

Immunohistochemical study of bcl-2 protein, Ki-67 antigen and p53 protein in epithelium of glandular odontogenic cysts and dentigerous cysts

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Abstract: The aim of the present study was the evaluation of the immunohistochemical expression of the apoptosis-inhibiting protein bcl-2, the cell-cycle-related antigen Ki-67 and the p53 protein, which is involved both in cell cycle and apoptosis regulation, in the lining epithelium of glandular odontogenic cysts of the jaws. Formalin-fixed and paraffin-embedded tissue sections of three glandular odontogenic cysts and six dentigerous cysts were immunostained with a standard avidin-biotin peroxidase procedure, after microwave antigen retrieval. The glandular odontogenic cysts showed immunoreactivity for bcl-2 protein in the basal and suprabasal layers, while staining in dentigerous cysts was basal or focal. Most mucous cells and superficial cuboidal cells were negative. The percentage of Ki-67- or p53-positive cells was lower in glandular odontogenic cysts compared with dentigerous cysts. The findings suggest that the biological behavior of glandular odontogenic cysts may be associated with deregulation of cell death in the lining epithelium, while cell proliferation and p53 status do not seem to play a significant role.

Key words: apoptosis; bcl-2; cell proliferation; dentigerous cyst; glandular odontogenic cyst; Ki-67 antigen; odontogenic cyst; p53 protein; sialo-odontogenic cyst

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The glandular odontogenic cyst is a developmental jaw cyst with characteristic histopathological features and biological behavior, established as a distinct entity by Gardner et al. in 1988 (1). The lesion has also been described as sialo-odontogenic cyst (2), mucoepidermoid odontogenic cyst (3), and polymorphous odontogenic cyst (4).

Glandular odontogenic cysts are rare, with a total of 47 cases reported up to 1998 (5). Their incidence in two series of jaw cysts was estimated to be 1.3% (6) and 0.012% (7), respectively. They occur within the bone of either jaw, with a predilection for the anterior mandible (5, 8). Middle-aged men are more commonly affected (5). The clinical findings are not specific and radiographic examination shows a well-defined, unilocular or multilocular lucency with scalloped borders (8).

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Histologically (1, 8–10), the cyst is lined with non-keratinized stratified squamous epithelium of varying thickness, presenting a flat interface with the underlying connective tissue. In more or less extensive areas, the superficial layer of the epithelium consists of eosinophilic cuboidal cells, columnar cells, or mucous cells that form papillary projections and fronds. Variable numbers of larger granular cells, ciliated cells and vacuolated cells may be found. Within the thickness of the epithelium there are intraepithelial gland-like structures consisting of mucous cells, and mucin-filled crypts or microcysts lined by cuboidal cells, that are presumed to result from folding of the lining epithelium. Focal thickenings or plaques of the epithelium, where the cells form whorls or spheres, are also seen. The subepithelial connective tissue is usually free of inflammation and may present irregular-shaped calcifications or islands of odontogenic epithelium. Rare findings include an association with ameloblastoma (1) and squamous odontogenic tumor-like hyperplasia (11), solid epithelial downgrowths into the cyst wall (8), satellite microcysts (12), hyaline bodies (13) and epithelial ghost cell calcification (14).

Clinical, radiographic and histopathologic similarities between the glandular odontogenic cyst and the botryoid odontogenic cyst have prompted many authors (10–12) to consider both cysts as variants of the same entity, a view not unanimously accepted (2, 5). Differential diagnosis from the rare central mucoepidermoid carcinoma may be difficult (5).

The histogenesis of the lesion is unclear. Derivation from intraosseous salivary gland tissue was initially suggested (2), but histopathological features, as well as the cytokeratin profile, advocate an odontogenic origin (1, 5, 8, 11, 13–15).

Glandular odontogenic cysts grow slowly, but may attain a large size and recur (8). Careful follow-up for at least 3 years is recommended, as most of the recurrences, the frequency of which is estimated to be 21%, appear after the third year (8).

The aggressive biologic behavior of the glandular odontogenic cyst and its propensity for recurrence might be associated with cell kinetics in the lining epithelium, as has been demonstrated in odontogenic keratocysts (10, 17). In fact, certain histopathological features of the glandular odontogenic cyst, i.e., infoldings, microcysts and plaques, are suggestive of active cell proliferation (5, 10). The aim of the present study was to evaluate the immuno-

histochemical expression of the apoptosis-inhibiting protein bcl-2, the cell-cycle-related antigen Ki-67 and the p53 protein, which is involved both in cell cycle and apoptosis regulation, in the lining epithelium of three glandular odontogenic cysts.

Material and methods

Tissues

Three cysts conforming to the histopathological description of glandular odontogenic cyst (9) were retrieved from the files of the Department of Oral Pathology, Faculty of Dentistry, University of Athens. Clinical and radiographic findings are summarized in Table 1. Cases 1 and 2 had been originally diagnosed as a lateral periodontal cyst and a dentigerous cyst, respectively, and were reclassified during a retrospective study of unusual odontogenic cysts accessioned during the period 1986–1990. According to the clinical information sheet of case 2, a “cyst” (the exact pathological diagnosis was not given) had been excised from the same site 7 years earlier. Case 3 was the recurrence of a glandular odontogenic cyst previously reported (8). Six dentigerous cysts from two men and four women with an average age of 42 years (range 8 to 64 years) were included for comparative purposes. The specimens were fixed in 10% formalin for at least 24 h, routinely processed and embedded in paraffin.

Immunohistochemistry

Immunohistochemical detection was performed by a standard avidin-biotin peroxidase procedure. In summary, 5 micrometer thick sections were mounted on siliconized glass slides, air dried and heated at 45°C overnight. After deparaffinization and rehydration, the sections were incubated in 0.01 M citrate buffer in a microwave oven for 15 min for antigen retrieval. The slides were then washed in phosphate-buffered saline (PBS) for 30 min at room temperature and incubated in 0.5% H₂O₂ in methanol for 10 min to block endogenous peroxidase activity. Nonspecific antibody binding was blocked with 3% normal horse serum in PBS. The primary monoclonal antibody to the oncoprotein bcl-2 (clone 124, Dako A/S, Glostrup, Denmark)

Table 1. Clinical and radiographic presentation and follow-up data in three cases of glandular odontogenic cyst

Case no.	Sex	Age(yr)	Radiographic presentation	Follow-up
1	M	26	2.0×2.0×1.0 cm radiolucency between teeth #34 and #35	Not available
2	F	45	2.0×1.0×0.5 radiolucency between teeth #31 to #33	A “cyst” had been excised from the same site 7 years earlier
3	M	34	Radiolucency in the right maxilla	Recurred 2 years and 5 months after excision

was diluted 1:50 in PBS, the monoclonal antibody to the cell proliferation-associated antigen Ki-67 (Mib-1, Biogenex, San Ramon, CA, USA) was diluted 1:30, and the monoclonal antibody to both wild-type and mutant human p53 protein (DO-7, Biogenex) was diluted 1:200. The primary antibodies were applied on tumor sections placed in a humidified chamber at 4°C overnight. The sections were subsequently washed in PBS and processed for detection of the positive immunohistochemical reaction using the avidin-biotin peroxidase system (Vectastain, Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine as the chromogen. Sections were finally counterstained with hematoxylin, cleared and mounted. A breast adenocarcinoma of known positivity to Mib-1, DO-7 and bcl-2 was utilized as a positive control, and sections where the primary antibodies were omitted served as negative controls.

Scoring

The percentage of epithelial cells demonstrating cytoplasmic positivity for bcl-2 protein was estimated in the full length of the lining epithelium and classified as previously described by Singh et al. (18): (–) fewer than 5% positive cells or no staining; (±) 5% to 9% positive; (+) 10% to 24% positive; (++) 25% to 50% positive; and (+++) more than 50% positive. Lymphocytes in each section served as the internal positive control. Epithelial cells with a clear brown reaction product in their nuclei were considered positive for Mib-1 and DO-7 antibodies, regardless of the staining intensity. The percentage of positive nuclei within at least 500 well-defined, consecutive epithelial nuclei, as well as the percentage of positive nuclei in the basal-parabasal cells, were calculated manually at a final magnification of ×400. No baseline values were established. Scoring was performed only in non-inflamed areas. Overlapping nuclei and cells in tangential sections were rejected.

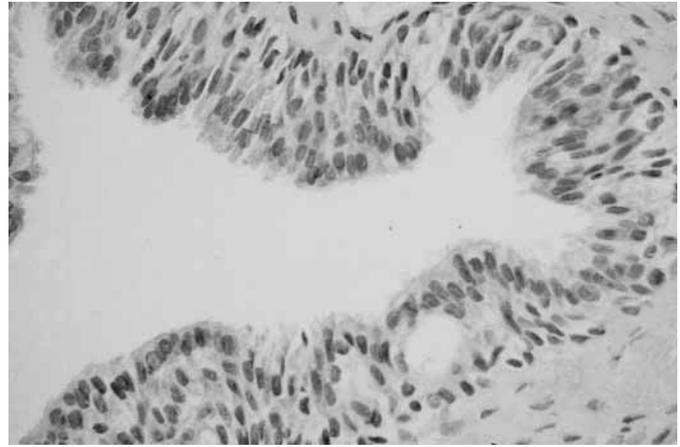


Fig. 1. Homogeneous immunoreactivity for bcl-2 protein in the lining epithelium of a glandular odontogenic cyst (case 1, magnification ×400).

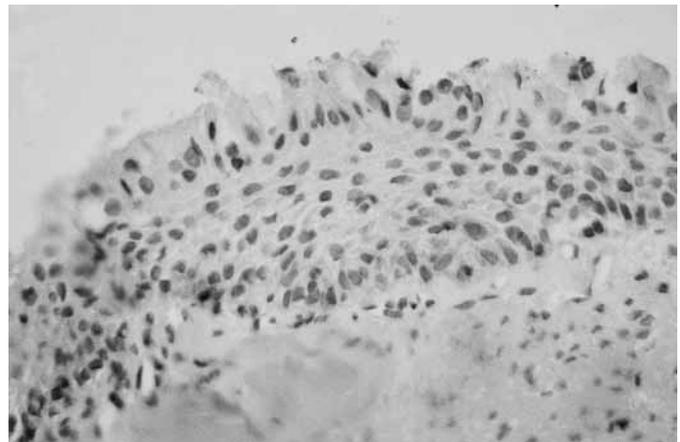


Fig. 2. Diminishing immunoreactivity for bcl-2 protein in the lining epithelium of a glandular odontogenic cyst from the basal layer to the surface. Most superficial cells are bcl-2-negative (case 2, magnification ×400).

Table 2. Immunohistochemical findings in glandular odontogenic cysts (GOC) and dentigerous cysts (DC)

	Case no.	bcl-2	Mib-1		DO-7	
			% of positive cells	% of positive cells in basal layer	% of positive cells	% of positive cells in basal layer
GOC	1	+++	0.78	100.00	0.49	100.00
	2	+++	1.36	85.71	1.27	64.29
	3	+++	3.87	85.71	1.38	100.00
DC	1	–	7.36	97.37	0.40	100.00
	2	–	6.60	97.30	0.10	0.00
	3	–	28.13	93.06	4.66	85.11
	4	++ ^a	8.41	70.45	2.40	83.33
	5	–	11.46	81.36	2.79	64.29
	6	+ ^b	7.82	70.73	0.70	14.29

^aStrong immunostaining in the basal layer and focally in suprabasal layers.

^bWeak immunostaining in the basal layer.

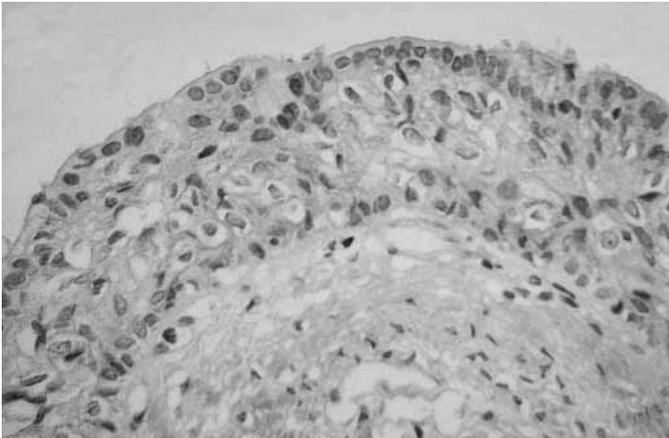


Fig. 3. Lack of immunoreactivity for bcl-2 protein in the superficial cuboidal cells of a glandular odontogenic cyst. Note positive reaction in cell membranes of vacuolated cells (case 3, magnification $\times 400$).

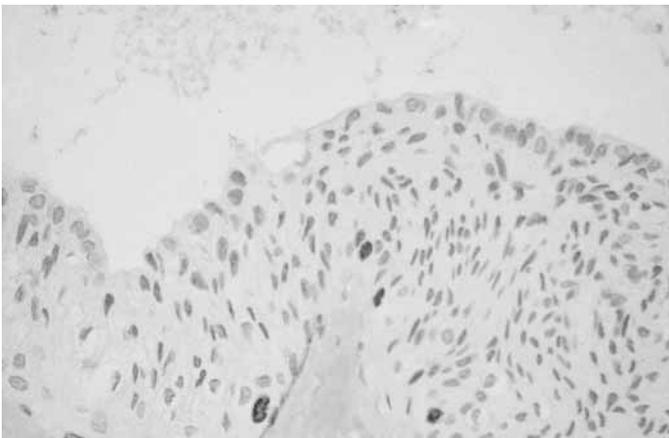


Fig. 4. Scattered Mib-1-positive cells in the basal layer of the lining epithelium of a glandular odontogenic cyst (case 3, magnification $\times 200$).

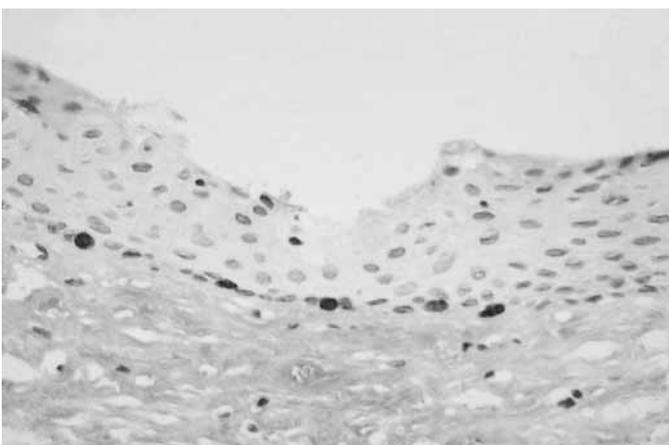


Fig. 5. Mib-1-positive cells in the basal layer of the lining epithelium of a dentigerous cyst (magnification $\times 200$).

Results

Immunohistochemical findings are summarized in Table 2. The glandular odontogenic cysts showed homogeneous immunoreactivity for bcl-2 protein either in the entire thickness of the epithelium (Fig. 1) or in the basal and suprabasal layers with a tendency to diminish towards the surface (Fig. 2). Most mucous cells and superficial cuboidal cells were negative, while vacuolated cells presented a positive reaction in their cell membranes (Fig. 3). A few cells, mainly in the basal layer, reacted for Mib-1 (Fig. 4) or DO-7.

Two dentigerous cysts disclosed faint basal immunostaining for bcl-2 protein, but in the other four cases only isolated foci of weakly or moderately stained basal and parabasal cells were identified. The average percentage of Mib-1-positive cells was $9.04 \pm 7.51\%$ (range 6.60% to 28.13%), and more than 70% of positive cells were basal or parabasal (Fig. 5). A variable number of weakly and strongly DO-7-positive cells, constituting less than 5% of the epithelial population (average percentage $1.44 \pm 1.33\%$, range 0.10% to 4.66%), was mostly seen in the basal area.

Discussion

Bcl-2 proto-oncogene is a member of a gene family that includes cell death suppressors and cell death promoters (19). Its gene product, bcl-2 protein, is a 26 kDa putative membrane-associated protein located on the mitochondrial membranes, the endoplasmic reticulum, the nuclear membrane, and the mitotic nuclei in epithelial cell lines (19). Bcl-2 protein acts as a cell death suppressor that facilitates cell survival by regulating apoptosis (19). Overexpression of bcl-2 protein has been reported in many human tumors (19).

Immunostaining for the apoptosis-inhibiting protein bcl-2 in dentigerous cysts was weak and restricted to the basal layer. Basal expression of bcl-2 protein is seen in normal adult epithelia, where it is associated with the presence of proliferating and stem cells in this layer (18, 19), and has been recently reported in odontogenic keratocysts (20). In the glandular odontogenic cysts, however, intense staining for bcl-2 protein was seen not only in the basal layer but also in the majority of suprabasal cells. This finding may suggest that the epithelial cells of the glandular odontogenic cyst have a prolonged life span (18, 19), whereas a comparable immunoreactivity pattern in hyperplastic odontogenic keratocysts and keratocysts of the nevoid basal cell carcinoma syndrome was considered to reflect the biological behavior of those lesions, including their potential for recurrence (20). However, bcl-2 protein is just one mem-

ber of a family of related genes that contribute to the regulation of apoptosis, and the ability of bcl-2 to inhibit apoptosis depends not only on its expression but also on the expression of other members of the bcl-2 family and their dimerization status (21). Furthermore, alternative bcl-2-resistant apoptotic pathways may also be functional (21).

Lack of expression of bcl-2 in mucous cells of glandular odontogenic cysts was anticipated, as bcl-2 protein is not usually expressed in terminally differentiated cells (18, 19). Bcl-2 protein absence from most superficial cuboidal cells is in accordance with the observation of van Heerden et al. (6) that these cells show nuclear ultrastructural features representative of an apoptotic process.

Ki-67 is a nuclear antigen, which is present in all active parts of the cell cycle (G₁, S, G₂, M) and absent in G₀ (22). Its expression increases with cell cycle progression and reaches its peak during the G₂ and M phases (22). Detection of Ki-67 antigen is considered a reliable marker of cell proliferation (22). Immunohistochemical studies have shown that the numbers of Ki-67/Mib-1-positive cells (23, 24) and proliferating cell nuclear antigen-positive cells (17, 25) are higher in odontogenic keratocysts than in dentigerous and radicular cysts, with the majority of positive cells located above the basal layer (17, 23–25). The quantitative and qualitative differences in the proliferative activity of the keratocyst epithelium are considered suggestive of an intrinsic growth potential that could play a role in its development and biological behavior (17, 24, 25). However, recurrence does not appear to be associated with increased proliferation (24, 25).

Although the glandular odontogenic cyst behaves in an aggressive manner and frequently recurs, the number of Ki-67-positive cells was lower than that of the indolent dentigerous cyst. Thus, it seems possible that the biological behavior of the glandular odontogenic cyst is not associated with cell proliferation. Incomplete removal of the cyst due to its multicystic configuration, tendency of the epithelium to separate from the connective tissue, or growth through the cancellous spaces of bone may account for its high recurrence rate (1, 6, 16).

P53 is a transcription factor (reviewed in Ref. 26) that functions as an integrator of cell responses to various cellular stresses. Adaptive responses elicited by p53 activation include growth arrest and apoptosis. Although positive immunohistochemical detection of p53 protein has been associated with gene mutations, regulatory defects of the *p53* gene may, in some cases, result in overexpression or stabilization of wild-type p53 protein (27).

An increased number of faintly or densely stained p53-positive cells was found in the epithelium of keratocysts in comparison with dentigerous and radicular cysts (28, 29). This accumulation of wild-type p53 protein was significantly correlated with the number and

distribution of Ki-67-positive cells (28, 29) and was not associated with mutations within exons 5-9, as shown by a combination of polymerase chain reaction and single-stranded conformation polymorphism analysis (29). Thus, overexpression of p53 protein was thought to result from overproduction and/or stabilization of wild-type p53 protein due to the increased proliferation of the epithelial cells (28, 29).

DO-7 antibody with antigen retrieval, as used in the present study, is considered the most sensitive and specific procedure for the immunohistochemical detection of p53 protein in formalin-fixed, paraffin-embedded archival tissue (30). DO-7-positive cells were shown in all cysts examined, as has been previously reported for various types of dentigerous cysts (29). However, the glandular odontogenic cysts may be considered p53-negative, as the small number of weakly p53-positive cells found could account for normal cell cycle fluctuations in wild-type p53 protein levels (27).

In conclusion, the immunohistochemical findings in the lesions investigated in the present study suggest that the biological behavior of the glandular odontogenic cyst may be associated with deregulation of cell death in the lining epithelium, indicated by increased expression of the anti-apoptotic protein bcl-2, while cell proliferation and p53 status do not seem to play a significant role. Data from additional cases, as well as the study of the expression of other bcl-2 family members, such as Bcl-x_S, Bcl-x_L, Bax, Bad, Bak and Bag, could provide a more accurate assessment of cell survival in the glandular odontogenic cyst.

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