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Proliferative potential in benign mixed salivary gland tumors and its value in primary and recurrent neoplasms

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SUMMARY

Objective. Mixed salivary gland tumors are characterized by a marked diversity in the cell proliferation. It course in the stromal component, and, especially in recurrent neoplasms, is not completely understood. This study evaluated cell proliferative potential, its value and the clinical course of primary and recurrent salivary gland pleomorphic adenomas (PA).

Materials and methods. 322 benign salivary gland tumors were used in this study. The cell proliferation was estimated by Ki-67 expression levels.

Results. Ki-67 immunoreactivity showed a wide range of spectra; in the epithelial and stromal type of PA the cell proliferation had the value from 0.07±0.03 (95% CI 0.01-0.14) to 4.81±0.60 (95% CI 3.61-6.02) and from 0 to 0.79±0.11 (95% CI 0.57-1.00), respectively. The Ki-67 value was higher in recurrent tumors compared with primary, and the mean number of Ki-67-positive cells per visual microscopic field constituted 2.14±1.60 (95% CI 1.47-2.47) comparing with 1.43 (95% CI 0.97-1.55) revealed in primary tumors.

Conclusion. Cell proliferation values correlate with a recurrence of neoplasm, and elevation of proliferation potential in the stromal component of recurrent PA is indicative of clinical course change for the worse.

Key words: cell proliferation, tumors, salivary glands.

INTRODUCTION

Salivary gland tumors constitute 2-6.5% of all head and neck neoplasms and show racial and geographic variations in the frequency and distribution. Benign salivary gland tumors often reveal morphological diversity between different tumor types and sometimes within individual tumor mass as well [1, 2]. Although there is a large volume of literature [3, 4, 5, 6] on correlation of tumor cell proliferation and the prognosis of the disease as well as metastasis in malignant salivary gland lesions, there are only few reports on prediction of clinical behavior of benign salivary tumors [1, 7, 8]. Pleomorphic adenoma (PA) is the most common salivary gland tumor and accounts for about 60% of all salivary gland neoplasms world-wide. The reported annual incidence of the PA is 2.4-3.05 per 100,000 in the world-wide population [9]. PA is found mostly in the parotid gland in middle-aged women. It progresses slowly and, left untreated, can produce significant morbidity and, rarely, death [10]. Local recurrence after surgical treatment is described in 1% to 5% of cases, whereas, malignant degeneration is reported in 2% to 9 % of cases of PA of salivary gland origin [7].

It is generally accepted by pathologists that both epithelial and stromal (mucoid (myxoid), hyaline, chondroid, osseous) elements of PA often arise from same cell clone, which may be a myoepithelial or ductal reserve cell, and tumors are more highly cellular in their early stages of development [8]. Moreover, an amount of chondromyxoid stroma increases with the duration of the neoplasm, and the recurrent PA is frequently rather hypocellular, and reveals an incomplete encapsulation.

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Some recent tissue engineering applications [11] suggest that salivary gland epithelial cells expressing the $\alpha 6\beta 1$ integrin may have stem cell capabilities. Moreover, [12] made a proposal that appearance of local salivary gland mediators, cytokines, like tumor necrosis factor-like weak inducer of apoptosis and contact with a receptor - Fn14 could lead to a general epithelial cell proliferation. Reserve cell hypothesis and its role for the induction of salivary gland tumors constantly were at the focus of interest for many researchers. It was stated by [13] that all cell types, including acinar cells, are at risk in the carcinogenic process, and by [14] that specialized myoepithelial cells must be considered one of the potential progenitor cells for human salivary gland tumors. The intercalated duct reserve cells are thought to be the epithelial source for adenoid cystic carcinoma, acinic cell carcinoma, benign mixed tumor and monomorphic adenoma. Recently [15] provided evidence based on quantitative Ki-67 measurements that intercalated duct cells of rats have the properties of tissue stem cells upon stimulation. Another type of cells - the myoepithelial cells play an important role in the composition and growth of several salivary gland tumors. But, with the exception of the myoepithelioma, it is doubtful that the myoepithelial cell is the primary cell of origin for any salivary gland tumor, however its participation in mixed tumor and several malignant salivary glands tumors cannot be denied. The studies of [16] show that the enzyme nitric oxide synthase and especially, its inducible form may be implicated in tumor-promoting activities in case of PA. These authors suggest that there is a strong correlation between the immunohistochemical expression of the inducible nitric oxide synthase and alpha-smooth muscle actin staining of myoepithelial cells in PAs.

Ki-67 has been considered to be a potent tool for making an easy and quick evaluation of the proportion of proliferating cell in tumor [3, 4, 17, 18]. The Ki-67 protein is expressed in all phases of the cell cycle except G0 [19]. It therefore has the potential to be a more sensitive biomarker for cellular proliferation than mitoses [20], and considered a useful tool in determining the aggressiveness of malignant neoplasm [5, 6]. The expression of Ki-67 has been correlated with mitotic activity, histological grade and clinical behaviour of tumor [1, 4]. Ki-67 proliferation marker has been absent [1] or had low positivity [21] in PAs indicating that these mixed tumors have low proliferative rate and good prognosis.

The aim of this study was to evaluate the expression of the proliferation marker Ki-67 in benign mixed salivary gland tumors using a quantitative approach, and apply these data for further understanding of proliferative peculiarities, pathological characteristics and clinical behaviour of primary and recurrent neoplasms. We suggest that elevation of proliferation potential in the stromal component of recurrent mixed tumors is indicative of clinical course change for the worse.

MATERIALS AND METHODS

322 patients with the histologically confirmed diagnosis of salivary gland tumor treated between 1996 and 2007 at the Oncology Center of Latvia were used in this study. In total 212 female and 110 male patients were recorded. The age range was 17 - 86 years. The clinical data of patients were obtained with respect to duration and type of the lesion at the time of presentation, clinical features, anatomic location, and course of the tumor. This study has been independently reviewed and approved by the Ethical Committee of Riga Stradins University. An immunohistochemical detection of tumor cell proliferation in the benign parotid, submandibular and palatinal salivary gland tissue using Ki-67 as a marker was performed. Control tissues were taken along the surgical removal, from histologically intact salivary gland tissue areas.

For conventional light microscopy and immunohistochemistry tissues were fixed in 10% formalin, processed through absolute ethanol and xylene, and embedded in Paraplast Plus wax (58°C). For diagnostic purposes, sections (5 μm) were stained routinely with haematoxylin and eosin. Additional paraffin sections were used for immunohistochemistry. The clone MIB-1 anti Ki-67 monoclonal antibody (DAKO A/S, Glostrup, Denmark), which reacts with the Ki-67 nuclear antigen associated with cell proliferation and which is found through the G1, S, G2 and M phases of the cycle, was used for immunohistochemical evaluation of proliferation in salivary gland tumors [1, 5, 6, 17, 19, 21, 22]. This antibody detects Ki-67 antigen on paraffin sections after exposure to microwave and has been established as a reference monoclonal mouse antibody for the demonstration of the Ki-67 antigen in formalin-fixed, paraffin embedded specimens [3, 4, 17]. Sections were dewaxed in xylene, immersed in absolute ethanol and then traditionally in graded alcohols, transferred to a methanol/0.3% hydrogen peroxide solution for 20 min in order to abolish endogenous peroxidase activity. After quenching of endogenous peroxidase activity sections were washed three times in double distilled water, im-

mersed in 0.01 M phosphate-buffered saline (PBS), pH 7.2-7.4, for 10 min and then incubated with primary antibody at a dilution 1:200 overnight at 4°C, and next day, with secondary biotinylated goat antibody to mouse immunoglobulins (Vector Laboratories, Burlingame, CA, USA) 1:500 dilution for 30 min and streptavidin-biotin-peroxidase preformed complex (BioGenex Laboratories, San Ramon, CA, USA) 1:250 dilution for 30 min. The immunological reaction was developed with 3, 3'-diaminobenzidine tetrahydrochloride (50 mg in 100 ml of PBS with 0.03% v/v hydrogen peroxide). Sections were counterstained with Harrys haematoxylin and mounted in Kaiser's glycerol gelatin. Cells labeled by the antibody displayed a brown nuclear staining pattern. Negative controls were performed by omitting the primary antibody on one of the two sections per slide. Lymph node sections were used as positive controls for the reaction with MIB-I.

Two observers independently evaluated and interpreted the immunostaining results without knowledge of the clinical data. The staining reaction was further estimated by counting Ki-67 positive tumor cells in all areas of the sample (magnification x400) and accessing the number of labeled cells. We determined Ki-67 positive cells within the visual microscopic field and compared these data with the total cell number appearing within the same field. Additionally, the cell proliferation index was calculated as the number of Ki-67-positive nuclei per 1000 cellular nuclei. The means, standard error means and confidence intervals of the major variables were determined. Statistical analysis was performed using the SPSS system (release 17.0 software) and the difference was considered at the 0.05 significance level. Microphotographs were obtained using Leitz DMRB bright field optics equipped with a digital camera DC 300F. Photography was made using x200 magnification.

RESULTS

The location of benign salivary gland tumors was distributed between the major (parotid and submandibular) and the minor (palatinal) salivary glands. The parotid gland accounted for 274 (85%) of all benign salivary gland tumors. There were variations in proportions of benign tumor lesions at different lobes of the parotid gland reflecting a predominant involvement of the superficial lobe of this gland. The submandibular and minor salivary glands revealed much less frequent involvement compared with the parotid gland – 35 (11%) and 13 (4%), respectively.

PA was the most common benign salivary gland neoplasm and accounted for 242 (75%). The majority of these tumors were in the parotid gland 198 (82%) and the remainder in the submandibular 27 (11%) and the minor palatinal 17 (7%) salivary glands. The second most common benign tumor was adenolymphoma which accounted for 45 (14%), whereas monomorphic adenoma was the third most common benign tumor – 29 (9%). The parotid gland was the exclusive site of involvement in case of adenolymphoma and monomorphic adenoma. Less common benign tumors including oncocytoma 2 (1%), myoepithelioma 2 (1%), and vascular tumors 2 (1%) were also reported.

The patient records showed no correlations between the duration of neoplasm, the tumor size, and the fact of recurrence. In a majority of cases PA tumors were small, well-circumscribed, encapsulated nodules measuring from 1 up to 10 cm. The size of most tumors varied from about 1-3 cm (47%), followed by 3-5cm (38%). Some reported cases showed much larger size 5-10cm (15%). On gross examination of PA tumors a homogeneous grayish-white to brown mass was appearing, the cut surface of myxoid and cartilaginous lesions sometimes provided a glistening appearance. Most recurrent tumors were multinodular, and the number of nodules varied. The myxoid subtype was predominant. The various forms of monomorphic adenoma tumors closely resembled PA. The cut surface of oncocytomas usually was brown and revealed a lobular appearance. Wartin's tumors often revealed a multicystic appearance with a fine granular cut surface.

We studied parotid, submandibular and palatinal PAs with the tumor history 1-3 years (55.5% of the cases), 3-5 years (19.8%), 5-10 years (9%) and more than 10 years (15.5%). Duration and size of these tumors had no association with pathological type of PA.

Nuclei stained for Ki-67 antigen were present in all tissue sections, including the control slides. Ki-67 expression was detected in stromal and epithelial components of salivary gland PA. The MIB-1 antibody also slightly stained the cytoplasm of cells constituting epithelial component in some tumors. In the groups with primary and recurrent tumors, MIB-1-positive nuclei were seen in various types of cells, including vascular endothelial cells.

The greatest cellularity and the number of Ki-67-positive cells per visual microscopic field was observed in recurrent PA, where the mean number

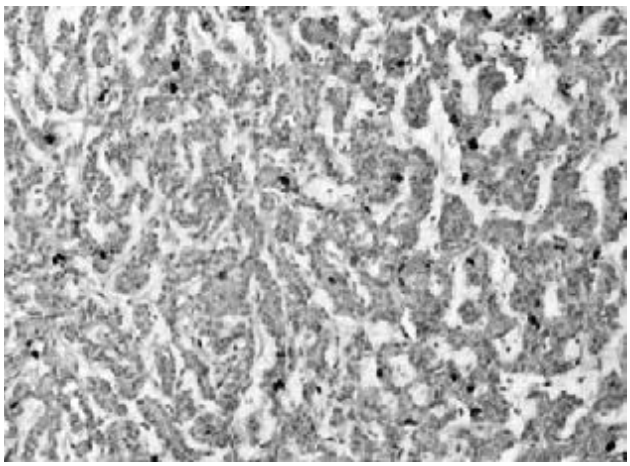

Fig. 1. Cell-rich epithelial area showing many Ki-67-positive cells stained with the MIB-1 antibody in case of pleomorphic adenoma. (original magnification x 200)

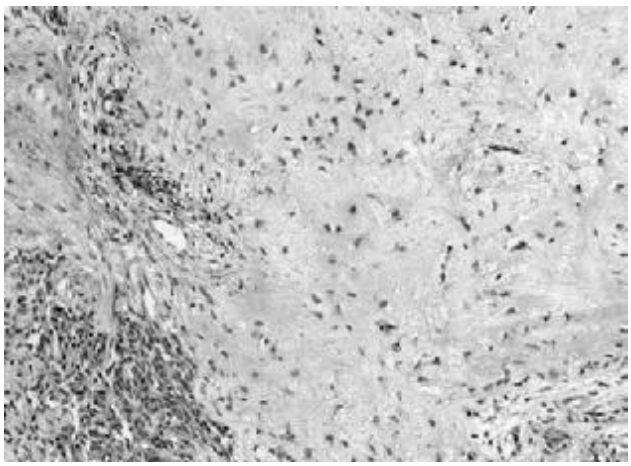

Fig. 2. Mucoid stroma and a small fragment of epithelial component lacking Ki-67-positive cells in case of pleomorphic adenoma. (original magnification x 200)

of Ki-67-positive cells per visual microscopic field constituted 2.14±1.60 (95% CI 1.47-2.47) comparing with 1.43 (95% CI 0.97-1.55) revealed in primary tumors when the whole material was taken into consideration. Still when the morphological peculiarities of the tumor type were estimated along with evaluation of the expression of proliferation marker, a wide range of expression became visible. The number of the Ki-67-positive cell nuclei per visual microscopic field in the epithelial and stromal component of PA varied from 0.07±0.03 (95% CI 0.01-0.14) to 4.81±0.60 (95% CI 3.61-6.02), and from 0 to 0.79±0.11 (95% CI 0.57-1.00), respectively. A great Ki-67 expression was observed in the epithelial tumor variants (Fig. 1). Much lower expression of the proliferation marker was detected in the stromal type of the tumor (Fig. 2). In the chondroid stroma the Ki-67 expression was almost nil, whereas, a richly developed mucoid stroma revealed higher cell proliferation marker expression. No significant differences were found between the mean number of the Ki-67-positive cells per visual microscopic field in the principally stromal variant of the tumor and a control tissue – 0.14±0.04 (95% CI 0.05-0.22) and 0.04±0.03 (95% CI 0-0.09), respectively.

The patients included in this study have had two and three previous surgeries due to the tumor, and with the last tumor developed in 1 year. All of the recurrences were developed in the parotid salivary gland. The estimated recurrence of the mixed tumor was about 4%, and recurrence rates were higher in previously recurrent tumors. Subsequent recurrence after an initial recurrence occurred at a rate of 25%. The next recurrence was reported at a shorter interval in cases with the history of recurrence.

Re-recurrent tumors revealed the highest Ki-67 expression. In the re-recurrent PA tumors cell proliferation demonstrated using Ki-67 as a marker was revealed in both – epithelial and stromal component (Fig. 3A, B), and the estimated cell proliferation index was 7.15, comparing with 3.27 revealed in the non-recurrent tumors. The predominantly epithelial and extremely epithelial types of re-recurrent tumor compared with the first recurrent tumor showed the mean value of Ki-67 equal to 1.87 (range 0.23-4.81) and 1.59 (range 1.33-1.86), respectively; whereas, the stromal types – 0.32 (range 0-0.79) and 0.24 (range 0.22-0.27), respectively. Moreover, there was a significant increase in the Ki-67 expression within the above mentioned stromal component in all recurrent PA comparing with this in the non-recurrent cases and a normal salivary gland tissue, and the mean number of the Ki-67-positive cells constituted 0.79±0.11 (95% CI 0.57-1.00); 0.14±0.04 (95% CI 0.05-0.22), and 0.04±0.03 (95% CI 0-0.09), respectively.

We haven't got an evidence of higher Ki-67 expression in Wartin's tumors and other types of monomorphic adenoma comparing with the PA.

DISCUSSION

PA with a parotid gland involvement was the most common salivary gland tumor and accounted for 75% of all benign salivary neoplasms. The reviewed incidence is comparable with an incidence of PA observed in the equal and larger cohorts of patients and ranging from 33% to 70% of all tumors and from 70.6% to 100% of benign tumors [2, 9, 22, 23].

The most common size of surgically removed benign salivary gland tumors was from 1 cm up to 3 cm, whereas, the next most common size was vary-

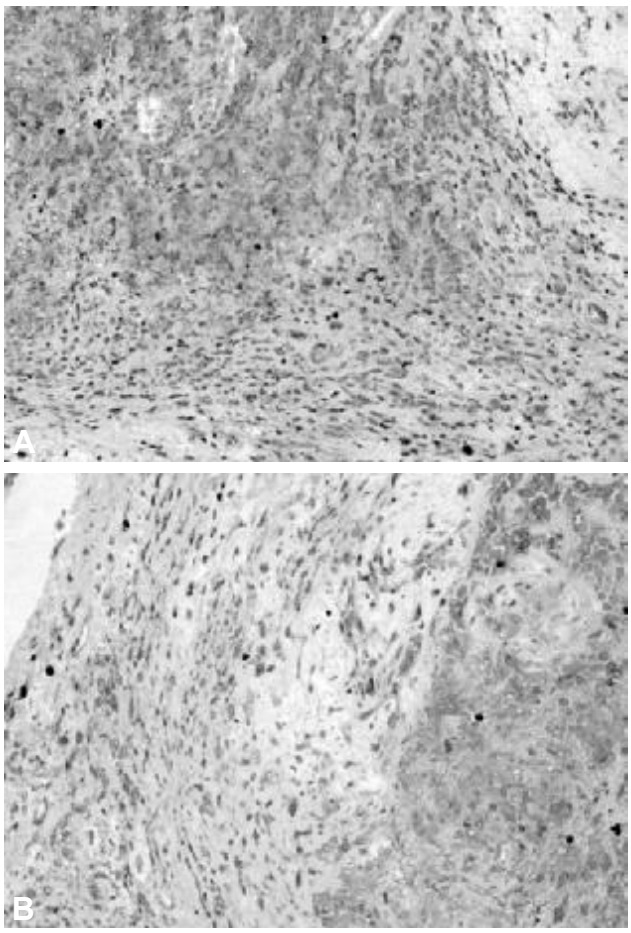

Fig. 3. Epithelial (A) and stromal (B) components revealing Ki-67-positive cells in case of recurrent pleomorphic adenoma. (original magnification x 200)

ing from 3 cm up to 5 cm. At the time of presentation, a major portion of benign salivary gland tumors used in this study revealed a duration varying from 1 up to 3 years. Our data on the size and duration of benign salivary gland tumors are in accordance with the literature data [2, 9, 24].

High Ki-67 expression has been reported to be a prognostic factor in numerous human cancers and also in some salivary gland carcinomas [3, 4, 6, 17, 21]. From these, [3] showed that none of the patients with low MIB-1 indices developed salivary gland acinic cell carcinoma recurrence during a long follow-up period. In malignant tumors of salivary glands (as reported for mucoepidermoid carcinoma) high Ki-67 expression showed correlations with increased histological grade, necrosis, cell anaplasia and mitotic index. In a number of publications [6, 25, 26] a common finding is no expression or minimum expression (1%) of Ki-67 in low-grade mucoepidermoid carcinomas with progressive increase from intermediate-grade (4%) to high-grade (10%) tumors. Some papers specify the Ki-67 expression in particular sites of involvement [1, 17]. According to the results obtained by [1] less than 5% of Ki-67-positive cells were present in case of PA, counting at least 1000 cells. The others reported that the Ki-67 value was significantly higher in large salivary gland tumors and in cases with treatment failure [17]. In study published by [5] the proliferative capacity of salivary gland tumor as measured by the volume corrected index of Ki-67 corresponding to Ki-67 / mm^2 of tumor tissue has been shown to be one of the most powerful indicators of tumor behavior. Our quantitative Ki-67 estimations agree with these of other authors [27]. Quantitative Ki-67 assessment is more superior as a measure of cell proliferation as compared to often used semiquantitative estimations. The search for novel sensitive proliferation markers including those useful for differential diagnosis between various types of salivary gland tumors is continued. Although the Ki-67 protein is well characterized on the molecular level and extensively used in a number of studies, the functional significance of it still remains unclear. Moreover, the Ki-67 value for prognosis of primary and recurrent salivary gland tumors with widely variable histopathologic and biologic characteristics has so far not been completely clarified. It has been generally accepted that the local recurrence of salivary gland PA is attributed to the vulnerability of the capsule. Recently, the presence of satellite tumors arising from capsular perforation of the primary tumor cells, and their proliferative peculiarities has been studied by [28]. Conducting Ki-67 estimations these authors were unable to find any evidence suggesting that primary PA with satellite tumors could be more biologically aggressive than those without. The results of the present clinicopathological study allow us to suggest that appearance and elevation of stromal proliferative activity in the recurrent salivary gland PA is an intrinsic event that may be responsible for more aggressive clinical behavior. Ezrin and inducible nitric oxide synthase are considered to be cell proliferation and PA malignant transformation promoting factors as suggested by some authors [29, 30].

CONCLUSIONS

This study extended our knowledge from structural and architectural composition of the mixed tumor that is important for behavior, and suggests that the clinical course and pathological evaluation of benign salivary gland tumors, and especially, recurrent tumors would benefit to be complemented with information on cell proliferation within the tumor mass based on accurate estimations using immunohistochemical labels. Further prospective

studies performed in large cohorts of patients are warranted to assess the value of proliferation markers as predictors of recurrence and survival.

The present study showed that varying architectural and cellular composition of the tumor in case of PA is characterized by a marked diversity in the cell proliferation reflected by Ki-67 expression levels. The increase of tumor cell proliferation in the stromal component correlates with recurrence of neoplasm and deterioration of its clinical course. In order to clarify the role of the elevated proliferation potential in the stromal component of the recurrent and re-recurrent tumors, further pathological and immunohistochemical data should be accumulated through more accurate clinical and pathological studies.

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AUTHOR CONTRIBUTIONS

A Kazanceva, V Groma and E Kornevs designed the study. A Kazanceva, V Groma and L Smane performed all morphological examinations. L Smane and U Teibe performed all statistical data analyses. V Groma drafted the manuscript and all authors participated in the editing and final preparation of this paper.

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