Recurrent salivary pleomorphic adenoma shows increased immunohistologic expression of bcl-2 oncoprotein

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Background. Internal cell biology, including apoptotic regulation, is presumed to play a key role in the development of recurrent pleomorphic adenoma (PA).
Aim. The aim of our study was to determine the relevance of B-cell lymphoma 2 (bcl-2) oncoprotein immunoexpression and distribution in primary PA, and its recurrence.
Methods. Ten primary-non-recurrent, 14 primary-to-recur, and 28 recurrences of parotid PA patients aged 19-73 (mean 40.7±16.7) years were enrolled. The bcl-2 expression was compared between groups using a semi-quantitative histoscore, defined as the multiple of the percentage of cells by the intensity of immunostaining.
Results. Widely varying bcl-2 immunoreaction was found in the epithelial areas of 91.7% of primary and 85.2% of recurrent PA. Similarly varying but much less, immunopositivity was found in the myxoid areas of 62.5% of primary and 71.4% of recurrent tumours. No obvious differences in the bcl-2 staining intensity or pattern of specific epithelial morphologic structures in either the primary-non-recurrent, primary-to-recur or recurrent tumours were found. In both the mesenchymal and epithelial areas of PA, the differences in bcl-2 immunohistoscore between the primary-non-recurrent and primary-to-recur groups were not statistically significant (P=0.62, respectively 0.51). In the mesenchymal areas, the study revealed a significantly increased histoscore in recurrent tumours compared to their corresponding primaries (P=0.01). Increased bcl-2 expression in recurrent PA suggests an exaggerated aggressiveness of that tumor.
Conclusion. The fact that a significant difference in the histoscore was found exclusively in the myxoid component seems to accord with the reported prevalence of the latter in recurrent and metastatic PA.

Key words: apoptosis, bcl-2 gene, recurrence, pleomorphic adenoma, mesenchymal stromal cells

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INTRODUCTION

Recurrent parotid pleomorphic adenoma is a constant challenge, associated with an increased incidence of iatrogenic facial nerve trauma at revision surgery[1], the risk of subsequent recurrences with potential malignant conversion in terms of carcin"oma ex pleomorphic adenoma or the extremely rare metastasing “benign” pleomorphic adenoma[2], that has recently been categorized among salivary carcinomas[3]. It is generally accepted that a recurrent pleomorphic adenoma originates from the tumour cells spilled after inadvertent intraoperative rupture ofthe (pseudo)capsule, as well as from extracapsular tumour projections and marginal parts of the tumour left behind during surgery [4-7].

A number of authors have assessed the impact of the histomorphological structure of pleomorphic adenoma on its recurrence. A significantly increased recurrence rate in myxoid tumours over those with a predominant epithelial-cell-rich component, with the former being associated with more frequent capsular slenderness, incompletness and rupture were reported in articles[8-12]. Similarly, Goudot et al.[12] and Skálová et al.[13] concluded that neoplastic myoepithelial cells were responsible for the recurrence. The views of these authors seem to be supported by the fact that most recurrent tumours are of the myxoid type[4].

However, some studies suggest[14-17] that not all remnants of pleomorphic adenoma give rise to a clinically apparent recurrence. Intrinsic cell biology, including anti-apoptotic processes, seems to be involved in the development of the latter. Generally, the failure of apoptotic control mechanisms, in which the B-cell lymphoma 2 (bcl-2) oncogene plays a very important role, is thought to prolong the cellular life span with the subsequnet occurrence of gene mutations resulting in the initiation and progression of tumours[18]. The expression of bcl-2 oncoprotein as a

Table 1. Bcl-2 histoscore of myxoid and epithelial areas of primary-non-recurrent (PrimNonRec) and primary-to- recur (PrimToRec) pleomorphic adenomas.

Myxoid area		Epithelial area	
PrimNonRec	PrimToRec	PrimNonRec	PrimToRec
10	5	160	30
10	0	180	180
60	5	140	160
5	5	120	100
5	5	90	120
0	0	200	180
0	0	80	20
0	0	160	0
5	30	20	160
100	120	120	0
	40		180
	0		10
	0		270
	40		5
$P = 0.62$		$P = 0.51$	

PrimNonRec: primary-non-recurrent tumours
PrimToRec: primary-to-recur tumours

Table 2. Bcl-2 histoscore of myxoid areas of primary-to-recur (PrimToRec) pleomorphic adenomas and corresponding first (RecTumour) and consequent recurrences.

PrimToRec	RecTumour			
	R I	R II	R III	R IV
5	5			
0	30			
*	0			
*	*	60	0	
*	80			
5	0			
*	20			
*	*	10		
5	30	*	60	
5	50	*	150	
0	10	0		
0	0			
*	*	20		
0	160			
30	30	0		
120	*			
40	60	10	*	240
0	*	*	0	0
0	160			
40	100			
*	15			
$P = 0.01$				

Please note, the PrimToRec group is identical in Table 1 and 2. Recurrences are marked as "R" followed by the number of recurrences.
PrimToRec: primary-non-recurrent tumours
RecTumour: recurrences
R I - R IV: 1st, 2nd, 3rd, 4th recurrences
* material not available

marker of apoptosis-free immortalized neoplastic stem cells was studied in both malignant and benign salivary tumours, suggesting the role of apoptosis inhibition in the oncogenesis of these neoplasms[19-26]. Debiec-Rychter detected immunohistochemical co-positivity of PLAG1 (pleomorphic adenoma gene 1) and bcl-2 gene in pleomorphic adenomas, suggesting their involvement in the initiation and recurrence of this tumour[24]. Similarly, Sunardhi-Widyaputra[27] considered bcl-2 immunopositive cells to be progenitors of the latter.

Recurrent pleomorphic adenoma typically consists of multiple nodules, spreading widely into the surrounding soft tissues[4]. Owing to this local invasiveness as well as the extremely rare development of metastatic foci, a recurrent pleomorphic adenoma may be considered more aggressive than its primary. A question is thus raised as to whether the adverse biological nature of the recurrence might be explained by the bcl-2 oncogene overexpression.

In a retrospective study, we evaluated the bcl-2 oncoprotein overexpression in both primary and recurrent pleomorphic adenomas.

MATERIALS AND METHODS

Materials

Tissue samples of parotid gland pleomorphic adenomas were obtained from the Tissue Archive of our Departments of Pathology. Included cases consisted of 10 primary-non-recurrent (PrimNonRec), 14 primary-to-recur pleomorphic adenomas (PrimToRec), and subsequent 16 first (RecTumour) and 12 further recurrences of parotid pleomorphic adenomas, making a total of 52 tumours. Table 1 compares the bcl-2 expression of primary non-recurrent and primary recurrent tumours (Table 1). Table 2 shows the bcl-2 values in patients with recurrent disease at different time points from primary surgery through each recurrence (Table 2). All surgeries were performed by 2 experienced surgeons, each having done over a 1000 parotid surgeries. The age of patients at the time of their primary surgery ranged from 19 to 73 (mean 40.7±16.7) years. The female-to-male ratio was 5:1. The time interval between the primary and consecutive first, second, third and fourth recurrence was 2-24 (mean 8.0±7.1), 5-11 (mean 8.3±3.1), 10-13 (mean 11.5±2.1) and 14-16 (mean 15±1.4) years, respectively. In primary-non-recurrent tumours the follow-up was 1-13 (mean 7.2±4.1) years.

Methods

Standard indirect immunohistochemistry on formalin-fixed, paraffin-embedded sections was used for the detection of bcl-2 (FLEX monoclonal mouse anti-human BCL-2 Oncoprotein, clone 124, Ready-to-use, code IR

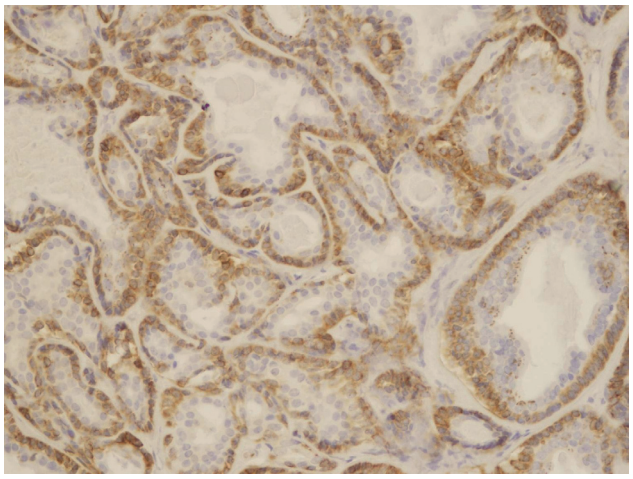

Fig. 1. Bcl-2 in the outer cells of excretory-duct like structures. Strong bcl-2 positivity in the outer cells of excretory-duct like structures. Original magnification, x 200.

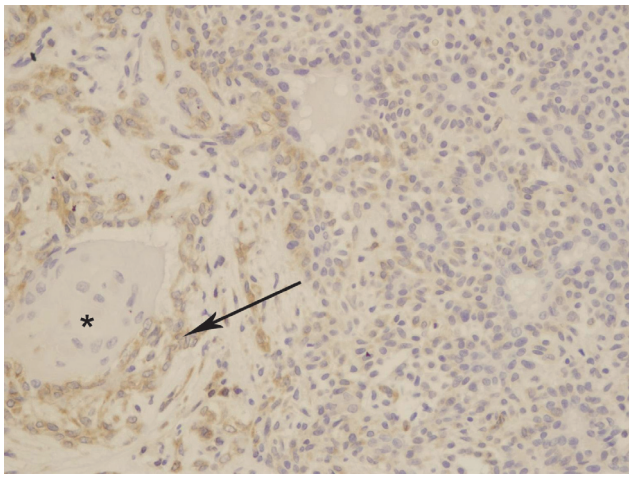

Fig. 2. Squamous cell metaplasia in pleomorphic adenoma. Negative bcl-2 immunoreaction of intercalated-duct like structures on the right side of the picture. Note a squamous cell metaplasia (asterisk), surrounded by bcl-2 positive intermediate cells (arrow). Original magnification, x 200.

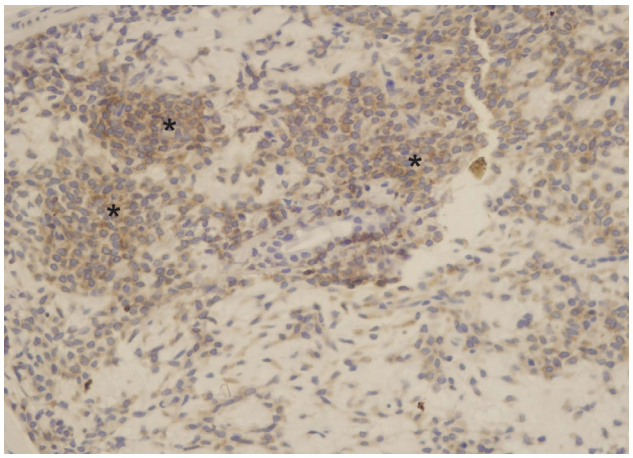

Fig. 3. Bcl-2 in intermediate cells. Moderate to strong bcl-2 immunoreaction of intermediate cells (asterisks) forming solid epithelial tumour areas. Original magnification, x 200.

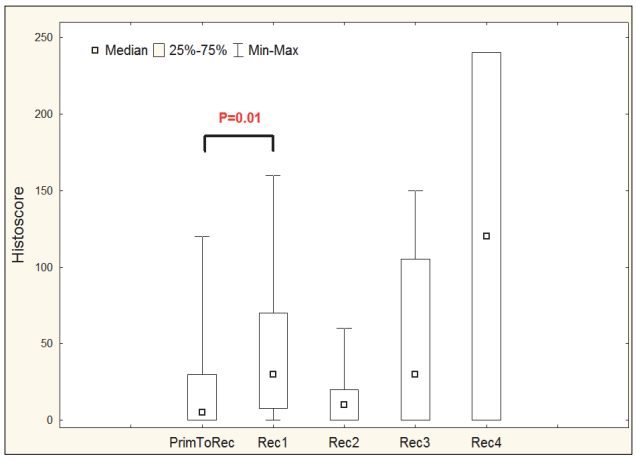

Fig. 4. Bcl-2 in aggressive recurrent pleomorphic adenoma. Aggressive multiple recurrent pleomorphic adenoma with a high bcl-2 histoscore. A myxoid nodule (asterisk) infiltrating soft tissue of the neck is composed almost exclusively of strong bcl-2 positive spindle and stellate cells. Bcl-2 positive tubular structures can be seen in the upper part of the fine tumour capsule. Original magnification, x 200.

614, DAKO, Glostrup, Denmark), using a high temperature epitope retrieval technique. A 4-point scale 0, 1+, 2+ and 3+ (no, weak, moderate and strong, respectively) was used for the evaluation of bcl-2 staining positivity, with the latter being arbitrarily considered equal to that of the infiltrating lymphocytes, present in the adjacent soft tissues and/or in the tumour tissues. Bcl-2 cytoplasmic staining was considered positive. The bcl-2 expression of cells of the epithelial tubulo-ductal and solid formation was assessed qualitatively, while for the spindle and stellate cells of myxoid regions, a semi-quantitative histoscore - defined as the multiple of the percentage of cells by the intensity of immunostaining - was applied[28]. Evaluation of the immunohistological reaction was performed by two experienced pathologists (AK, JD). The differences in the histoscore between PrimNonRec and PrimToRec groups were statistically evaluated using the Mann-Whitney U-test. The comparison between PrimToRec and RecTumour group was done using the Wilcoxon paired test.

RESULTS

Overall bcl-2 positivity

Widely varying bcl-2 immunoreaction was found in the epithelial areas of 91.7% of primary and in 85.2% of recurrent pleomorphic adenomas. Again, greatly varying, although much lower, immunopositivity was found in the myxoid areas (62.5% of primary and 71.4% of recurrent tumours).

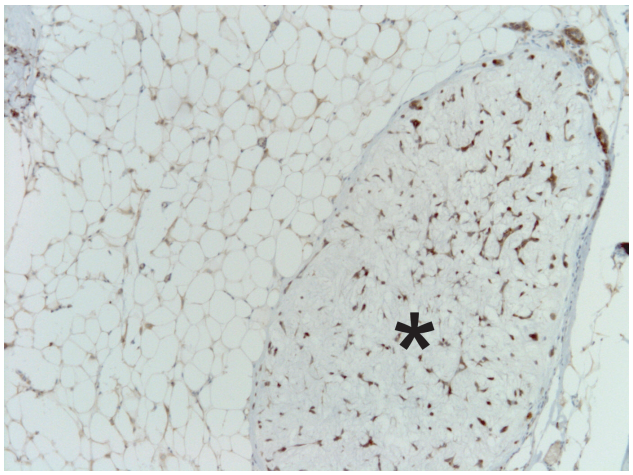

Fig. 5. Box&whiskers graph of histoscore in recurrent and non-recurrent primary tumours. Histoscore in mesenchymal cells of primary and recurrent pleomorphic adenomas shows a significant difference between the PrimToRec group and the first recurrence group (RecTumour).

Pattern of bcl-2 positivity

The most intensive 3+ immunostaining was found focally in the outer cells of well-differentiated excretory duct resembling formations. The cells of the inner layer were always negative (Fig. 1). Nearly all cells of the two-layered intercalated duct-like structures revealed negative bcl-2 immunostaining. Only rarely were some outer elements weakly stained with the antibody, the occasional foci of squamous cell metaplasia were bcl-2 negative (Fig. 2). In some tumours, strong staining was present in a cluster of cells, present in or close to the capsule. Weak to moderate reaction showed cells at the periphery of excretory-like ductal formations as well as "intermediate" cells in some unstructured solid regions (Fig. 3). No obvious differences in the bcl-2 staining intensity and the pattern of specific epithelial morphologic structures in primary-non-recurrent, primary-to-recur and recurrent tumours were found.

Quantity of the stroma according to Seifert's classification

Four out of 10 (40.0%) in the PrimNonRec Group; 10 out of 14 (71.4%) tumours in the primary-to-recur group and 10 out of 16 (62.5%) first and all second and further recurrences were classified as a stroma-rich subtype by Seifert[29].

Bcl-2 expression in epithelial areas

The differences in the bcl-2 histoscore between the primary-non recurrent group and the primary-to-recur group were not statistically significant (P=0.51, Mann-Whitney, Table 1).

The histoscores in the epithelial cells did not change from primary tumours to subsequent recurrences (P>0.05, Wilcoxon).

Bcl-2 expression in "stromal" areas

Positive bcl-2 immunoreaction of the mesenchymal areas (composed of spindle and stellate cells) was present in 7 out of 10 (70.0%) primary-non recurrent and 8 out of 14 (57.1%) primary-to-recur tumours, and in 20 of all 28 recurrent lesions (71.4%), respectively. The immunostaining of particular cells varied greatly fromow this negative to strongly positive. The highest bcl-2 histoscore of 240 was recorded in the fourth recurrence of a parapharyngeal pleomorphic adenoma, aggressively infiltrating the soft tissues of the neck (Fig. 4). The differences in the bcl-2 histoscore between the primary-non-recurrent group and the primary-to-recur group were not statistically significant (P=0.62, Mann-Whitney, Table 1). The histoscores in mesenchymal cells increased from primary tumours to subsequent recurrences. Statistical significance was demonstrated when comparing samples from primary-to-recur tumours and from their corresponding first recurrences (Table 2, Fig. 5, P=0.01, Wilcoxon). Despite the clear tendency in Fig. 5, subsequent recurrences were not statistically significant.

DISCUSSION

In our study, the majority (>90%) of all primary pleomorphic adenomas showed the presence of bcl-2 positive cells. Gordon-Nunez[25] found 37% and Al-Rawi[26] only 30% of their cases to be bcl-2 positive. However, Cruz-Perez[22], Aoki[20] and Yanez[19], reported immunoreaction in 87%, 94% and 100% of tested tumours, respectively, which agrees with our data. As such, we agree with the Yanez[19] that the bcl-2 gene plays an important role in the oncogenesis of pleomorphic adenoma.

In our cases, the most intensively bcl-2 stained cells were localized almost exclusively in the outer layer of the excretory-duct-like formations, while the inner cells, along with all the cells of the intercalated-duct-resembling neoplastic structures, were bcl-2 negative. The immunoreaction of cells located at the periphery of the neoplastic excretory ducts and those present in some solid clusters, varied widely from negative to 2+ positive. A similar bcl-2 pattern of cells in the epithelial areas of pleomorphic adenomas was also reported by other authors. Debiec-Rychter[24] demonstrated intensive immunolabelling in duct-like structures, solid tumour regions as well as in the cells adjacent to reaction-free intercalated-duct-like structures. Yanez[19] found strong bcl-2 positivity mainly in duct-like structures and less in solid and trabecular formations. Pammer[23] detected intense expression in basal cells of duct-like structures and no expresion in intercalated-duct-like structures, while the immunoreaction of solid and trabecular formations was present to various degrees.

The intensity of bcl-2 immunostaining of the individual spindle and stellate cells and the histoscore of "stromal" components of the tumours in our study greatly varied in all test groups, with the majority showing minimal expression. Similar findings were reported by others, demonstrating either no[25] or very weak[19,23] immunoreaction in the myxochondroid areas of primary pleomorphic adenomas.

The strongest bcl-2 immunoreaction was observed in the outer cells of neoplastic excretory duct-like structures. This finding supports the unicellular theory of the oncogenesis of pleomorphic adenoma, thought to arise from multipotential reserve (stem) cells of normal salivary tissue, located at the periphery of excretory ducts[30]. Moreover, this staining pattern is in agreement with Dardick's histogenetic model of pleomorphic adenoma[31], in which these (strongly bcl-2 positive) immortalized progenitor cells give rise to normally differentiated (hence bcl-2 negative) inner cells of neoplastic ducts as well as to „stromal" cells. The latter, however, lose their epithelial features in favour of some mesenchymal ones, follow "abnormal" differentiation pathways[26] and thus show a very inconsistent bcl-2 reaction.

Sunardhi-Widyaputra studied the distribution of bcl-2 positive cells in a group of primary, primary-to-recur and recurrent pleomorphic adenomas. In the former, uniform immunoreaction was present in the cells of the outer layer of the tubulo-ductal structures, as well as in adjacent cells[27]. More remote spindle cells forming the mesenchymal "stroma" of tumors were partly bcl-2 positive. The tumours of the latter two groups were composed predominantly of myxoid component, with the majority of cells showing a strong bcl-2 reaction. Unfortunately, that study lacks statistical evaluation of the immunoreaction between particular tumour groups. However, the results correspond with those of our study, revealing significantly higher bcl-2 expression in stromal areas of the recurrent tumours compared to the PrimToRec group. The highest bcl-2 histoscore was found in our patient with a locally aggressive, largely myxoid pattern showing recurrent pleomorphic adenoma.

As the myxochondroid pattern is typical for a recurrent pleomorphic adenoma[32], we can speculate that it originates from the bcl-2 positive spindle or stellate cell progenitors, protected from the apoptotic processes. Moreover, these long-lived bcl-2 positive elements might eventually undergo hitherto unknown molecular events responsible for their invasiveness[32], culminating in metastasis.

The significantly increased bcl-2 histoscore in the "stromal" cells of recurrent pleomorphic adenomas, amongst which one greatly invasive tumour revealed extraordinarily high bcl-2 values, implies that the latter might be a marker of aggressiveness of these tumours. However, the impact of bcl-2 overexpression on the clinical behaviour of tumors varies considerably. While in prostate carcinoma strong immunoreaction was demonstrated to be an important predictor of recurrence and poor prognosis[33], in colorectal[34] and breast cancers[35] it marks a more favourable outcome. Currently, we have only limited data regarding the prognostic significance of bcl-2 expression in salivary carcinomas. Manjunatha[36], reported a greater and more intense reaction in malignant versus benign salivary neoplasms. In adenoid cystic carcinoma no correlation was demonstrated between the bcl-2 expression and the clinical stage or histological grade[37,38].

The possible role of bcl-2 positive "stromal" cells as progenitors of tumour recurrences seems to be further supported by the demonstration of dysregulation of adhesion proteins[39,40]. Individual cells may therefore, be easily widely seeded in adjacent tissue after capsular rupture, causing the typical multiplicity of a recurrent pleomorphic adenoma. Moreover, the reported minimal proliferative activity of these cells might explain why some recurrent tumours manifest themselves clinically many decades after the incomplete resection of the primary[41,42].

In our study, as well as in that of Sunardhi-Widyaputra[27,43], intensely bcl-2 stained cell formations were focally present in or adjacent to the capsule of pleomorphic adenomas. These peripheral foci, in addition to the "stromal" bcl-2 positive cells, may also give rise to a tumour recurrence, providing they were left behind perioperatively.

CONCLUSION

The results of our study confirm a significant role of the bcl-2 oncogene in the tumourigenesis of salivary pleomorphic adenoma. In the development of a recurrence, bcl-2 positive "stromal", easily implantable cells seem to play a primary role, thus explaining the prevalence of the myxoid component in recurrent pleomorphic adenomas. We can hypothesize that the immortalized, more aggressive bcl-2 positive cells may undergo currently unknown molecular events, associated with a definite metastatic potential. Further molecular research is necessary to elucidate the biological reality of recurrent salivary pleomorphic adenoma.

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Author contributions: KO, IS: manuscript writting and literature search; AK: figures; KO, IS, MS: study design; KO: data collection; KO, IS, RS: data analysis and interpretation; RS: statistical analysis; AK, JE, JD: pathological evaluation; KO: final approval.

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