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DNA ploidy analysis in salivary gland tumours by image cytometry

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AIM: To determine whether DNA ploidy by image cytometry is a good diagnostic tool to distinguish benign and malignant salivary gland tumours.

METHODS: A total of 62 salivary gland tumours were studied. Cases were histologically diagnosed [haematoxylin and eosin (H&E)]. According to the World Health Organization (WHO) classification, there were 14 mucoepidermoid carcinomas (MEC), 11 adenoid cystic carcinomas (ACC), 10 pleomorphic adenomas (PA), 10 carcinoma ex PA (CEPA), 9 acinic cell carcinomas (ACCa), 3 polymorphous low-grade adenocarcinomas (PLGA), 2 papillary cystadenocarcinomas (PC), 1 myoepithelial carcinoma (MC), 1 undifferentiated carcinoma (UC) and 1 mucinous adenocarcinoma (MA). Paraffin sections (40 μm) were micro-dissected to isolate tumour areas; cell nuclei were extracted and Feulgen-stained cytospin monolayers were analysed using a DNA image cytometry system. For each case, DNA index (DI) was calculated relative to internal controls (lymphocytes; DI = 1.0). Cases were categorized as diploid or aneuploid and the proportion of cells over 5c was also calculated.

RESULTS: Fifty-three of 62 salivary gland tumours were uniformly diploid. Only nine cases were aneuploid: five CEPA, one low-grade MEC, one PC, one UC and one MA.

CONCLUSIONS:The vast majority of salivary gland tumours were diploid. High-grade malignancies may be aneuploid, and ploidy may be useful to identify malignant change in atypical PA. Further, larger studies are needed to confirm our results and to further evaluate the usefulness of the technique in high-grade lesions.

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Introduction

Salivary gland tumours are rare with an overall incidence in the Western world of about 2.5–3.0 per 100 000 persons per year (1). About 80% of all tumours are benign, and hence malignancies are particularly rare; comprising less than 5% of cancers of the head and neck (2–5). The parotid gland is the most common site, but the submandibular and sublingual glands can also be affected. About 15% of salivary gland neoplasms arise in intraoral glands (2). Histologically, malignant salivary neoplasms are a heterogeneous group causing some difficulties in diagnosis and prognosis assessment. Additionally, there are no good markers for prediction of their clinical behaviour (1).

Ploidy analysis by flow or image cytometry is able to detect gross genomic aberrations and has been used to determine prognosis in pre-cancerous and cancerous lesions in the mouth, cervix and oesophagus (6–9). For example, diploid oral squamous cell carcinomas (SCC) have a better prognosis than aneuploid cases (7).

According to some authors, DNA flow cytometric parameters have prognostic value in some salivary gland carcinomas (10–12). However, there are few series published in the literature focusing on ploidy analysis for prediction of tumour aggressiveness, prognosis, and for differentiating benign from malignant salivary gland tumours (12, 13).

The purpose of the present study was to use image cytometry to determine whether DNA ploidy analysis is a good diagnostic tool to distinguish benign and malignant salivary gland tumours as well as to correlate the ploidy status with the clinical and histopathological features of these lesions.

Patients and methods

Patient population

Sixty-two salivary gland tumours [52 malignant and 10 pleomorphic adenomas (PA)] were retrieved from the files of the Department of Oral Pathology, University of Sheffield. Information about age, gender, tumour

Table 1 Clinical data and histopathological types of the salivary gland tumours (n = 62)

Tumours	*No. of cases*	*Gender M/F*	*Age (years) mean (range)*	*Size (cm) mean (range)*
Mucoepidermoid carcinoma	14	1 M/13 F	48.1 (25–73)	1.8 (0.7–6.5)
Adenoid cystic carcinoma	11	5 M/6 F	53.4 (26–75)	2.4 (0.7–4.0)
Pleomorphic adenoma	10	4 M/6 F	44.5 (22–68)	3.1 (1.5–5.5)
Carcinoma ex pleomorphic adenoma	10	3 M/7 F	54.4 (43–67)	3.8 (1.3–9.0)
Acinic cell carcinoma	9	4 M/5 F	45.5 (17–77)	3.7 (1.4–8.5)
Polymorphous low-grade adenocarcinoma	3	1 M/2 F	71.6 (61–78)	2.7 (1.4–3.8)
Papillary cystadenocarcinoma	2	2 M	77.5 (74–81)	4.9 (3.5–6.3)
Myoepithelial carcinoma	1	1 M	83	4
Undifferentiated carcinoma	1	1 F	73	1.8
Mucinous adenocarcinoma	1	1 F	44	3
Total	62	21 M/41 F		

No, number; M, male; F, female.

location and size was obtained from the pathology records. The histopathology of all cases was reviewed on haematoxylin and eosin (H&E) stained sections, and tumours were classified according to the World Health Organization (WHO) guidelines (14) by two oral pathologists (PAV and PMS). The types of tumour and data relating to age, gender and size are summarized in Table 1. The study was approved by the South Sheffield Research Ethics Committee.

There were ten pleomorphic adenomas Seven were located in the parotid gland and three in the palate. There were 14 mucoepidermoid carcinomas (MEC). Eleven occurred in the minor salivary glands, two in the parotid and one in the submandibular gland. Eleven were histologically classified as low grade, two as intermediate grade and one case as high grade.

There were eleven adenoid cystic carcinomas (ACC), six from the palate, three in the buccal mucosa, one in the floor of mouth and one in the right nasofacial fold. The predominant histological subtype of ACC was cribriform (eight cases) followed by the solid variant (three cases).

Ten cases of carcinoma ex pleomorphic adenoma (CEPA) were studied, six cases located in the parotid gland, one in the submandibular gland, one in the upper lip, one in the left maxillary tuberosity and one in the nose. Two CEPAs were intracapsular. Nine acinic cell carcinomas (ACCa) were selected. Seven cases occurred in the parotid and two in the submandibular gland. There were three polymorphous low-grade adenocarcinomas (PLGA). Two cases occurred in the soft palate and one in the right cheek. Other malignant tumours studied were two papillary cystadenocarcinomas (PC), which occurred in the left parotid and floor of the mouth, one myoepithelial carcinoma (MC) from the left cheek, one undifferentiated carcinoma (UC) from the buccal mucosa, and one mucinous adenocarcinoma (MA) from the posterior tongue.

DNA image cytometry

DNA image cytometry (IC) was performed on formalin-fixed paraffin-embedded samples of all 62 tumours. Representative tumour areas were identified in an H&E stained section then identified and trimmed in the respective blocks. Three 40 μm sections were obtained of each case. The method described by Hedley (15) was used for cell nuclei extraction from the paraffin sections. The sections were digested with bacterial protease XXIV (Sigma) releasing isolated nuclei, which were then prepared into a monolayer by cytospin. Nuclei were stained with Feulgen's stain and periodic acid Schiff (PAS). The samples were evaluated using a cytometric image analysis system (Fairfield Imaging Ltd, Medical Solutions, Nottingham, UK) that consists of an automated Zeiss Axioscop microscope attached to a digital camera (Hamamatsu C4742/95) connected to a computer that runs two analysis programmes: DNA ploidy v1.3 and Histogram Draftsman v1.4 (Fairfield Imaging Ltd).

Over 2000 nuclei were scanned and a minimum of 400 images of tumour cell-nuclei were manually selected avoiding artefact, folded, overlapping or repeated nuclei. Lymphocytes were used as the standard internal controls. Criteria for ploidy classification were taken from the guidelines of the European Society for Analytical Cellular Pathology (ESACP) (16). DNA histograms were categorized as diploid if the histogram presented a single peak (2c) in the G0-G1 area and the cell nuclei population in 4c did not exceed 10% in the G2 region (4c). A sample was considered aneuploid if clear aneuploid peaks (3c, 5c, 7c and 9c) were present and/or if there were more that 1% nuclei over 5c (5cER) (16). For each case, coefficient of variance (CV) and DNA index (DI) was calculated relative to internal controls (lymphocytes; DI = 1.0). A DI between 0.9 and 1.20 was considered diploid.

The ploidy status was correlated to the benign and malignant status of salivary gland tumours, clinical data and histopathological grade.

Results

DNA content of tumours

The DNA ploidy data are summarized in Tables 2 and 3. Fifty-three of 62 salivary gland tumours were uniformly diploid (Fig. 1). All the PAs were diploid and only 17.4% of malignant salivary gland tumours were aneuploid (8 high-grade lesions and 1 low-grade

Table 2 DNA ploidy status in benign and malignant salivary gland tumours

Tumours	*No. of cases*	*Diploid (%)*	*Aneuploid (%)*
Benign tumours			
Pleomorphic adenoma	10	10 (100%)	0
Malignant tumours	52	43 (82.6%)	9 (17.4%)
Mucoepidermoid carcinoma	14	13 (92.8%)	1 (7.2%)
Low-grade	11	10	1
Intermediate-grade	2	2	0
High-grade	1	1	0
Adenoid cystic carcinoma	11	11 (100%)	0
Cribriform variant	8	8	0
Solid variant (high-grade lesions)	3	3	0
Carcinoma ex pleomorphic adenoma (high-grade lesions)	10	5 (50%)	5 (50%)
Acinic cell carcinoma (low-grade lesions)	9	9 (100%)	0
Polymorphous low-grade adenocarcinoma	3	3 (100%)	0
Papillary cystadenocarcinoma (high-grade lesions)	2	1 (50%)	1 (50%)
Myoepithelial Carcinoma (low-grade lesion)	1	1 (100%)	0
Undifferentiated Carcinoma (high-grade lesion)	1	0	1 (100%)
Mucinous Adenocarcinoma (high-grade lesion)	1	0	1 (100%)
Total	62	53 (85.4%)	9 (14.6%)

Table 3 DNA index (DI) and 5cER values of the nine aneuploid cases (n = 62). All nine cases showed at least two clear distinct aneuploid peaks.

Aneuploid malignant salivary gland tumours	*No. of cases*	*DI*	*5cER (%)*
Carcinoma ex pleomorphic adenoma (high-grade lesions)	5	1.32	1.52
		1.40	2.50
		1.40	3.26
		1.72	0.24
		1.78	14.29
Mucoepidermoid carcinoma (low-grade lesion)	1	2.03	7.55
Papillary cystadenocarcinoma (high-grade lesion)	1	1.86	31.51
Undifferentiated carcinoma (high-grade lesion)	1	1.21	1.32
Mucinous adenocarcinoma (high-grade lesion)	1	1.20	1.86
Total	9		

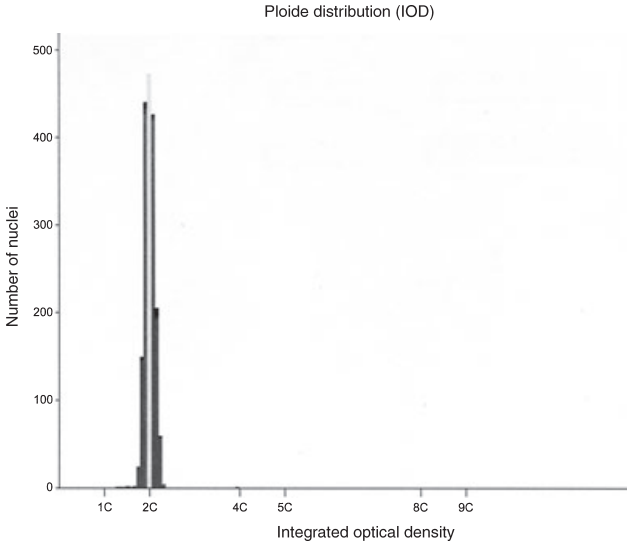

Figure 1 Histogram of a DNA diploid acinic cell carcinoma displaying one clear peak on 2c and scarce cells before 2c representing nuclear fragments and apoptotic cells (DI = 1.0, 5CEr = 0).

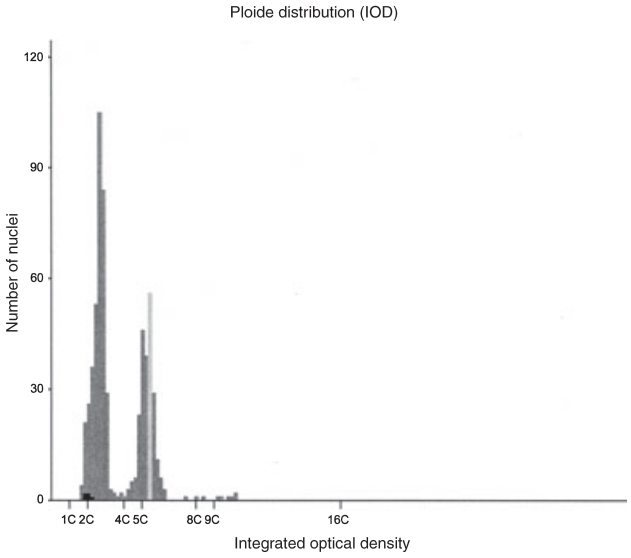

Figure 2 Histogram of a DNA aneuploid papillary cystadenocarcinoma showing two clears distinct peaks in 3c and 5c (DI = 1.86, 5CEr = 31.51).

MEC). Eight of 18 high-grade malignant lesions analysed were aneuploid (44%) and five of 10 CEPAs (50%) were aneuploid (Figs 2 and 3).

CV and DI

The DI of the 53 diploid cases was between 0.95 and 1.19. The DI from the aneuploid cases is shown in Table 3. The CV of the integrated optical density values from the reference cells in G0-G1 fraction was lower than 5% in all the 62 salivary gland tumours. The mean CV and standard deviation for MEC (n = 14) was 1.65% ± 0.78, ACC (n = 11) 2.35% ± 0.88, CEPA (n = 10) 1.35% ± 0.46, PA (n = 10) 0.99% ± 0.67, ACCa (n = 9) 1.51% ± 0.65, PLGA (n = 3) 1.29% ± 0.93, PC (n = 2) 2.26% ± 0.27, MC (n = 1) 1.09%, UC (n = 1) 3.33% and MA (n = 1) 3.32.

Ploidy status and clinicopathological correlation

There was no correlation between clinical data (gender, age, location or tumour size) and ploidy status in our study. Only five cases were known to have recurred. One was a CEPA, which was aneuploid, but the other four were diploid (three cribriform ACC and one MC).

Two of the 52 malignant salivary gland tumours studied have metastasized to cervical lymph nodes. One was an aneuploid MA and the other a diploid ACCa.

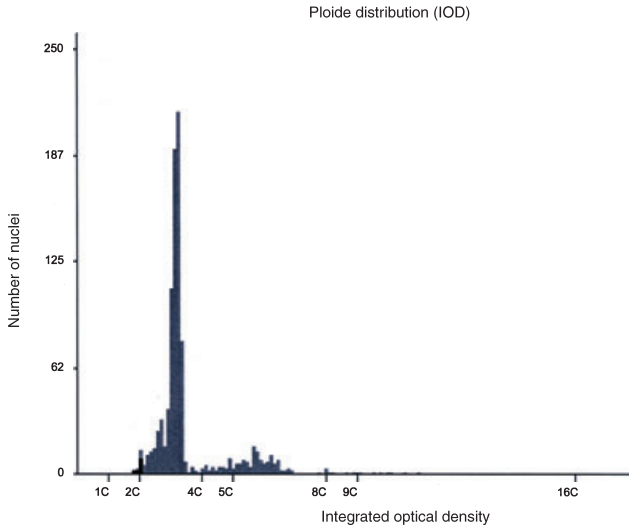

Figure 3 Histogram of a DNA aneuploid CEPA intracapsular showing two clears distinct peaks between 2c and 4c (DI = 1.78, 5CEr = 14.29).

Discussion

DNA content can be assessed by flow or image cytometry, which are able to detect gross genomic aberrations (17, 18). According to some authors, flow cytometry (FCM) of solid tumours has drawbacks because of the process of cell separation and that fractions of aneuploid cells could be lost (19). IC has the advantage of analysing the whole nuclei of tumour cells and may be more sensitive than FCM for analysing DNA content (19–21).

A vast majority of studies have used FCM to evaluate DNA content in salivary gland tumours, and the samples have been obtained from fresh tumour tissue or paraffin blocks (10–13, 18, 22–34). Takashima et al. (13) suggested that a combination of magnetic resonance, cytology and FCM is optimal for diagnosing malignancies of parotid gland, and fine needle aspiration biopsy (FNAB) derived materials can replace the surgical specimens in FCM analysis.

There are few large series published in the English language focusing on ploidy analysis for the prediction of tumour aggressiveness, prognosis, or to distinguish benign and malignant salivary gland tumours (12, 17, 18, 23, 28). Some authors reported that DNA diploidy may be seen in both benign and malignant lesions, but aneuploidy is mainly seen in malignant lesions (12, 24). Three of the four recent large series published by Driemel et al. (18) (n = 279), Pinto et al. (12) (n = 97) and Enamorado et al. (23) (n = 46) have studied the ploidy status on fresh samples of salivary gland tumours by FCM. These authors have worked with different diagnostic criteria (CV, DI, histogram peaks and minimum number of tumour cell-nuclei) to interpret the histograms as diploid or aneuploid.

According to Pinto et al. (12), the most common aneuploid tumour in their series was CEPA (four cases) followed by salivary duct carcinoma (two cases) and undifferentiated adenocarcinoma (one case). Fifty per cent of CEPA (n = 8) were aneuploid, which coincides with our IC results. Driemel et al. (18) reported three aneuploid CEPA (60%, n = 5), which had been initially diagnosed as PA, but the ploidy results aided the oral pathologists to reclassify them to CEPA. In our study, two out of five aneuploid CEPA were intracapsular, which validates the use of ploidy analysis in these tumours.

According to scientific literature in the English language, there is no consensus about the DNA content of the MEC. Several authors have shown a diploid status for MEC using FCM (12, 18, 27). However, other papers detected aneuploid MEC and concluded that diploid MEC had a better prognosis than aneuploid lesions (13, 27, 28). van Heerden et al. (28) studied paraffin samples of 55 MEC using FCM and found 30 aneuploid lesions, 89% of high-grade MEC, were aneuploid. Gemryd et al. (27) studied the ploidy of 28 MEC and found 22 diploid and 6 aneuploid. Five of the six aneuploid MEC recurred, compared with only 1⁄22 diploid cases.

Franzen et al. (11) studied 51 ACC and found 12 aneuploid cases. Enamorado et al. (23) analysed 46 ACC (31 cribriform and 15 solid) and found 15 aneuploid lesions (10 solid and 5 cribriform). Franzen et al. (11) and Enamorado et al. (23) showed a correlation between aneuploidy and high-grade ACC. Driemel et al. (18) studied the ploidy status in 18 ACC and found only two aneuploid cases. Takashima et al. (13) reported one diploid and one aneuploid ACC (n = 2). Pinto et al. (12) detected only diploid ACC (n = 6) in their series. All the ACC articles mentioned have used FCM to study the DNA content. In our study (IC), the 11 ACC (eight low-grade and three high-grade) were uniformly diploid.

Driemel et al. (18) found one aneuploid ACCa (n = 5). El-Naggar et al. (29), using FCM from paraffin samples, reported eight aneuploid ACCa (n = 15), four of which had caused death to the patients. All seven diploid ACCa presented a good clinical course. Pinto et al. (12) detected only diploid ACCa (n = 2).

Driemel et al. (18) found aneuploidy in one of three MC. Pinto et al. (12) and our series (IC) reported only diploid MC.

Kelsch et al. (30) and Carrillo et al. (31) found aneuploid cells in three of 10 and five of 22 PLGA, respectively. Other authors reported only diploid PLGA cases similar to our findings (12). None of these studies (FCM) demonstrated a correlation between aneuploidy, histopathological grade and prognosis.

IC analysis can use paraffin blocks or cytological slides from FNAB or cytospin (17). Only a few studies have analysed salivary gland tumours using IC (17, 35–40). Three of these were single case reports documenting aneuploidy in an ACCa (38) and only diploid findings in a MEC (36) and an ACCa (39). The present series and Gerstner et al. (17) have studied the DNA content by IC. Gerstner et al. (17) analysed the ploidy status on FNAB samples of primary epithelial salivary gland tumours (n = 27) using laser scanner cytometry (LSC). Cells or nuclei with $0.95 < DI < 1.05$ were defined as

DNA diploid; 1.9 < DI < 2.1 was defined as DNA tetraploid; any other DI was defined as DNA aneuploid. The LSC sample was classified as malignant if DNA aneuploid peaks were detected or if the 5cER exceeded 5%. Gerstner et al. (17) reported that all PA ($n = 22$) and one myoepithelioma had a diploid DNA content. However, all four (one ACCa, one MEC, one adenocarcinoma and one carcinosarcoma) epithelial primary malignant salivary gland tumours were aneuploid. Thus, a larger number of malignant primary epithelial salivary gland tumours should be studied by LSC to confirm this high index of aneuploidy.

Di Palma et al. (38), using IC and paraffin samples, displayed both diploid and aneuploid tumour cells in a case of dedifferentiated ACCa. Gerstner et al. (17) found one aneuploid ACCa, and our series only detected diploid ACCa ($n = 9$).

Hamper et al. (35) studied the ploidy status of 46 MEC by IC. They found 32 diploid and 14 aneuploid cases and reported a correlation between aneuploid or atypical high-grade MEC and an unfavourable clinical course. These authors only analysed 100 nuclei per case on 8 μm paraffin sections stained for Feulgen. In our series, only one of 14 MEC was aneuploid and it was a low-grade lesion. Thus, there was no correlation between ploidy status and histopathological grade. Similarly, Diwakar et al. (41) reported that ploidy is closely related to nuclear pleomorphism but not to grade in oral SCC.

Larger and more recent studies (FCM and IC) have been unanimous about the diploid status of all benign salivary gland tumours including recurrent lesions (12, 13, 17, 18, 23, 31). This is similar to our findings and consistent with the benign course of these tumours. However, some authors using FCM or IC found a small number of aneuploid cases occurring in recurrent PA or in lesions with cytological atypia (24, 27, 40).

The present study (IC) and three FCM series (12, 18, 23) found a lower incidence of DNA aneuploidy in primary malignant salivary gland tumours (17.4%, 24%, 27% and 32%, respectively). Nevertheless, when high-grade tumours were analysed, this incidence considerably increased to 44% (present paper), 47% (12) and 66% (23).

An important point to highlight is tumour heterogeneity reported in salivary gland tumours and oral SCC when assessing the ploidy status (27, 38, 41). Diwakar et al. (41) recommended the repetition of ploidy analysis to truly exclude spurious diploid cases (41). In our study, we selected large representative tumour areas, and at least three paraffin sections (40 μm) per case were collected. Thus, in our opinion, the tumour heterogeneity might only be a factor in diploid CEPAs because of their high histological heterogeneity.

The data regarding the prognostic value of DNA ploidy in salivary gland tumours are inconclusive regardless of the technique (FCM or IC) or criteria used to assess the DNA content. Some authors affirm that DNA ploidy is helpful to predict tumour aggressiveness (aneuploid cases) in ACC (11), ACCa (29), MEC (28, 35, 37) and salivary duct carcinoma (42). On the contrary, several authors did not find a significant correlation between aneuploid tumours, histopathological grade, recurrence and bad prognosis (12, 18, 22, 32–34). Therefore, the prognostic value of ploidy in malignant salivary gland tumours remains uncertain.

The different ploidy findings reported in the literature could be explained because of detection limits (FCM or IC), sample variation, tumour heterogeneity and different diagnostic criteria. However, the recent large series (FCM or IC) have detected a small index of aneuploidy in salivary gland tumours.

In conclusion, DNA ploidy by image analysis is not a good method to distinguish PA from malignant salivary gland tumours because a majority of malignant salivary gland tumours are also diploid. High-grade malignancies may be aneuploid and 50% of CEPA were aneuploid, including two intracapsular lesions, suggesting that ploidy by IC may be useful in assisting the diagnosis of malignant change in PA. Diwakar et al. (41) recommended that a minimum of five samples are needed from each case to make a precise diagnosis of diploid oral SCC. This may be especially relevant to CEPA, which are known to be histologically heterogeneous lesions. Further larger studies are needed to confirm the usefulness of IC in CEPA and high-grade lesions.

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