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The Role of DNA Ploidy and Ki-67 in the Grading of Mucoepidermoid Carcinomas

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Abstract. *Background: The grading of mucoepidermoid carcinomas (MEC) is based on subjective microscopic evaluation of the prevalence of cell types as well as features of atypia and aggressiveness. Our study was aimed at evaluating the role of high-resolution DNA flow cytometry and Ki-67 expression in the grading of MEC. Materials and Methods: Fifty-five cases of intraoral and major salivary gland tumours, diagnosed as MEC, were retrieved and the grading system proposed by Brandwein et al. applied. Results: Forty-nine per cent of our sample was graded as high, 35% as intermediate and 16% as low. Eighty-nine per cent of the high-grade MEC showed aneuploid DNA cell populations, while 88% of the diploid tumours were graded as intermediate or low. The mean Ki-67 positivity was significantly different between the high and intermediate grade tumours and between the aneuploid and diploid tumours. Conclusion: This study showed that high-resolution DNA flow cytometry of archival paraffin-embedded tissue is accurate in the grading of MEC and can be used with Ki-67 expression as an additional diagnostic tool.*

Mucoepidermoid carcinoma (MEC) constitutes almost 10% of all salivary gland tumours and is one the most common malignant salivary gland neoplasms, comprising approximately 35% of malignant salivary gland tumours (1). MECs are characterised by the presence of mucous cells, epidermoid cells and intermediate cells and vary histologically from solid, mainly epidermoid tumours to cystic, predominantly mucous cell tumours. Grading of MEC is based on subjective microscopic evaluation of the prevalence of the different cell types as well as features of atypia and aggressiveness (2-5). A more objective, weighted histological grading method was recently proposed (6), which was modified by Brandwein *et al.* (7), who evaluated the interobserver reproducibility of this method.

Even though the prognosis of MEC correlates with the histological grade, the occasional presence of metastases in low-grade tumours (2, 8, 9) demonstrates that low-grade histological features are not always indicative of low-grade biological behaviour. DNA ploidy status was found to be an important parameter in predicting the biological behaviour of MECs (10, 11), while conflicting results regarding the prognostic capability of the proliferation index through immunohistology were established (7, 12, 13).

The objective of this study was to evaluate the role of high-resolution DNA flow cytometry and Ki-67 expression in the grading of MEC using the modified grading method proposed by Brandwein *et al.* (7).

Materials and Methods

Intraoral and major salivary gland tumours, diagnosed as MEC, were retrieved from the files of the departments of Oral Pathology and Anatomical Pathology at the University of Pretoria and the department of Oral Pathology at the Medical University of Southern Africa. The diagnosis of all cases was reviewed and the grading system proposed by Brandwein *et al.* applied.

Sections from the same tumour block used for grading were used for DNA ploidy analysis. The sections were prepared according to the modified Hedley method (14). In short, four to six 40-μm sections were cut, wrapped in 50-μm nylon mesh, placed in a histocasssette and manually dewaxed and hydrated in distilled water. The sections were left in distilled water overnight, before being digested in subtilisin Carlsberg solution at 37˚C for 120 minutes. The cell suspension was then stained with DAPI (4'6 diamidino 2 phenylindole) (Research Organics, Cleveland, OH, USA). Flow cytometry was carried out using a PAS II flow cytometer equipped with a high-pressure 100W mercury lamp (Partec, Münster, Germany). DNA histograms of at least 10,000 cells were plotted. The diploid cell population was used as an internal reference standard for the identification of aneuploid clones.

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Table I. *Anatomical site, mean age of patients and male to female ratio of the MECs.*

Tumour site	No. (%)	Mean age	M:F
Major salivary glands	30 (55)	52.7	16:14
Parotid	25 (45)	49.4	13:12
Submandibular	5 (9)	65.4	3:2
Minor salivary glands	24 (43)	47.3	12:12
Palate	7 (13)	43.8	3:4
Retromolar area	5 (9)	60.4	3:2
Buccal mucosa	4 (7)	48.7	3:1
Floor of mouth	3 (5)	41.3	0:3
Tongue	1 (2)	41	0:1
Tuberositas	1 (2)	9	1:0
Not specified	3 (5)	64	2:1
Intra bony	1 (2)	57	1:0

M: male, F: female.

Table II. *Histological grading and DNA ploidy of the different MEC.*

Tumour site	N	Grade 3	Grade 2	Grade 1	Ploidy Aneuploid	Ploidy Diploid
Major salivary glands	30	15 (50%)	12 (40%)	2 (10%)	18 (60%)	12 (40%)
Minor salivary glands	24	11 (46%)	6 (25%)	7 (29%)	11 (46%)	13 (54%)
Intra bony	1	1			1	
Total	55	27 (49%)	18 (35%)	9 (16%)	30 (55%)	25 (45%)

N: number.

Table III. *Relationship between the histological grading and DNA ploidy status.*

	Aneuploid	Diploid	Total
Grade 3	24	3	27
Grade 2	4	15	19
Grade 1	2	7	9
Total	30	25	55

Ki-67 (MM1 clone, prediluted, Novocostra, Newcastle-upon-Tyne, UK) expression was determined following heat antigen retrieval, according to the manufacturer's instructions. The most stained areas in each section were evaluated and positivity expressed as a percentage of cells counted. A craticule was used to prevent double counting of positive cells. A minimum of 400 cells were counted for each section.

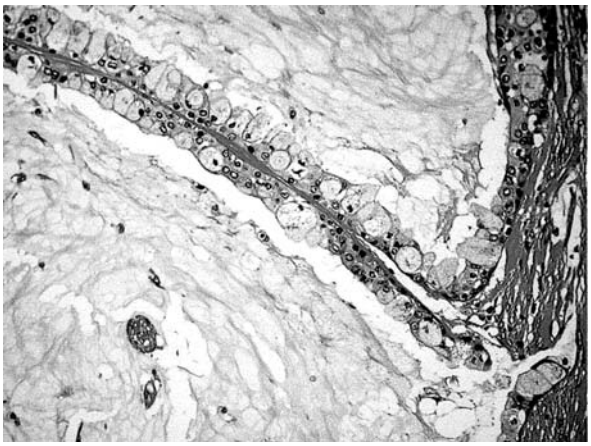

Figure 1. *Grade 1 MEC showing a prominent cystic component consisting mainly of mucous cells. This tumour has metastasised to the cervical lymph nodes. (x250)*

The DNA ploidy status was related to the histological grading in 2X2 contingency tables. The Chi-square with Yates correction and Fischer's exact tests were used for the analysis of the categorical data. The Ki-67 scores between the different histological grades and DNA content were evaluated using the Student's *t*-test. Correlations with $p<0.05$ were considered statistically significant.

Results

Fifty-five cases of MEC were retrieved. Thirty were in the major salivary glands and 24 in the minor glands. One MEC had a primary intra bony origin. The different locations and patient information are shown in Table I.

Forty-nine per cent of our sample was graded as high (grade 3), 35% as intermediate (grade 2) and 16% as low (grade 1) (Table II). Eighty-nine per cent of the grade 3 MECs showed aneuploid DNA cell populations, while 88% of the diploid tumours were graded as 2 or 1 (Table III). This difference was statistically highly significant ($p<0.001$). The coefficient of variance (CV) of the flow cytometry measurements varied between 2.3 and 6.5, with a mean of 3.9 (±0.9). Two of the grade 1 tumours (Figure 1) presented with an aneuploid cell population in a peri-diploid position (Figure 2). One of these had cervical lymph node metastases at the time of presentation.

Both cytoplasmic and nuclear positivity were detected with the Ki-67 antibody. Only nuclear staining was regarded as positive Ki-67 expression. Ki-67 positivity was only seen in 21 of the 55 cases. The positivity ranged from 0.8 to 68. Lack of Ki-67 staining was seen in all grades of MEC. The mean Ki-67 positivity was significantly different ($p<0.001$) between the grade 2 and 3 tumours and between the aneuploid and diploid tumours (Table IV).

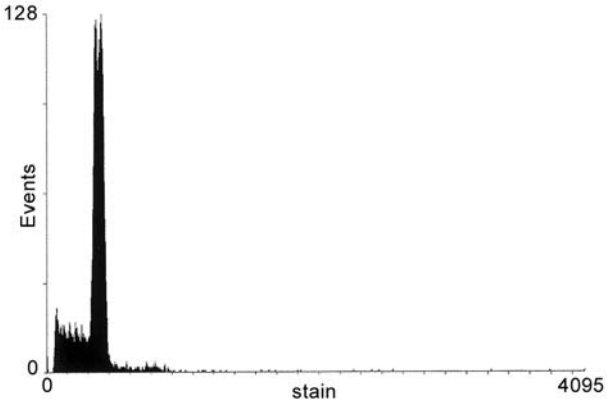

Figure 2. *Aneuploid cell population present in a peri-diploid position of the grade 1 MEC (in Figure 1) presenting with cervical lymph node metastases.*

Table IV. *Ki-67 expression in the different MEC groups expressed as a percentage of cells counted.*

		Mean Ki-67 (number of cases)	Range of positivity	SD
Grade	3	38.3 (10)	9.2-68	18.4
	2	3.5 (8)	0.8-13	4.2
	1	18.7 (3)	9-36	15.0
DNA	Aneuploid	38.3 (10)	9.2-68	18.4
	Diploid	7.3 (11)	0.8-36	9.9

SD: standard deviation.

Discussion

The distribution of MEC and the age group of patients in this study are in agreement with large reported series (15). The parotid was the most commonly affected gland, followed by the minor salivary glands where most MECs occurred on the palate, followed by the retromolar area.

It was difficult to compare the grading of MEC in our study with other studies in the literature due to the different grading methods used. We were in agreement with Brandwein *et al.* (7) in that previously reported grading systems (2-6) tend to underestimate the histological grade of some MECs. Thirty-five per cent of the MECs in the study of Brandwein *et al.* (7) were classified as grade 1, 34% as grade 2 and 30% as grade 3. The percentage of MEC in the intermediate group in our study (35%) was similar to their findings, with the difference being the lower percentage of cases (16%) classified as grade 1. This was mainly the result of only two low-grade MECs in the major salivary glands. A contributing factor might be that, in our experience, patients with head and neck tumours generally tend to present at a late stage for treatment. It is conceivable that longstanding low-grade MECs might develop more solid areas or invading tumour nests and islands that change the grading to grade 2 MEC.

The DNA ploidy status, as determined by flow cytometry, has been shown to be a valuable parameter for evaluating the biological behaviour of MECs (10, 11). A significant correlation between DNA ploidy and histological grading was observed in this study, regardless of the fact that paraffin-embedded tissue was used instead of fresh tumour tissue. One of the tumours in the retromolar area, histologically classified as a grade 1 MEC, presented with cervical lymph node metastases. The primary tumour was cystic in nature, although the metastases contained solid areas. The tumour had, however, shown a peridiploid aneuploid cell population on flow cytometry analysis, even though no criteria of a higher histological grade could be seen on several deeper sections of the primary tumour. DAPI staining contributed to the relatively low CV obtained for the paraffin-embedded material to enable the detection of this peridiploid population. This case would otherwise have been reported as diploid if it were not possible to detect the peridiploid cell population through high-resolution flow cytometry. The DNA ploidy status should not be used to influence the histological grading, but might be an important additional factor in staging of MECs.

Ki-67 expression is generally associated with aggressive behaviour and poor prognosis in MECs (12, 16-18). The absence of nuclear staining for Ki-67 in a large proportion of the tumours (34 out of 55) was also observed by Brandwein *et al.* (7), who found no nuclear staining in 62% of the MECs evaluated. The absence of Ki-67 staining should not be interpreted as low or no proliferative activity or absence of the target antigen. Lack of staining was observed in all histological grades of MECs and was, in all probability a technical drawback; most likely the inability to unmask the required epitope of the target antigen through the prescribed technique. The mean Ki-67 positivity, however, differed significantly between die aneuploid and diploid MECs, as well as between the grade 3 and grade 2 tumours. The three low-grade MECs (Table IV) that showed Ki-67 positivity were all diploid and, although their mean Ki-67 count was higher than the intermediate group of MEC, the number was too small for statistical analysis. It would appear that Ki-67 staining, especially higher than 20% positivity, might be supportive of a high-grade MEC.

This study showed that high-resolution DNA flow cytometry of archival paraffin-embedded tissue of MEC correlated with the grading method proposed by Brandwein *et al.* (7) and, together with Ki-67 expression, could add additional information about tumour behaviour.

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