous epithelium like cells and a part of solidly proliferative cells, pleomorphic adenoma. $\times 100$

Fig. 5 Weak positive immunoreaction of S-100 protein is localized in myoepithelial cells surrounding acini and excretory ducts, normal submandibular gland. $\times 200$

Fig. 4 The expression of vimentin is observed in myxoma like cells, but is negative in squamous epithelium like cells, pleomorphic adenoma. $\times 200$

Fig. 6 Intensive reaction of S-100 protein is observed in myoepithelial type cells, but is negative in duct lining cells, pleomorphic adenoma. $\times 200$

observed in several solidly proliferative cells, and slight positive reaction was seen in a small portion of the cells similar to myoepithelial cells, but a negative reaction was observed in those cells resembling cartilage cells.

3. *Vimentin*

In the normal salivary gland, vimentin showed localization in the cytoplasm of myoepithelial cells. Moreover, vimentin was observed in the smooth muscle cells and in the endothelial cells.

In the pleomorphic adenomas, vimentin stained intensively cells similar to both myoepithelial and myxoma cells (**Fig. 4**), and solidly proliferative cells. Cells similar to cartilage cells were stained moderately. A negative reaction, however, occurred in duct lining cells and cells resembling squamous epithelial cells.

4. *S-100 protein*

In the normal salivary gland, localization of S-100 protein was seen in the myoepithelial cells

surrounding acini and excretory ducts (**Fig. 5**).

In the pleomorphic adenomas, S-100 protein was observed as a relatively intensive reaction in the cells resembling myoepithelial cells (**Fig. 6**), myxoma and cartilage cells, and solidly proliferative cells. A portion of the cells similar to squamous epithelial cells showed rather weaker reactions.

5. *Desmin*

In this study, desmin was not observed in any tumor cells, although it was distributed in the smooth muscle cells of vessels.

Discussion

Several hypotheses have been reported concerning the origin of pleomorphic adenoma. Recently, investigators have suggested that this tumor is of ectodermal origin and that the myoepithelial cells might play an important role^{2,3,8,9,12,13}. The discussion regarding the origin of pleomorphic adenoma has been focused on the morphogenesis of the mesenchymal elements. Several ultrastructural studies have demonstrated tonofilaments in tumor cells, even in the mesenchymal components^{1,2}, and supported the ectodermal origin hypothesis of this tumor.

Actin is a microfilament and the major contractile protein of the cells. It was believed that actin was a muscular-cell specific protein, but now it has been found even in non muscular cells, and related to their motility function^{17,18}. In our study, normal myoepithelial cells showed positive immunoreactivity for actin. However, in salivary gland tumors, only a few cells of pleomorphic adenomas showed localization of actin in the outer layer of the ductal elements, i.e. those similar to myoepithelial cells. These findings support previous electron microscopic studies^{1~6} and reveal a more qualitative observation. The authors observed many cells morphologically similar to myoepithelial cells that showed negative immunoreactivity with the actin antibody. This suggests that these tumor cells are undergoing neoplastic alterations in the cytoskeleton resulting in loss or change in antigen determinants of actin, if these tumors are derived from the myoepithelial cell.

Intermediate filaments are 9-11 nm in diameter, and they were presumed to reveal the origin of each germ layer^{19~21}. However, recent pathological studies have shown a number of exceptions^{22~24}. Thus, the cell origin can not be discussed only with reference to the intermediate filament. The quantitative and qualitative alternations of the intermediate filament, however, are still important as potential markers of neoplastic transformation²⁵. Keratin is one of the intermediate filaments which locates mainly in ectodermal derived epithelium. Keratin is composed of 19 subunits²⁶, which have molecular weights of 40-68 kD²⁷. The antibody used in this study showed a positive reaction mainly with the stratum spinosum and granulosum of the skin. In the current study, normal myoepithelial cells showed a negative reaction, but ductal epithelium was positive for keratin antibody. Other researchers, however, have reported the localization of keratin in normal myoepithelial cells^{8,28~30}. This discrepancy might be due to the difference in antibody specificity. On the other hand, keratin was widely localized in morphologically epithelial-differentiating cells, and also in mesenchymal-differentiating cells of pleomorphic adenomas. This result suggests that these tumor cells of pleomorphic adenoma might

contain immunohistochemically identical keratin types in some degree as that of the duct epithelium in normal salivary glands, and not the keratin type of normal myoepithelial cells.

Vimentin is constituted of a single protein, having a reported molecular weight of 52 kD³¹⁾, 53 kD³²⁾, or 57 kD¹⁴⁾. As mentioned above, this intermediate filament localizes in cells of mesenchymal origin, but recent studies have shown that vimentin is demonstrated simultaneously with keratin, desmin, or glial fibrillary acidic protein in various tissues unrelated to the germ layer²²⁻²⁵⁾. In our study, vimentin was observed in normal myoepithelial cells. If the authors accept the results of others, normal myoepithelial cells show simultaneous localization of two kinds of intermediate filaments. This is rare in the mature normal cells and indicates a characteristic of myoepithelial cells. In pleomorphic adenomas, vimentin was observed in the cells showing mesenchymal morphological changes and also ectodermal changes. Adding the distribution of keratin, these tumor cells are likely to express both vimentin and keratin. The appearance of vimentin in vivo is reported to correlate with decreasing intercellular attachment. However, our results showed no remarkable difference of vimentin immunoreactivity between scattered and clustered tumor cells in pleomorphic adenoma.

S-100 protein is an acidic Ca²⁺ binding protein with a molecular weight of approximately 20,000^{33,34)}. This protein is widely distributed in the central and peripheral nervous systems of all vertebrates³⁵⁾. However, its function has not been elucidated. S-100 protein is said to be a neuron specific protein, but recently it has also been demonstrated in several non-neural tissues^{36,37)}. In our study, S-100 protein was observed in normal myoepithelial cells, and almost all tumor cells of pleomorphic adenomas, except for duct lining cuboidal cells. The distribution of S-100 protein was approximately akin to that of keratin.

Desmin is a single acid protein having a reported molecular weight of 52 kD³²⁾, 53 kD¹⁴⁾, or 50-55 kD³¹⁾. Desmin distributes in skeletal, cardiac, and smooth muscle. In our study, desmin was not seen in normal myoepithelial cells, nor in any tumor cells, but only in the smooth muscle cells of small blood vessels. It is interesting to note that myoepithelial cells reveal characteristics resembling those of smooth muscle cells morphologically and functionally. The intermediate filaments of normal myoepithelial cells consist of keratin and vimentin, although smooth muscle cells exhibit desmin and vimentin. On the other hand, over a large part of the amino-acid sequence, desmin was reported to show strong homology with vimentin³⁸⁾.

According to the results of our study, normal myoepithelial cells revealed the localization of actin, vimentin and S-100 protein. These observations indicate that normal myoepithelial cells show mesenchymal characteristics in spite of ectodermal origin. Tumor cells of pleomorphic adenoma showed positive immunoreactivity for keratin, vimentin, and S-100 protein, but only a few tumor cells showed localization of actin. This reveals that these tumor cells show bipolar differentiation in a tumor simultaneously. Considering these points, it is likely that pleomorphic adenoma tumor cells might be the result of the dedifferentiation of the myoepithelial cell, with the essential features retained, altered or lost.

References

- 1) Mills, S.E. and Cooper, P.H.: An ultrastructural study of cartilaginous zones and surrounding epithelium in mixed tumors of salivary glands and skin. *Lab. Invest.*, **44**, 6-12, 1981.
- 2) Dardick, I., van Nostrand, A.W.P., Jeans, M.T.D., Rippstein, P. and Edwards, V.: Pleomorphic adenoma. I. Ultrastruc-

- tural organization of epithelial regions. *Hum. Pathol.*, **14**, 780–797, 1983.
- 3) Dardick, I., van Nostrand, A.W.P., Jeans, M.T.D., Rippstein, P. and Edwards, V.: Pleomorphic adenoma. II. Ultrastructural organization of stromal regions. *Hum. Pathol.*, **14**, 798–809, 1983.
- 4) Dardick, I., van Nostrand, A.W.P. and Phillips, M.J.: Histogenesis of salivary gland pleomorphic adenoma (mixed tumor) with an evaluation of the role of the myoepithelial cell. *Hum. Pathol.*, **13**, 62–75, 1982.
- 5) Gustafsson, H., Carlsöö, B., Kjörrell, U. and Thornell, L.E.: Ultrastructural and immunohistochemical aspects of carcinoma in mixed tumors. *Am. J. Otolaryngol.*, **7**, 218–230, 1986.
- 6) McClure, J., Smith, P.S. and Jamieson, G.G.: Mixed salivary type adenoma of the human female breast. *Arch. Pathol. Lab. Med.*, **106**, 615–619, 1982.
- 7) Nakazato, Y., Ishizeki, J., Takahashi, K., Yamaguchi, H., Kamei, T. and Mori, T.: Localization of S-100 protein and glial fibrillary acidic protein-related antigen in pleomorphic adenoma of the salivary glands. *Lab. Invest.*, **46**, 621–626, 1982.
- 8) Caselitz, J., Löning, T., Staquet, M.J., Seifert, G. and Thivolet, J.: Immunocytochemical demonstration of filamentous structures in the parotid gland. *J. Cancer Res. Clin. Oncol.*, **100**, 59–68, 1981.
- 9) Ogawa, I., Takada, T., Nikai, H., Ogura, M., Ijuin, N. and Ito, H.: Immuno-histochemical studies on salivary gland tumors. II. Localization of secretory epithelial markers in various benign tumors. *Jpn. J. Oral Biol.*, **26**, 1200–1209, 1984.
- 10) Egan, M., Crocker, J. and Nar, P.: Localization of salivary amylase and epithelial membrane antigen in salivary gland tumors by means of immunoperoxidase and immunogold-silver techniques. *J. Laryngol. Otol.*, **102**, 242–247, 1988.
- 11) Caselitz, J., Lunau, U., Hamper, K., Walther, B. and Schmiegelow, P.: The pleomorphic adenoma of salivary glands transplanted on athymic mice; A light microscopical and immunohistochemical investigation. *Virchows Arch. [A]*, **408**, 191–209, 1985.
- 12) Shirasuna, K., Sato, M. and Miyazaki, T.: A myoepithelial cell line established from a human pleomorphic adenoma arising in minor salivary gland. *Cancer*, **45**, 297–305, 1980.
- 13) Batsakis, J.G.: Salivary gland neoplasia; An outcome of modified morphogenesis and cytodifferentiation. *Oral Surg.*, **49**, 229–232, 1980.
- 14) Osborn, M. and Weber, K.: Tumor diagnosis by intermediate filament typing; A novel tool for surgical pathology. *Lab. Invest.*, **48**, 372–393, 1983.
- 15) Miettinen, M., Lehto, V.P. and Virtanen, I.: Antibodies to intermediate filament proteins in the diagnosis and classification of human tumors. *Ultrastruct. Pathol.*, **7**, 83–107, 1984.
- 16) Batsakis, J.G.: Intermediate filaments and salivary gland tumors. *Am. J. Otolaryngol.*, **7**, 231–232, 1986.
- 17) Buckley, I.K.: Fine-structural and related aspects of non-muscle cell motility. "Cell and Muscle Motility 1" (Dowben, R.M. and Shay, J.W. eds.), p. 135–203, Plenum Publ. Corp., New York, 1981.
- 18) Tsukita, S.: Cytoskeleton; Actin filament. *Pathol. Clin. Med.*, **5**, 374–384, 1987.
- 19) Cooper, D., Schermer, A. and Sun, T.-T.: Classification of human epithelia and their neoplasms using monoclonal antibodies to keratins; Strategies, applications, and limitations. *Lab. Invest.*, **52**, 243–256, 1985.
- 20) Gown, A.M. and Vogel, A.M.: Monoclonal antibodies to human intermediate filament proteins. III. Analysis of tumors. *Am. J. Clin. Pathol.*, **84**, 413–424, 1985.
- 21) Miettinen, M., Lehto, V.P., Badley, R.A. and Virtanen, I.: Expression of intermediate filaments in soft tissue sarcomas. *Int. J. Cancer*, **30**, 541–546, 1982.
- 22) Franke, W.W., Schmid, E., Winter, S., Osborn, M. and Weber, K.: Widespread occurrence of intermediate-sized filaments of the vimentin-type in cultured cells from diverse vertebrates. *Exp. Cell Res.*, **123**, 25–46, 1979.
- 23) Virtanen, I., Lehto, V.P., Lehtonen, E., Vartio, T., Stenman, S., Kurki, P., Wager, O., Small, J.V., Dahl, D. and Badley, R.A.: Expression of intermediate filaments in cultured cells. *J. Cell Sci.*, **50**, 45–63, 1981.
- 24) Ramaekers, F.C.S., Haag, D., Kant, A., Moesker, O., Jap, P.H.K. and Vooijs, G.P.: Coexpression of keratin- and vimentin-type intermediate filaments in human metastatic carcinoma cells. *Proc. Natl. Acad. Sci. USA*, **80**, 2618–2622, 1983.
- 25) Tsutsumi, Y.: Cytoskeleton—application in pathology—keratin, vimentin, desmin. *Pathol. Clin. Med.*, **5**, 445–461, 1987.
- 26) Fuchs, E., Marchuk, D. and Tyner, A.: Differential expression of the genes encoding the keratins of cultured human epidermal cells. "Cell and Molecular Biology of the Cytoskeleton" (Shay, J.M. ed.), p. 85–107, Plenum Press, New York, 1986.
- 27) Moll, R., Franke, W.W., Schiller, D.I., Geiger, B. and Krepler, R.: The catalog of human cytokeratins; Patterns of expression in normal epithelia, tumors and cultured cells. *Cell*, **31**, 11–24, 1982.
- 28) Mori, M., Sumitomo, S., Iwai, Y. and Meenagh, M.A.: Immunolocalization of keratins in salivary gland pleomorphic adenoma using monoclonal antibodies. *Oral Surg. Oral Med. Oral Pathol.*, **61**, 611–616, 1986.
- 29) Achtstätter, T., Moll, R., Anderson, A., Kuhn, C., Pitz, S., Schwechheimer, K. and Franke, W.W.: Expression of glial filament protein (GFP) in nerve sheaths and non-neural cells re-examined using monoclonal antibodies; With special emphasis the co-expression of GFP and cyto-keratins in epithelial cells of human salivary gland and pleomorphic adenomas. *Differentiation*, **31**, 206–227, 1986.
- 30) Caselitz, J., Osborn, M. and Seifert, G.: Intermediate-sized filament proteins (prekeratin, vimentin, desmin) in the normal parotid gland and parotid gland tumors-immunofluorescence study. *Virchows Arch. [A]*, **393**, 273–286, 1981.
- 31) Lazarides, E.: Intermediate filaments; A chemically heterogeneous, developmentally regulated class of proteins. *Annu.*

- Rev. Biochem., **51**, 219~250, 1982.
- 32) Steinert, P.M., Steven, A.C. and Roop, D.R.: The molecular biology of intermediate filaments. *Cell*, **42**, 411~419, 1985.
- 33) Isobe, T. and Okuyama, T.: The amino acid sequence of S-100 protein and its relation to the calcium-binding proteins. *Eur. J. Biochem.*, **89**, 379~388, 1978.
- 34) Moore, B.W.: A soluble protein characteristic of the nervous system. *Biochem. Biophys. Res. Commun.*, **19**, 739~744, 1965.
- 35) Nakamura, Y., Becker, L.E. and Marks, A.: Distribution of immunoreactive S-100 protein in pediatric brain tumors. *J. Neuropathol. Exp. Neurol.*, **42**, 136~145, 1983.
- 36) Hirano, T.: Immunohistochemical study of malignant melanoma. *Acta Pathol. Jpn.*, **36**, 733~743, 1986.
- 37) Takahashi, K., Isobe, T., Ohtsuki, Y., Akagi, T., Sonobe, H. and Okuyama, T.: Immunohistochemical study on the distribution of α and β subunits of S-100 protein in human neoplasm and normal tissues. *Virchow Arch. [B]*, **45**, 385~396, 1984.
- 38) Mckeon, F.D., Kirschner, M.W. and Caput, D.: Homologies in both primary and secondary structure between nuclear envelope and intermediate filament proteins. *Nature*, **319**, 463~468, 1986.

(Received for publication, October 6, 1989)