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Immunohistochemical analysis of the biological potential of odontogenic keratocysts

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BACKGROUND: The aim of this study was to analyse the usefulness of detecting important apoptosis and proliferation markers in assessing the biological potential of odontogenic keratocysts (OKC) and thus selecting the optimal diagnostic algorithm for these lesions.
METHODS: Indirect immunohistochemistry and relevant statistical methods were used for analysis of formalin-fixed and paraffin-embedded samples from 98 patients.
RESULTS: Nevoid basal cell carcinoma syndrome (NBCCS) keratocysts were characterized by higher expression of Bcl-2, $p27^{Kip1}$ and c-erbB-2 as well as by lower proliferative activity measured by Ki-67 in basal cell epithelium and by a lower inflammatory response in comparison with sporadic keratocysts. Dentigerous, radicular and non-specified odontogenic cysts differed from both NBCCS and sporadic keratocysts in a wide spectrum of apoptosis and/or cell cycle-related protein expressions, higher proliferation in the basal cell layer, and vice versa, lower proliferation in the suprabasal cell layer.
CONCLUSIONS: The NBCCS keratocysts have a different immunophenotype from sporadic keratocysts and both types are distinguishable from dentigerous, radicular and non-specified odontogenic cysts. These findings confirm the separate biological potential of these lesions and the results of the immunohistochemical analysis have diagnostic and prognostic implications.
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Keywords: apoptosis; diagnosis; immunohistochemistry; odontogenic keratocysts; oncogenes; prognosis; proliferation

Introduction

Odontogenic keratocysts (OKC) are considered to be developmental abnormalities arising from derivatives of the embryonic dental lamina. Some have the potential for aggressive behaviour and local recurrence (reviewed in Ref. 1). The typical histology of these cysts includes lesions whose epithelial lining is uniformly thin, ranging from 8 to 10 cell layers. The basal layer exhibits a characteristic palisaded pattern with uniform nuclei and the luminal epithelial cells are often parakeratinized. Orthokeratinized foci, however, can also be found. Occasionally budding of the basal cell layer into surrounding connective tissue and the formation of microcysts can be seen. The fibrous cyst wall is relatively thin and usually lacking inflammatory cell infiltrate. In comparison with this parakeratotic type of keratocyst, orthokeratinized cysts with a prominent granular layer lying immediately below the flat surface have also been described. The latter are less aggressive and have a lower rate of recurrence. Recently, a few new solid variants of OKCs (2) and peripheral OKCs (3) have been reported. OKCs can arise sporadically or in association with the nevoid basal cell carcinoma syndrome (NBCCS) also called the Gorlin syndrome (4, 5). OKCs are unique among odontogenic cysts because of their specific histological features, clinical characteristics and biological behaviour (6–8). They are still a serious clinical and diagnostic problem. Apart from the abnormal developmental origin of these cysts there is some evidence supporting their neoplastic origin (9). Malignant transformation of OKCs is rare but possible (10, 11). Some authors have described an abnormal expression of tumour-suppressor genes and oncogenes in the cystic epithelium but relatively few have focussed on apoptotic mechanisms and proliferation regulation (12–14). For this reason we aimed at more detailed analysis of some apoptotic (Bcl-2, Bax) and cell cycle-related ($p21^{Waf1}$, $p27^{Kip1}$, proliferating cell nuclear antigen (PCNA), Ki-67) markers, the common tumour suppressor (p53) and oncogene (c-erbB-2/HER-2/neu) in a larger cohort of patients sharing this diagnosis in comparison with other studies.

Materials and methods

Specimens

Formalin-fixed and paraffin-embedded samples of lesions were obtained by orofacial surgery from a total

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of 98 patients. The lesions included 57 OKCs (18 samples represented keratocytes arising from five patients sharing Gorlin's nevoid basal cell syndrome; the rest were keratocysts from 39 cases with sporadic OKCs), 10 dentigerous cysts, 29 radicular cysts, 11 non-specified odontogenic cysts involving cases lacking the typical morphology of true OKCs or dentigerous/radicular cysts, where the pathologist was unable to distinguish between them, two epidermoid carcinomas arising inside cysts and two cystic ameloblastomas. Histopathological diagnosis was confirmed by an experienced pathologist using haematoxylin and eosin (H & E) stained sections. Clinical data including the aggressivity of lesions and local recurrence were recorded.

Immunohistochemistry

The expressions of p53, p53 phosphorylated, Bcl-2, Bax, $p21^{Waf1}$, $p27^{Kip1}$, c-erbB-2/HER2/neu and proliferation antigens PCNA and Ki-67 were determined by indirect immunohistochemistry on paraffin sections from tissue blocks using a microwave antigen retrieval method. The monoclonal antibodies used were: anti-Bcl-2 [clone 100; Biogenex, San Ramon, CA, USA; antibody:phosphate-buffered saline (PBS), v:v, 1:50], anti-$p27^{Kip1}$ (clone 1B4, Novocastra, Newcastle-upon-Tyne, UK; 1:25), anti-p53 (clone DO-7; DakoCytomation, Glostrup, Denmark; 1:50), anti-p53 phosphorylated (clone FP3.1, which recognizes the phosphorylated form of p53 at serine 392, kindly provided by Dr B. Vojtesek, Masaryk Memorial Cancer Institute Brno, Czech Republic; 1:200), anti-$p21^{Waf1/Cip1}$ (clone 118, kindly provided by Dr B. Vojtesek, MMCI Brno, Czech Republic; 1:100), anti-PCNA (clone PC10; DakoCytomation; 1:200), anti-Ki67 (clone MIB1; DakoCytomation; 1:25). The polyclonal antibodies used were: rabbit anti-Bax (N-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:100) and rabbit anti-c-erbB-2 (A 0485; DakoCytomation; 1:100). The immunohistochemical results were estimated by two independent histopathologists and were validated using computer image analysis (Laboratory Imaging, Prague, Czech Republic). The percentage of positive cells was calculated from a minimum of 1000 cells and the *H*-score was expressed (% positive cells × intensity of staining: 1, weak; 2, moderate; 3, strong).

Intensity of inflammation

The density of inflammatory cells (lymphocytes) was estimated on the basis of the H & E stain and graded as: 0, without inflammatory cells; 1, focal lymphocyte infiltration; 2, diffuse lymphocyte infiltration.

Staining procedures

Sections were deparaffinized in xylene, rehydrated and endogenous peroxidase was blocked in 5% hydrogen peroxide for 15 min. Following antigen retrieval procedures (10 mM citrate buffer, pH 6.0, 100°C for 15 min in a microwave generator, 750 W), the sections were kept in a container for 30 min to reach room temperature. After washing in distilled water (1 min) and PBS (1 min) sections were incubated in blocking milk buffer (5% dry skimmed milk in PBS, pH 7.6 for 30 min) and incubated with primary antibody at 4°C overnight. Following washing in PBS (3 × 5 min) sections were incubated with horseradish peroxidase (HRP)-labelled polymer conjugated with secondary antibody for 60 min at room temperature (mouse and/or rabbit EnVision System HRP, DakoCytomation). Visualization was performed by the standard diaminobenzidine reaction. The nuclei were counterstained by haematoxylin. Positive and negative controls were included in all runs. The primary antibodies were omitted as negative controls and sections from selected tissue specimens were used as positive controls.

Statistical analysis

The results were analysed using the following: chi-square test for testing independence of variables, and homogeneity with eventual Yates correction. Differences between the expressions of variables were analysed using ANOVA/plus *post hoc* tests/Mann–Whitney *U*-test (Department of Biometry, Faculty of Medicine, Palacký University, Olomouc). Differences which reached significance at the 0.05 level are presented in Tables 1–5.

Results

A significant results are summarized in Tables 1–5. There were significant differences in expression of some markers as between NBCCS keratocysts and sporadic keratocysts, as well as between NBCCS/sporadic keratocysts and dentigerous cysts, between NBCCS/sporadic keratocysts and radicular cysts, between NBCCS/sporadic keratocysts and non-specified keratocysts and between non-specified keratocysts and dentigerous cysts.

Significant differences between NBCCS keratocysts and sporadic keratocysts

The NBCCS keratocysts showed significantly higher expression of Bcl-2, $p27^{Kip1}$, c-erbB-2, and significantly lower expression of proliferation antigen Ki-67 in basal cells than sporadic keratocysts. NBCCS keratocysts also

Table 1 Significant differences between nevoid basal cell carcinoma syndrome (NBCCS) keratocysts and sporadic keratocysts

Marker	*P-value*	*NBCCS keratocysts (mean ± SD; %)*	*Sporadic keratocysts (mean ± SD; %)*
Bcl-2 (basal cells)	0.006	78.6 ± 63.91	44.6 ± 43.89
$p27^{Kip1}$ (basal cells)	0.018	41.3 ± 42.49	17.1 ± 18.85
c-erbB-2 (basal cells)	0.029	61.7 ± 79.09	30.5 ± 45.12
Ki-67 (basal cells)	0.011	6.2 ± 13.59	16.6 ± 22.32
		Cases of grade (0/2; %)	
Intensity of inflammation	0.010	55.6/16.7	28.1/34.4

Table 2 Significant differences between nevoid basal cell carcinoma syndrome (NBCCS)/sporadic keratocysts and dentigerous cysts

Marker	*P-value*	*NBCCS + sporadic keratocysts (mean ± SD; %)*	*Dentigerous cysts (mean ± SD; %)*
Bcl-2 (basal cells)	0.008	57.4 ± 54.25	13.6 ± 28.17
Bax (cytoplasm)	0.007	72.5 ± 30.43	38.0 ± 31.55
PCNA (suprabasal cells)	0.012	46.3 ± 26.09	16.0 ± 19.17
		NBCCS keratocysts	
Bcl-2 (basal cells)	0.0003	78.6 ± 63.91	13.6 ± 28.17
Bax (cytoplasm)	0.017	77.5 ± 26.59	38.0 ± 31.55
Bax (nuclei)	0.044	45.0 ± 65.95	7.7 ± 7.68
$p27^{Kip1}$ (basal cells)	0.017	41.3 ± 42.49	13.6 ± 19.03
c-erbB-2 (basal cells)	0.048	61.7 ± 79.09	24.4 ± 22.56
PCNA (suprabasal cells)	0.014	48.6 ± 27.06	16.0 ± 19.17
		Sporadic keratocysts	
Bax (cytoplasm)	0.015	70.6 ± 32.17	38.0 ± 31.55
PCNA (suprabasal cells)	0.019	45.0 ± 26.01	16.0 ± 19.17

Table 3 Significant differences between nevoid basal cell carcinoma syndrome (NBCCS)/sporadic keratocysts and radicular cysts

Marker	*P-value*	*NBCCS + sporadic keratocysts (mean ± SD; %)*	*Radicular cysts (mean ± SD; %)*
Bcl-2 (basal cells)	0.00001	57.4 ± 54.25	7.5 ± 19.10
Bax (nuclei)	0.011	31.9 ± 45.46	4.0 ± 7.05
$p27^{Kip1}$ (basal cells)	0.008	29.7 ± 34.87	8.8 ± 12.11
c-erbB-2 (basal cells)	0.034	43.5 ± 62.75	18.3 ± 17.86
PCNA (basal cells)	0.006	20.7 ± 23.40	37.7 ± 23.70
PCNA (suprabasal cells)	0.005	46.3 ± 26.09	23.5 ± 19.19
		NBCCS keratocysts	
Bcl-2 (basal cells)	0.0001	78.6 ± 63.91	7.5 ± 19.10
Bcl-2 (suprabasal cells)	0.043	42.5 ± 28.72	11.2 ± 11.17
Bax (nuclei)	0.004	45.0 ± 65.95	4.0 ± 7.05
$p27^{Kip1}$ (basal cells)	0.0005	41.3 ± 42.49	8.8 ± 12.11
c-erbB-2 (basal cells)	0.003	61.7 ± 79.09	18.3 ± 17.86
PCNA (basal cells)	0.01	16.4 ± 25.12	37.7 ± 23.70
PCNA (suprabasal cells)	0.011	48.6 ± 27.06	23.5 ± 19.19
Ki-67 (basal cells)	0.013	6.2 ± 13.59	19.0 ± 29.05
		Sporadic keratocysts	
Bcl-2 (basal cells)	0.001	44.6 ± 43.89	7.5 ± 19.10
PCNA (basal cells)	0.024	22.7 ± 22.74	37.7 ± 23.70
PCNA (suprabasal cells)	0.013	45.0 ± 26.01	23.5 ± 19.19

Table 4 Significant differences between nevoid basal cell carcinoma syndrome (NBCCS)/sporadic keratocysts and non-specified odontogenic cysts

Marker	*P-value*	*NBCCS + sporadic keratocysts (mean ± SD; %)*	*Odontogenic cysts (mean ± SD; %)*
Bcl-2 (basal cells)	0.0004	57.4 ± 54.25	3.1 ± 9.46
PCNA (basal cells)	0.002	20.7 ± 23.40	47.5 ± 31.56
		NBCCS keratocysts	
p53 (nuclei)	0.038	8.6 ± 13.24	1.4 ± 2.07
Bcl-2 (basal cells)	0.00001	78.6 ± 63.91	3.1 ± 9.46
Bax (nuclei)	0.02	45.0 ± 65.95	7.1 ± 11.47
$p27^{Kip1}$ (basal cells)	0.005	41.3 ± 42.49	30.0 ± 28.28
c-erbB-2 (basal cells)	0.029	61.7 ± 79.09	22.0 ± 14.57
PCNA (basal cells)	0.003	16.4 ± 25.12	47.5 ± 31.56
Ki-67 (basal cells)	0.004	6.2 ± 13.59	24.1 ± 28.56
		Sporadic keratocysts	
Bcl-2 (basal cells)	0.006	44.6 ± 43.89	3.1 ± 9.46
PCNA (basal cells)	0.007	22.7 ± 22.74	47.5 ± 31.56
		Cases of grade (0/2; %)	
		NBCCS keratocysts	*Odontogenic cysts*
Intensity of inflammation	0.003	55.6/16.7	0.0/72.7
		Sporadic keratocysts	*Odontogenic cysts*
Intensity of inflammation	0.048	28.1/34.4	0.0/72.7

Table 5 Significant differences between dentigerous cysts and non-specified odontogenic cysts

Marker	*P-value*	*Dentigerous cysts (mean ± SD; %)*	*Odontogenic cysts (mean ± SD; %)*
PCNA (basal cells)	0.029	23.2 ± 23.85	47.5 ± 31.56

revealed significantly lower intensity of inflammation than sporadic keratocysts (Table 1).

Significant differences between NBCCS/sporadic keratocysts and dentigerous cysts
Combined keratocysts (NBCCS plus sporadic) had a higher percentage of Bcl-2 positivity in basal cells and Bax (cytoplasmic location) as well as PCNA in suprabasal cells than the cells of dentigerous cysts. Separated NBCCS keratocysts showed besides the same differences also a higher number of positive basal cells expressing Bax in nuclei, $p27^{Kip1}$ and c-erbB-2. On the contrary, sporadic cysts contained only a higher number of Bax-(cytoplasmic staining) and PCNA (suprabasal layer)-positive cells (Table 2).

Significant differences between NBCCS/sporadic keratocysts and radicular cysts
Combined sporadic and NBCCS keratocysts compared witho radicular cysts were significantly different in higher expression of Bcl-2, $p27^{Kip1}$, c-erbB-2, nuclear localization of Bax and in expression of PCNA in suprabasal cells and in a lower expression of PCNA in basal cell layer. When NBCCS keratocysts were analysed separately, significant differences between expression of the same markers plus a higher expression of Bcl-2 in suprabasal cells and lower expression of Ki-67 in basal cell layer were found. Sporadic keratocysts showed against radicular cysts differences only in higher expression of Bcl-2 and PCNA in suprabasal, and lower expression of PCNA in basal cells (Table 3).

Significant differences between NBCCS/sporadic keratocysts and non-specified odontogenic cysts
Numerous considerable differences between expression of markers (p53, Bcl-2, Bax, $p27^{Kip1}$, c-erbB-2, PCNA, Ki-67 and intensity of inflammation) were found between NBCCS keratocysts and non-specified odontogenic cysts. With the exception of proliferative markers PCNA and Ki-67 in basal cell layer and intensity of inflammation, all other markers were significantly higher in NBCCS keratocysts. On the contrary, the number of significantly different markers between combined NBCCS/sporadic keratocysts and non-specified odontogenic cysts and sporadic keratocysts and non-specified odontogenic cysts was less extensive. Generally, proliferation of basal cell layer and intensity of inflammation were lower and percentage of Bcl-2-positive basal cells was higher in keratocysts compared with non-specified odontogenic cysts (Table 4).

Significant differences between non-specified odontogenic cysts and dentigerous cysts
Only one significant relationship in expression of PCNA between non-specified odontogenic cysts and dentigerous cysts was found (Table 5). Dentigerous cysts revealed a lower level of basal cell proliferative activity measured by expression of PCNA than odontogenic cysts.

Discussion

The significance of immunohistochemical analysis of epithelial cell markers in OKCs has been adequately reviewed by Shear (15). It is generally accepted that some cytokeratins, namely cytokeratin 10, can be valuable markers for differentiation of odontogenic cysts (16–18). Recent research has confirmed the importance of p53, MDM2 and some other markers for histopathological diagnosis (17, 19, 20). Tumour-suppressor p53 plays a key role in processes of DNA repair. Overexpression of p53 in tumour tissues usually demonstrates mutation or changes in the p53 molecule leading to higher stability. The stability of the p53 molecule is also dependent on degree of phosphorylation and therefore the detection of phosphorylated p53 by specific antibody could be useful to evaluate the functional status of this protein. However, we did not find significant differences in expression of the phosphorylated p53 between groups. Effort has also been directed to the study of the basement membrane component (18, 21) and immunocompetent CD1a-positive cells in cystic epithelium (21, 22). It is assumed that proteins of the basement membrane fibronectin and collagens I/III are expressed in OKCs in a non-fibrillar aspect and that laminin and collagen IV reveal discontinuous staining. These results are consistent with the presumption of defective adjacent connective tissue forming the envelope of OKCs as well as with a larger number of antigen-presenting Langerhans cells in better-differentiated orthokeratotic OKCs. However, with the exception of cytokeratin 10, none of the above-mentioned studies presented any useful diagnostic tools for histopathologists.

Much more useful in this respect is a number of studies dealing with expression of cell cycle and apoptosis-related proteins. A few authors have analysed the significance of proliferation antigen expression like PCNA and Ki-67 (12, 19, 23–25). Lo Muzio et al. (12) for example, studied the expression of PCNA in NBCCS and sporadic keratocysts. They found no significant difference between these two groups of lesions not only for PCNA but also for Bcl-2. On the contrary, they reported higher expression of p53 and cyclin D1 in NBCCS keratocysts. Our results confirm their finding for PCNA but not for p53 or Bcl-2. We found p53 expression in sporadic OKCs as well but there was no significant difference between the groups. We discovered that Bcl-2 positivity in NBCCS is significantly higher. These results may have been caused by the higher sensitivity of our detection system (EnVision, Dako-Cytomation) in the case of p53 and by use of a more

specific antibody against Bcl-2 (clone 100; Biogenex). In addition, we have described a new finding of significantly higher expression of endogenous inhibitor of cyclin-dependent kinases $p27^{Kip1}$ and oncogene c-erbB-2 in basal cells of NBCCS keratocysts as well as lower expression of proliferation antigen Ki-67.

Tosios et al. (19) describe higher expression of Bcl-2 and lower expression of Ki-67 in glandular odontogenic cysts compared with dentigerous cysts. Kim et al. (23) reported a greater proliferative potential measured by expression of Ki-67 and higher apoptotic rate in OKCs than in dentigerous cysts. Generally, we can confirm these results as we found a higher expression of antiapoptotic as well as proapototic proteins Bcl-2 and Bax, cell cycle-related protein $p27^{Kip1}$, oncogene c-erbB-2 and proliferative potential measured by PCNA in OKCs than in dentigerous cysts. We also confirmed the direct relationship between proliferative activity and inflammation in odontogenic cystic lesions as did by de Paula et al. (24), Nickolaychuk et al. (26) and Kaplan and Hirshberg (27).

In 1999, Wagner et al. (17) published a study on the expression of p53 in OKCs. However, this promising report was not confirmed later by Carvalhais et al. (20) who described only a higher positivity for MDM2 protein, which is member of the p53-induced family of proteins with regulatory effect against p53 or by Lo Muzio et al. (12) who found the expression of p53 only in NBCCS OKCs. In our study, we confirmed the significance of p53 expression only for differentiation between NBCCS OKCs and non-specified odontogenic cysts. We conclude that NBCCS keratocysts have distinct immunophenotype from sporadic keratocysts. This is characterized by higher expression of Bcl-2, $p27^{Kip1}$ and c-erbB-2 as well as lower proliferative activity measured by Ki-67 in basal cell epithelium and by lower inflammatory response. Dentigerous, radicular and non-specified odontogenic cysts differ from NBCCS OKCs in a wider spectrum of apoptosis and/or cell cycle-related protein expressions (lower expression of Bcl-2, Bax, $p27^{Kip1}$, c-erbB-2) and show higher proliferation in basal cell layers and vice versa lower proliferation in suprabasal cell layers. Sporadic OKCs by contrast, preserve only some NBCCS keratocyst features. However, they can also be used to advantage for precise differentiation from the other above-mentioned types of cysts. We are aware that there are many other apoptotic as well as cell cycle-related markers, which may be useful for estimating the biological potential of OKCs. However, in the present study the relationships described could be a first contribution to better understanding of the pathogenesis of OKCs and to improving histopathological diagnostics.

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