Differential Immunohistochemical Staining in Polymorphous Low Grade Adenocarcinoma and Adenoid Cystic Carcinoma: Bcl-2, MIB-1 and TP53

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Purpose: Overlapping clinicopathological and immunopathological features of polymorphous low-grade adenocarcinoma (PLGA) and adenoid cystic carcinoma (ACC) may present a diagnostic problem, particularly with respect to small tissue biopsies from intraoral lesions. The aim of this study was to compare expression of Bcl-2, MIB-1 and TP53 in PLGA and ACC.

Materials and Methods: Ten PLGA, 11 ACC and 19 normal salivary gland controls were stained with antibodies Bcl-2, MIB-1and TP53. Bcl-2 and TP53 immunostaining was subjectively assessed using semi-quantitative methods previously described in the literature, while a novel objective approach using a digital imaging system was used to assess MIB-1 staining.

Results: There was increased expression of TP53, Bcl-2 and Ki-67 (MIB-1) in ACC, although the differences were not significant. However, MIB-1 was significantly increased in the solid pattern of ACC.

Conclusions: MIB-1 staining may be of value in discriminating between more and less aggressive tumours. Further study of larger series is needed to elucidate differences between PLGA and ACC.

Key words: polymorphous low-grade adenocarcinoma, adenoid cystic carcinoma, MIB-1Bcl-2, TP53

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INTRODUCTION

Polymorphous low-grade adenocarcinoma (PLGA) and adenoid cystic carcinoma (ACC) of salivary glands share several overlapping histological growth patterns, including cribriform, tubular and solid patterns. Additionally, overlapping clinicopathological and immunopathological features of PLGA and ACC may present a diagnostic problem, particularly with respect to small tissue biopsies from intraoral lesions. PLGA occurs almost exclusively in minor salivary gland sites where it is found more frequently than ACC and is exceeded only by pleomorphic adenoma and mucoepidermoid carcinoma (Ellis and Auclair, 1996). The tumour may arise in major glands within a pre-existing pleomorphic adenoma (Wenig and Gnepp, 1991). It accounts for 7-11% of all salivary gland tumours and about 19-26% of all malignancies in minor salivary glands, with a slight female predilection (2:1) and an average age of 59 years (Ellis and Auclair, 1996; Waldron et al, 1998).

ACC constitutes about 4% of all benign and malignant epithelial salivary gland tumours (Tomich, 1991; Ellis and Auclair, 1996). A relatively equal sex distribution is found and the tumour occurs in adults in the fifth, sixth and seventh decades. The parotid gland,

submandibular gland and accessory glands of the palate, in descending order, are the sites of most frequent occurrence (Tomich, 1991; Ellis and Auclair, 1996).

ACC has a much poorer prognosis than PLGA, making differentiation important for therapeutic and prognostic purposes. Although PLGA and ACC have many features in common, PLGA is uncommon in the major salivary glands. Histopathological distinction is therefore mainly a problem in tumours of minor salivary gland origin where small biopsies often contribute to diagnostic difficulties. A number of immunohistochemical studies have attempted to differentiate between PLGA and ACC (Gnepp et al, 1988; Chen et al, 1988; Simpson et al, 1991; Regezi et al, 1991; Lazarro and Cleveland, 2000) but the evidence has been equivocal and inconclusive. A recent review provides an overview and summary of the literature with respect to immunostaining and different immunohistochemical markers found in PLGA and ACC (Darling et al, 2002). The potential discriminating value of subjectively assessed immunohistochemical profiles between cases of PLGA and ACC remains controversial.

The objective of this study was to determine whether the application of various immunohistochemical markers, viz. Bcl-2, TP53 and MIB-1, could demonstrate significant differences in the immunostaining of PLGA and ACC respectively. In particular, MIB-1 expression would be determined using a novel approach by means of an objective measuring system. If such differences could be determined, they would be of practical use in the differential diagnosis of these tumours. Furthermore, significant differences in the expression of these cell growth associated proteins could contribute to the understanding of the molecular pathways that are involved in the development of PLGA and ACC respectively.

MATERIAL AND METHODS

A retrospective study was done of archived material available in the departments of Oral Pathology and Anatomical Pathology of the University of Stellenbosch at Tygerberg Academic Hospital, Tygerberg, South Africa. Representative sections from paraffin embedded blocks of resected specimens were stained with haematoxylin and eosin using routine histochemical techniques. The diagnoses of PLGA and ACC were established based on criteria published in the Atlas of Salivary Gland Tumours, Armed Forces Institute of Pathology (Ellis and Auclair, 1996).

Representative sections from each tumour were immunostained using a routine streptavidin-biotin complex technique and various antibodies that are commonly applied in studies of salivary gland tumours (Table 1). Nineteen cases of normal salivary gland tissue served as controls.

The sections were incubated for 5 minutes with 3% hydrogen peroxide in distilled water, to quench endogenous peroxidase activity. They were then rinsed with distilled water and placed in phosphate buffered saline (PBS) for 5 minutes. Subsequently they were incubated in 5% fat free milk diluted 1:5 in PBS for blocking of non-specific background. The milk was then tapped away and the sections were incubated for 45 minutes with primary antibody diluted optimally in PBS. The antibody was then tapped off and the sections placed in a PBS bath for 10 minutes. The sections were then incubated for 30 minutes with either rabbit anti-mouse (1:250; Dakopatts code No. E354, for MIB-1 and Bcl-2), or swine anti-rabbit (1:500; Dakopatts code No. E353, for TP53) biotinylated immunoglobulins and diluted in PBS. The biotinylated antibody was then tapped off and the sections were placed in a PBS bath for 5 minutes. They were then incubated with ABComplex/HRP (1:500; Dakopatts code No. K0377) for 30 minutes. The complex was then tapped off and the sections were placed in a PBS bath for 10 minutes, incubated for 10 minutes with peroxidase substrate solution, rinsed with distilled water and counterstained with Mayer's haematoxylin. DAB was used as chromogen.

Criteria as documented by Lee and Chou (1998) were used to assess TP53 immunostaining:

Table 1 Antibodies used for immunostaining

Antibody directed against:	Clone	Dilution	Antigen retrieval	Source
p53	CM1, polyclonal rabbit	1:1000	Microwave	Novocastra, UK
Bcl-2	Bcl2/100/D5	1:50	Microwave	Novocastra, UK
Ki-67	MIB1	1:50	Microwave	Dako, Glostrup, Denmark

- No expression (N)=no cells staining
- Weak (W) expression=<30% of tumour cells with strong nuclear staining
- Strong (S) expression=>30% of tumour cells with strong nuclear staining
- Cells with very weak equivocal staining were considered to be negative.

A positive control for TP53 was done according to standard laboratory practice. Strong nuclear staining was deemed present when the stain seen in the tumour cells was equivalent to or greater than the nuclear staining seen in the positive control.

The following criteria were used to assess Bcl-2 immunostaining (Nakopoulou et al, 1998):

0=No staining, staining weaker than in control cells, or <10% of cells with staining at least equal to control cells

1=staining at least equal in intensity to control cells in 10-50% of cells

2=staining at least equal in intensity to control cells in >50% of cells.

For descriptive and comparative purposes, positive immunostaining was grouped into the following categories:

- Weak (score 0)
- Moderate (score 1)
- Strong (score 2).

Intensity of immunostaining in the cytoplasm of tumour cells was compared to intensity of staining of duct cells in normal salivary gland control tissue. Only those tumour cells with at least equal staining intensity were counted.

The staining was assessed by a single examiner, and controlled in order to reduce intraexaminer variability.

Quantification of Ki-67 staining was performed with a Zeiss image analysis system, consisting of a Zeiss Axioskop 2 (Carl Zeiss, Jena, Germany) microscope fitted with a Zeiss Axiocam (Carl Zeiss, Jena, Germany) digital camera and connected to a pentium III 750 MHz computer (Mecer, Johannesburg, South Africa). Image digitalization, enhancement and analysis were performed using the KS 300 release 3.0 image processing software (Carl Zeiss, Jena, Germany). Images for image analysis were captured using a Zeiss AcroPlan x40 objective. Microscope light intensity of the Zeiss Axioskop 2 is automatically temperature corrected to 3200K. Image black and white colour correction was standardised for each specimen. The system was calibrated with a 1mm slide objective micrometer (Nikon Corp, Tokyo, Japan) with 0.01mm (10 μm) divisions using the ×40 Zeiss Acroplan objectives. A macro was created which used RGB segmentation to discriminate between positive and negative stained cells within a defined area. Overlapping cells were resolved by binary erosion and dilation. The number of cells staining positively was counted, expressed as a percentage of the total number of cells in a field. Varying numbers of high power fields (40× objective) per tumour pattern were scored. The average number of fields scored was 15. Acquired data of the different parameters measured were stored in a KS 300 database and exported into Excel 97 (Microsoft Corp, USA) for calculation and basic statistical evaluation.

Pearson's chi-square tests were used to determine the statistical significance of the results for TP53 and Bcl-2, and a Mann-Whitney U test was used to evaluate the results for MIB-1.

RESULTS

Meaningful statistical comparisons could not be drawn between differences in staining in different tumour patterns for Bcl-2 and TP53, so only overall staining (positive and negative) and graded staining (intensity) of the two tumours were compared.

For TP53, only 9 cases of PLGA could be examined because of technical problems. All cases of ACC stained positively, although 8 demonstrated low intensity (weak) staining and only 2 demonstrated high intensity (strong) staining. Four PLGA cases were negative, and 5 cases demonstrated low intensity (weak) staining. This result indicated a difference between the two tumours when graded staining (i.e. low intensity and high intensity) was compared, which approached significance ($p=0.04$; Pearsons chi-square test). In a 2×2 table where only the number of positively staining cases was compared, $p=0.07$ (Pearsons chi-square test), which is of more importance than staining intensity (as suggested by Lazarro and Cleveland, 2000). The latter finding suggests that there is no difference between the 2 tumours with respect to TP53 staining, and further studies including larger numbers of cases are warranted to resolve this issue.

For Bcl-2, only 9 cases of PLGA could be evaluated because of technical problems. Ten cases of ACC stained positively, with all 10 showing high intensity (strong) staining. Five cases of PLGA stained moderately, while 2 showed high intensity (strong) staining, and 2 cases were negative. When the staining intensity between the 2 tumours was compared, the difference was significant ($p=0.002$; Pearson's chi-square test) showing that ACC stains more intensely for Bcl-2. In a 2×2 table where only positive staining between the 2

tumours was compared, the difference was not significant (p=0.4; Pearson's chi-square test). Again, the latter is of more importance than staining intensity.

In 9 cases of PLGA, an average of 6.2% (range 0.2–14.3%; standard deviation 5.4) of cells stained positively for MIB-1, compared with 13.9% (range 1.3–37.3%; standard deviation 11.7) of cells in 11 cases of ACC. A Mann-Whitney U test showed that this difference approached significance (p=0.06). T-tests for independent patterns showed no difference in MIB-1 expression for the duct and cribriform patterns (p=0.73 and 0.21 respectively) of PLGA and ACC, but its expression was increased in the solid pattern of ACC when compared with PLGA (p=0.03). This is significant in that the solid variant of ACC generally demonstrates behaviour that is more aggressive.

DISCUSSION

Many premalignant and malignant lesions of human tissues express TP53 oncoprotein. In the current study, TP53 levels were higher in ACC than in PLGA, with the difference approaching statistical significance. This finding may correlate with ACC being the more aggressive tumour. It is also consistent with the fact that mutant TP53 expression does not direct malignant cells to undergo apoptosis. However, the results indicate that because the marker is expressed by both tumours, it could not be used as a differentiating factor between PLGA and ACC. Lazarro and Cleveland (2000) expressed the opinion that well-differentiated tumours did not show differential expression of TP53 nor of Bcl-2, and that these markers may be useful for tumours showing dedifferentiation and more aggressive behaviour. Nordqvist et al (2000) previously reported that TP53 could be useful for distinguishing between benign and malignant salivary gland tumours. In contrast to the current study, these authors found that a higher prevalence of TP53 overexpression (nuclear staining exceeding 10%) was observed in polymorphous low grade adenocarcinoma than in ACC. However, TP53 involvement in the development of both PLGA and ACC is controversial, as some authors report a high prevalence (Yamamoto et al, 1998; Castle et al,1999) while others report a low prevalence (Lazarro and Cleveland, 2000; Kiyoshima et al, 2001).

The Bcl-2 gene family plays an important role in the regulation of cell death and survival, without affecting cell proliferation. Overexpression of Bcl-2 protects epithelial cells from death, but it cannot immortalise cells nor cause tumorigenesis in immortalised cells (Lu et al, 1996). However, overexpression of the apoptosis inhibitor Bcl-2 and subsequent cooperation with cellular and viral oncogenes appears to be an early event in carcinogenesis (Coultas and Strasser, 2003). The difference in the intensity of expression of Bcl-2 by PLGA and ACC seen in the current study may offer a potential benefit in establishing a diagnosis of ACC versus PLGA and warrants further investigation. Other studies which have reported Bcl-2 overexpression in PLGA include Perez-Ordonez et al (1998) in 17 cases (100%), and Castle et al (1999) in 39 cases (100%). These studies did not include ACC and further studies of comparative Bcl-2 expression are required.

Several investigators have examined the expression of the cell proliferation marker MIB-1 (Ki-67). Skalova et al (1997) found that MIB-1 expression was significantly higher in ACC (mean value of 21.4%; range 11.3–56.7) than in PLGA (mean MIB-1 value of 2.4%; range 0.2–6.4). Lazarro and Cleveland (2000) found a relatively low percentage of cells expressing MIB-1 in PLGA and ACC (0% and 11.8% respectively, compared with 6.2% and 13.9% in the current study) although much of the positive staining was high intensity, and suggested that the difference would only be of benefit in identifying more aggressive lesions. Castle et al (1999) found that 74.4% of PLGA expressed MIB-1 in their study. In the current investigation, MIB-1 expression was seen in a higher percentage of cells in ACC than in PLGA, in line with the findings of Lazarro and Cleveland (2000), and Skalova et al (1997). Logical thought suggests that ACC, being a more aggressive tumour, should show a higher cellular expression of MIB-1. This is in some way confirmed in the current study by the increased expression of MIB-1 in the solid variant of ACC, when compared with the non-solid variants and to its expression in PLGA. It is generally accepted that the solid variant of ACC is more aggressive, and has a poorer prognosis. The morphometric analysis using the KS 300 release 3.0 image processing software provided a rapid and accurate objective means of assessing the percentage of cells staining for MIB-1. It was considered an advantage over an 'eyeballed' subjective semi-quantitative method, both in terms of time saved and precision of quantification. To our knowledge, this method has not been used previously for similar studies.

In conclusion, the potentially useful differences of immunostaining in PLGA and ACC are increased Bcl-2 and MIB-1 expression in ACC; these markers could provide useful information pertaining to the prognostication and management of patients. The number of cases examined in the current study probably precludes

a definitive conclusion, and the overlap between PLGA and ACC is a limitation. Perhaps it is necessary to accept that the final diagnosis of such tumours rests on careful microscopic examination of the entire resected specimen, and that these markers may aid in discriminating between more and less aggressive tumours, as Lazarro and Cleveland (2000) suggested. Even the recently suggested potentially useful marker CD117 (the product of the c-kit proto-oncogene) does not distinguish unequivocally between PLGA and ACC (Edwards et al, 2003). Further research using larger numbers of cases is warranted for clarification of these issues.

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