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ORIGINAL ARTICLE

DNA content in malignant salivary gland tumours

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OBJECTIVE: Our aim was to evaluate the DNA content in malignant salivary gland tumours using image cytometry and its possible relationships with clinical and morphologic findings, disease course and prognosis.
PATIENTS AND METHODS: The study sample comprised 31 patients diagnosed and treated for primary malignant salivary gland tumours. Formalin-fixed, paraffin-embedded surgical specimens of all patients were Feulgen-stained for DNA content analysis by image cytometry. Statistical analysis was used to investigate possible relationships between DNA content variables and clinical and histological findings, disease course and patient survival.
RESULTS: Seventeen (55%) cases of our sample were graded as DNA diploid, four (13%) as DNA aneuploid and 10 (32%) as DNA multiploid. In 15 (48%) cases, the 5c exceeding rate (5cER) was higher than 1.7%. DNA ploidy correlated with N stage and tumour size. DNA ploidy and 5cER had a statistically significant prognostic influence on overall and disease-free survival in univariate analysis. However, in multivariate analysis, stage classification was the only parameter with an independent prognosis value.
CONCLUSION: Abnormal DNA content is a common finding in salivary gland cancers. Our results suggest an important role of DNA content analysis in the evaluation of these tumours.
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Keywords: salivary gland cancers; DNA ploidy; 5cER; image cytometry

Introduction

Salivary gland malignancies constitute a heterogeneous group of tumours that are completely different, taking into consideration their natural history, histological pattern, clinical behaviour and therapeutic strategy. Reliable indicators of their biologic aggressiveness are still lacking and, therefore, the clinical outcome is difficult to predict (Ellis and Auclair, 1995). They are rare, representing only 4% of head and neck malignancies and $<1\%$ of all malignant diseases (Kokemueller *et al*, 2004).

It is well known that carcinogenesis is frequently associated with genomic changes, chromosomal alterations, DNA content changes and variations in the cell cycle of neoplasic cells (Lee *et al*, 1994). The last two variables can be measured quantitatively and reliably through image cytometry and flow cytometry analysis. In image cytometry, nuclear DNA content is measured using a non-fluorescent compound which attaches itself to the DNA. The integrated optical density measured in each stained nuclei is proportional to the amount of DNA present in that same nuclei. This DNA content analysis provides us with two different types of biological information: data regarding the presence of abnormalities in the DNA cell content, i.e., DNA aneuploidy, as well as the distribution of a certain cell population through the different phases of cell cycle (Oliveira *et al*, 2005). The cell percentage with DNA content above 5c (5cER) is another DNA ploidy related parameter studied in some cancers (Santos *et al*, 2003).

DNA content analysis has been studied in salivary gland tumours but with conflicting results. Some reports showed an overall low proportion of cases with DNA aneuploidy (Pinto *et al*, 1999; Vargas *et al*, 2007). Others, however, reported a high frequency of DNA aneuploidy in this type of tumour (Tytor *et al*, 1993). Abnormal DNA content has been related to aggressive behaviour in mucoepidermoid carcinomas, adenoid cystic carcinomas, acinic cell carcinomas and oncocytomas (Pinto *et al*, 1999).

Some reports have suggested that image cytometry analysis parameters have also prognostic utility in some histological types of malignant salivary gland tumours, yet the findings are not concordant among studies

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(Lewis *et al*, 2001; Enamorado *et al*, 2004; Suzzi *et al*, 2005).

The objective of this study was to evaluate the DNA ploidy and 5cER in different malignant salivary gland tumours using image cytometry analysis, and to correlate them with clinical and pathological characteristics, as well as with their outcome.

Patients and methods

This study included 31 unselected patients diagnosed and treated for malignant salivary gland tumours of oral cavity and pharynx at Instituto Português de Oncologia, Porto, Portugal, between 1992 and 2002. Clinical and follow-up data were obtained by reviewing patients' records.

Haematoxylin–eosin-stained slides were available for all tumours. One representative block was selected from each case for image cytometric study.

Patients who had undergone radiotherapy or chemotherapy prior to surgery, had no clinical or follow-up information available and no paraffin blocks containing tumour tissue were excluded. From each case, data regarding patients, such as age, gender, tumour location, stage classification, primary lesion treatment, histological type, grade, surgical margin status and follow-up information, were obtained.

All specimens were reclassified following the World Health Organization classification (Barnes *et al*, 2005) and staged according to the AJCC system (AJCC, 2002).

Regarding clinical behaviour, neoplasms were subclassified into two groups: 'low potential malignancy (LPM)' (acinic cell carcinoma, polymorphous low grade adenocarcinoma, epithelial-mioepithelial carcinoma and low grade mucoepidermoid carcinoma) and 'high potential malignancy (HPM)' (adenocarcinoma NOS, adenosquamous carcinoma, adenoid cystic carcinoma, salivary duct carcinoma, carcinoma ex-pleomorphic adenoma, moderate or poorly differentiated squamous cell carcinoma, moderate or high grade mucoepidermoid carcinoma and carcinossarcoma). Surgical margins were classified as suggested by Sutton *et al* (2003), in clear margin, close margin and involved margin.

Image cytometry analysis

The nuclear DNA content of the cancer cells was measured with CAS 200 Image Analysis System (Cell Analysis Systems, Inc., Elmhurst, IL, USA). The sections from paraffin-embedded blocks were cut at 6 μm and were deparaffinized and rehydrated. The slides were stained using Feulgen's method with the DNA staining kit (Cell Analysis System, Inc.), according to the manufacturer's instructions. Briefly, the slides were hydrolysed in a 5 N HCl solution for 60 min and then transferred to the CAS DNA stain solution for 1 h. After staining, the slides were placed in three consecutive CAS rinse solutions for 30 s, 5 and 10 min, respectively, washed in deionized water and placed in an acid–alcohol solution for 5 min. The slides were then dehydrated and mounted. The Feulgen reaction with this kit produced a blue staining of nuclear DNA, reflecting stoichiometric binding of the stain to the DNA. CAS staining of rat hepatocyte was used as control slide. These cells, having a known quantity of DNA, were used as external control, allowing for instrument calibration prior to DNA image analysis. Twenty-five lymphocytes of each case and a minimum of 120 non-overlapping and well-preserved tumour nuclei were measured using the Quantitative DNA Analysis software program (Cell Analysis System, Inc.). The integrated optical density (OD) of each Feulgen-stained nucleus was considered proportional to the amount of DNA present in the nucleus. The OD of lymphocyte nuclei from each section served as internal control (diploid reference). The resultant DNA histograms were analysed by previously used methods (Santos *et al*, 2003). For each tumour G0/G1 visually identifiable peak, mean, standard deviation (s.d.) and coefficient of variation (CV) values were calculated. The control CV provides an indicator of overall precision for the imaging technique. The DNA index (DI) was evaluated as the ratio of tumour G0/G1 peak mean value divided by the internal control lymphocytes G0/G1 peak mean value. Peaks having a DI >3 s.d. from the internal control lymphocytes were considered aneuploid. When more than one aneuploid peak was observed, the DNA histogram was classified as multiploid. The 5cER was also evaluated, being defined as the percentage of tumour nuclei with DNA values above 5n. For statistical purposes, this variable was divided into two groups with the average of 5cER values obtained serving as 'cut-off' value.

Statistical analysis

In this descriptive study, the associations between variables were evaluated by chi-square or Fisher's exact test. Overall survival was defined as the interval (months) between the date of primary treatment and the date of death or the last follow-up date. Disease-free survival was defined as the interval (months) between the date of primary treatment and the date of the first recurrence (whether local, regional or distant), and included only patients without evidence of disease for a minimum of 3 months after primary treatment. The Kaplan–Meier method was used to plot survival curves and the prognostic effect was tested using the log-rank test. Variables with significant effects in the univariate analyses were entered into Cox proportional hazards model to investigate the independent effects of these variables. Differences were considered statistically significant at $P < 0.05$.

Results

Clinicopathological findings

The study group included 31 patients (21 men and 10 women), whose ages ranged from 16 to 86 years, with a median of 57 years. The parotid gland was affected in 14 patients, the submandibular gland in 1 and the minor salivary glands in 16 patients.

Tumour size ranged from 1.4 to 10.0 cm, with an average of 3.2 cm in the 28 cases where this information was available. Eleven patients had nodal metastasis. No

patient presented any signs of distant metastasis at the time of diagnosis.

Five patients presented stage-I tumours, three patients had stage-II tumours, three patients displayed stage-III tumours and 20 patients had tumours in stage-IV. All patients underwent primary surgery. Eighteen received postoperative radiotherapy and one also received chemotherapy.

Adenoid cystic carcinoma was the most frequent histological type. Others are listed in Table 1.

Twenty-five tumours were HPM and six were LPM. Surgical margins with tumour were observed in 12 patients, in the 24 cases where this variable was available.

Table 1 Distribution of DNA content variables in malignant salivary gland tumours by histological type

		DNA ploidy		*5cER*	
Histology	n	*Diploid*	*Aneuploid*	*< 1.7*	*≥ 1.7*
Adenocarcinoma (NOS)	1	1	0	0	1
Acinic cell carcinoma	1	1	0	1	0
Polymorphous low grade adenocarcinoma	3	1	2	1	2
Salivary duct carcinoma	2	0	2	0	2
Adenosquamous carcinoma	4	0	4	0	4
Mucoepidermoid carcinoma	4	4	0	4	0
Adenoid cystic carcinoma	10	8	2	8	2
Carcinoma ex-pleomorphic adenoma[a]	2	1	1	0	2
Carcinossarcoma[a]	1	1	0	1	0
Squamous cell carcinoma	3	0	3	0	3
Total	31	17	14	15	16

[a]With a adenocarcinoma malignant epithelial component.

DNA content analysis

From the 31 cases studied, 17 (54.8%) were DNA diploid, four (12.9%) were DNA aneuploid and 10 (32.3%) were DNA multiploid. For statistical purposes, DNA multiploid cases were considered as DNA aneuploid cases. Fifteen tumours (48.4%) had a 5cER ≥ 1.7% (Figure 1). DNA ploidy and 5cER pattern distributed by histological types are summarized in Table 1.

The relationship between tumour DNA ploidy, 5cER and the clinicopathological findings is shown in Table 2. No significant correlations were found between DNA content analysis and gender, age, potential malignancy group, stage, treatment or margin status. DNA ploidy was, however, significantly related to cervical lymph

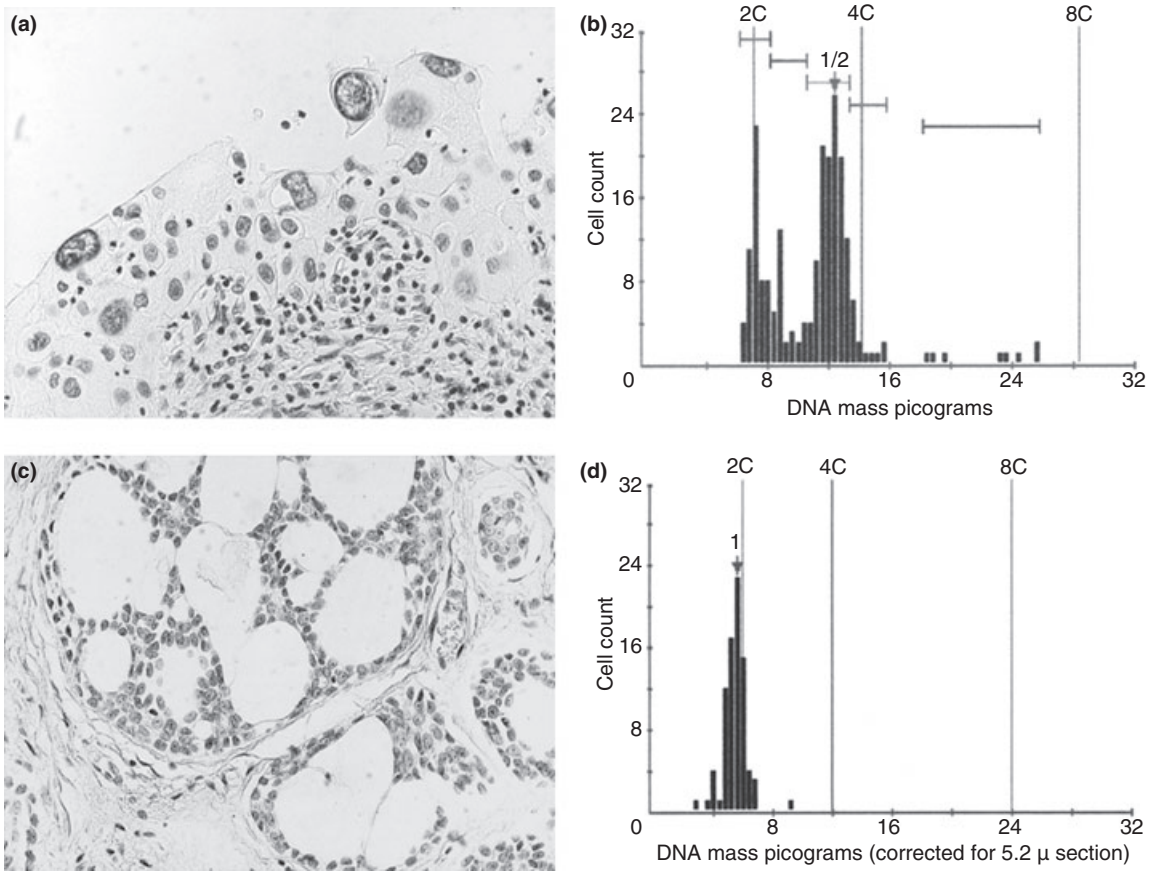

Figure 1 Poorly differentiated squamous cell carcinoma (**a**) (Feulgen stain; 400×) corresponding to a DNA aneuploid histogram (**b**); Adenoid cystic carcinoma (**c**) (Feulgen stain; 200×) corresponding to a DNA diploid histogram (**d**)

Table 2 Correlation between DNA content variables and clinicopathologic characteristics

Factor	*Group*	n	*Diploid*	*Aneuploid*	P-*value*	*5cER < 1.7*	*5cER ≥ 1.7*	P-*value*
Sex	Male	21	10	11	0.24	9	12	0.372
	Female	10	7	3		6	4	
Age	< 57 years	16	10	6	0.38	9	7	0.37
	≥ 57 years	15	7	8		6	9	
N status	0	20	14	6	0.022	12	8	0.081
	+	11	3	8		3	8	
Tumour size	< 3.2 cm	11	10	1	0.001	10	1	< 0.001
	≥ 3.2 cm	17	5	12		3	14	
Stage	I	5	5	–	0.101	5	–	0.092
	II	3	2	1		1	2	
	III	3	2	1		1	2	
	IV	20	8	12		8	12	
Tumour malignancy	LPM	6	4	2	0.664	4	2	0.394
	HPM	25	13	12		11	14	
Margin status	Free of tumour	6	3	3	0.777	1	5	0.2
	Close tumour	6	4	2		4	2	
	With tumour	12	6	6		6	6	
Treatment	SG	9	5	4	0.482	4	5	0.513
	SG + RT	18	11	7		10	8	
	SG + RT + QT	1	–	1		–	1	

SG, surgery; RT, radiotherapy; QT, chemotherapy; LPM, low potential malignancy; HPM, high potential malignancy.

Table 3 Prognostic factors and survival in patients with malignant salivary gland tumours

Factor	*Group*	n	*Overall survival (%) (3 years)*	P-*value*	n	*Disease-free survival (%) (3 years)*	P-*value*
Sex	Male	21	47	0.93	15	59	0.46
	Female	10	40		8	37	
Age	< 57 years	16	44	0.82	12	50	0.92
	≥ 57 years	15	46		11	52	
N status	0	20	65	0.0009	19	58	0.36
	+	11	14		4	0	
Tumour size	< 3.2 cm	11	72	0.0044	11	72	0.036
	≥3.2 cm	17	28		11	23	
Stage	I	5	100	0.0125	5	100	0.015
	II	3	66		3	67	
	III	3	66		3	33	
	IV	20	24		12	30	
Tumour malignancy	LPM	6	100	0.009	6	100	0.012
	HPM	25	32		17	35	
Margin status	Free of tumour	6	62	0.41	6	67	0.98
	Close tumour	6	50		6	50	
	With tumour	12	42		7	57	
Treatment	SG	9	55	0.99	7	71	0.78
	SG + RT	18	49		15	46	
	SG + RT + QT	1	0		1	0	
DNA ploidy	Diploid	17	52	0.0339	14	64	0.0371
	Aneuploid	14	18		9	15	
5cER	< 1.7%	15	53	0.0441	12	67	0.0405
	≥1.7%	16	20		11	17	

SG, surgery; RT, radiotherapy; QT, chemotherapy; LPM, low potential malignancy; HPM, high potential malignancy.

node status and tumour size. The 5cER was significantly related to tumour size.

Clinical follow-up

The follow-up period ranged from 2 to 157 months. At the time of the last contact, 10 patients were alive without disease, one patient was dead due to other causes (cardiovascular disease) and 20 died of their disease with a median survival of 16 months (range, 2–120 months). The median follow-up among living patients was 83 months (range, 26–157 months).

Recurrence was detected in 13 patients in a median time of 16 months (range, 3–64 months), in the form of local recurrences in 10 patients and distant metastasis in six (three patients in lung, two patients in bone and one patient in lung and liver).

The 3-year overall survival rate was 45%. In univariate analysis, for the clinicopathological factors analysed, tumour size ≥ 3.2 cm, positive N status, advanced stage and HPM tumours were statistically correlated with worse overall survival. The same happened in disease-free survival for tumour size ≥ 3.2 cm, advanced

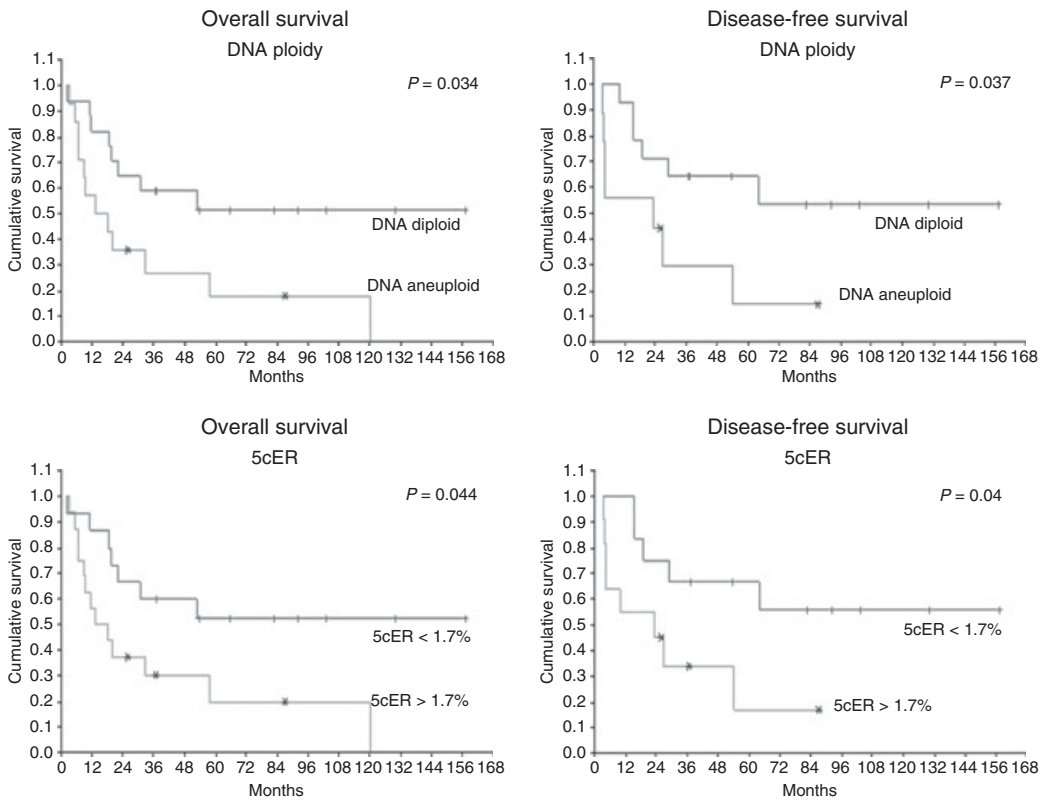

Figure 2 Overall survival and disease-free survival plots for DNA ploidy and 5cER

stage and HPM tumours (Table 3). The 3-year disease-free survival rate was 51%.

DNA ploidy had a statistically significant prognostic influence on overall survival and disease-free survival, with aneuploid tumours presenting the worst prognosis. The same was observed with the 5cER, where tumours with 5cER $\geq$ 1.7 presented the worst prognosis (Table 3, Figure 2).

However, in multivariate analysis, only positive N status was the independent predictor of low overall survival (*Exp*(β)-4.29; 95% CI: 1.521–12.124; $P = 0.006$) and stage III + IV of low disease-free survival (*Exp*(β)-12.75; 95% CI: 1.548–105.14; $P = 0.018$).

Discussion

DNA aneuploidy, which reflects an abnormal DNA and chromosome content, has been found in various human cancers, sometimes associated with a more aggressive behaviour as well as poorer prognosis (Santos *et al*, 2003).

In our series, 45% of the tumours were DNA aneuploid, which is in line with observations stated by Tytor *et al* (1993), Luna *et al* (1990) and El-Naggar *et al* (1990). Nevertheless, Driemen *et al* (2005) observed 24% of aneuploid cases, Pinto *et al* (1999) reported 27% of aneuploid cases and Vargas *et al* (2007) observed that only 17.4% of malignant tumours were aneuploid. These differences may depend on the histological type of biological samples selected, and size or grade of tumours included in the study. Moreover, in these studies different techniques were used to evaluate the DNA content, such as flow cytometry or image cytometry. In this study we used image cytometry. Compared with flow cytometry, image cytometry has limitations in distinguishing cell populations with sssmall DNA content alterations (near-diploid DNA pattern), due to the smaller number of cells measured and the higher coefficient of variation (CV) obtained using this technique, also the techique is more time-consuming. On the other hand, it requires a lower number of cells, allowing the study of small tumour samples. Furthermore, microscopic selection of tumour cells allows the rejection of artefacts and non-tumour cells like infiltrated lymphocytes (diploid population), along with the detection of aneuploid cells, which may represent a small proportion of the whole tumour cell population (Bañez *et al*, 1992).

In this study, DNA content varied among histological type. Tumours, such as mucoepidermoid carcinomas or adenoid cystic carcinomas, had a predominantly DNA diploid pattern, as observed by Vargas *et al* (2007), Pinto *et al* (1999), Bang *et al* (1994), Saka *et al* (1991) and also Franzén *et al* (1991). In adenoid cystic carcinoma (20% of DNA aneuploid cases in our series), there has been great disparity in the findings, with DNA aneuploid rates ranging from 0 to 78% (Eibling *et al*, 1991; Saka *et al*, 1991; Fonseca, 1994). These differences may depend on the selection of biological samples, size or grade of tumours included in the study. When we ascertained the tumour reclassification, most of the adenoid cystic carcinomas had both solid and cribiform or tubular patterns. Taking into consideration the small number of cases we decided, for statistical purposes, not to subdivide

the histological variable into subtype histological pattern. Regarding clinical behaviour, we considered adenoid cystic carcinoma a HPM because, as suggested by some authors (Vander Poorten *et al*, 1999; Kokemueller *et al*, 2004), these tumours are indolent, but with many recurrences and poor disease-free survival in a long follow-up analysis (Sur *et al*, 1997; Kokemueller *et al*, 2004). On the other hand, adenosquamous carcinoma, salivary duct carcinoma and squamous cell carcinoma were DNA aneuploid, which is in concordance with the reports by Pinto *et al* (1999), Grenko *et al* (1995), Lewis *et al* (1996) and Félix *et al* (1996).

Abnormal DNA content has been related to aggressive behaviour in adenoid cystic carcinoma, acinic cell carcinoma, mucoepidermoid carcinomas and oncocytomas (Pinto *et al*, 1999). In our series, we found a statistically significant correlation between DNA ploidy and cervical lymph node status ($P = 0.022$), and tumour size ($P = 0.001$). No other clinicopathological factor was able to correlate with this variable. Tytor *et al* (1993) observed an augmented average in tumour size in DNA aneuploid cases compared with the DNA diploid ones. The association between nodal metastasis and aneuploidy was also observed by Gemryd *et al* (1997) and Luna *et al* (1990).

The 5cER is a DNA content related variable very few reported in salivary gland tumours studies (Vargas *et al*, 2007). Our results showed a correlation between tumours with 5cER $\geq$ 1.7% and tumours $\geq$ 3.2 cm ($P < 0.001$). Among DNA content variables, 5cER was shown to be the variable representing the less time-consuming procedure, if we had previously set-up the morphometric image filter to select only these aneuploid cell nuclei (Santos *et al*, 2003).

Our results indicate that DNA content alteration is not an early event in salivary gland carcinogenesis but only appears in later stages of tumour progression, perhaps as a secondary effect, caused by the accumulation of critical genetic alterations in the neoplasic cells.

DNA ploidy has proven to be a useful prognostic indicator in a variety of tumours. In our study with salivary gland tumours, DNA aneuploid tumours and/or with 5cER $\geq$ 1.7% had poor overall and disease-free survival in the univariate analysis. These results are similar to those observed by Enamorado *et al* (2004), Bang *et al* (1994) and El-Naggar *et al* (1990). However, in this study on the multivariate analyses, these DNA content variables were not independent prognostic factors. Positive N status and stage III + IV were the only independent predictors of low overall survival and disease-free survival, respectively. As previously referred, the prognostic value of the DNA content variables in these pathologies is not already established. Franzén *et al* (1995) had observed DNA ploidy as an independent survival predictor factor. On the other hand, other reports have not shown any prognostic value of DNA content variables in salivary gland neoplasms (Lewis *et al*, 1996, 2001; Pinto *et al*, 1999; Suzzi *et al*, 2005).

In this study, we conclude that abnormal DNA content is a frequent finding in salivary gland neoplasms and is correlated with the disease progression variables, such as nodal metastasis and tumour size. We believe that DNA content alterations are not an early event in salivary gland cancers. Tumours with DNA aneuploidy and/or 5cER $\geq$ 1.7% had worse prognosis at univariate analysis, but we were unable to demonstrate the independent prognostic value of these variables as we observed with nodal status and stage classification.

Conflict of interest

We wish to declare that the submitted work is original and has not been submitted or published elsewhere. Also, all authors have read and approved the manuscript and agree with the current submission. Finally, there are no potential conflicts of interest.

References

American Joint Committee on Cancer (2002). *AJCC – Cancer Staging Handbook. Classification of malignant tumors*, 6th edn. Springer: New York, 27–87.

Bañez EI, Krishnan B, Ansari MQ, Carraway NP, McBride RA (1992). False aneuploidy in benign tumors with a high lymphocyte content: a study of Warthin's tumor and benign thymoma. *Hum Pathol* **23:** 1244–1251.

Bang G, Donath K, Thoresen S, Clausen OP (1994). DNA flow cytometry of reclassified subtypes of malignant salivary gland tumors. *J Oral Pathol Med* **23:** 291–297.

Barnes L, Everson JW, Reichart P, Sidransky D (2005). *World health organization classification of tumours: pathology and genetics of head and neck tumours*. IARC Press: Lyon, France.

Driemen O, Maier H, Kraft K, Haase S (2005). Flow cytometric DNA ploidy in salivary gland tumours. *Oncol Rep* **13:** 161–165.

Eibling DE, Johnson JT, McCoy JP *et al* (1991). Cytometry evaluation of adenoid cystic carcinoma: correlation with histologic subtype and survival. *Am J Surg* **162:** 367–372.

Ellis GL, Auclair PL (1995). *Tumors of the salivary glands. Atlas of tumor pathology*. 3rd series Armed Forces Institute of Pathology: Washington, DC.

El-Naggar AK, Batsakis JG, Luna MA, McLemore D, Byers RM (1990). DNA flow cytometry of acinic cell carcinomas of major salivary glands. *J Laryngol Otol* **104:** 410–416.

Enamorado I, Lakhani R, Korkmaz H *et al* (2004). Correlation of histopathological variants, cellular DNA content, and clinical outcome in adenoid cystic carcinoma of the salivary glands. *Otolaryngol Head Neck Surg* **131:** 646–650.

Félix A, El-Naggar AK, Press MF *et al* (1996). Prognostic significance of biomarkers (c-erbB-2, p53, proliferating cell nuclear antigen and DNA content) in salivary duct carcinoma. *Hum Pathol* **27:** 561–566.

Fonseca I (1994). *Adenocarcinomas salivares com participação mioepitelial. Morfologia, diferenciação e proliferação*. Doctoral thesis, Faculdade de Ciências Médicas da Universidade Nova de Lisboa.

Franzén G, Klausen OG, Grenko RT, Carstensen J, Nordenskjöld B (1991). Adenoid cystic carcinoma: DNA as a prognostic indicator. *Laryngoscope* **101:** 669–673.

Franzén G, Nordgard S, Boysen M, Larsen PL, Halvolsen TB, Clausen OP (1995). DNA content in adenoid cystic carcinomas. *Head Neck* **17:** 49–55.

Gemryd P, Lundquist PG, Tytor M, Hellquist HB, Nordenskjöld B (1997). Prognostic significance of DNA ploidy in mucoepidermoid carcinoma. *Eur Arch Otorhinolaryngol* **254:** 180–185.

Grenko RT, Gemryd P, Tytor M, Lundqvist PG, Boeryd B (1995). Salivary duct carcinoma. *Histopathology* **26:** 261–266.

Kokemueller H, Swennen G, Brueggemann N, Brachvogel P, Eckardt A, Hausamen JE (2004). Epithelial malignancies of the salivary glands: clinical experience of a single institution – a review. *Int J Oral Maxillofac Surg* **33:** 423–432.

Lee S, Tolmachoff T, Marchevsky AM (1994). DNA content analysis (''ploidy'') by image analysis: clinical applications and comparison with flow cytometry. In: Marchevsky AM, Bartels PH, eds. *Image analysis: a primer for pathologists*. Raven Press, Lta: New York, NY, pp. 261–307.

Lewis JE, McKinney BC, Weiland LH, Ferreiro JA, Olsen KD (1996). Salivary duct carcinoma. Clinicopathologic and imunohistochemical review of 26 cases. *Cancer* **77:** 223–230.

Lewis JE, Olsen KD, Sebo TJ (2001). Carcinoma ex pleomorphic adenoma: pathologic analysis of 73 cases. *Hum Pathol* **32:** 596–604.

Luna MA, El-Naggar A, Batsakis JG, Weber RS, Gernsey LA, Goepfert H (1990). Flow cytometric DNA content of adenoid cystic carcinoma of submandibular gland. Correlation of histologic features and prognosis. *Arch Otolaryngol Head Neck Surg* **116:** 1291–1296.

Oliveira PA, Palmeira C, Lourenço LM, Lopes CA (2005). Evaluation of DNA content in préneoplastic changes of mouse urinary bladder induced by *N*-Butyl-*N*-(4-hydroxybutyl) nitrosamine. *J Exp Clin Cancer Res* **24:** 609–616.

Pinto AE, Fonseca I, Soares J (1999). The clinical relevance of ploidy and S-phase fraction determination in salivary gland tumors. A flow cytometric study of 97 cases. *Cancer* **85:** 273–281.

Saka T, Yamamoto Y, Takahashi H (1991). Comparative cytofluorometric DNA analysis of pleomorphic adenoma and adenoid cystic carcinoma of salivary glands. *Virchows Arch B Cell Pathol Incl Mol Pathol* **61:** 255–261.

Santos L, Lameiras C, Afonso J *et al* (2003). Is DNA content alteration a consequence of proliferative and differentiation changes in urothelial bladder tumours? *Acta Urol* **20:** 9–17.

Sur RK, Donde B, Levin V *et al* (1997). Adenoid cystic carcinoma of the salivary glands: a review of 10 years. *Laryngoscope* **107:** 1276–1280.

Sutton DN, Brown JS, Rogers SN, Vaughan JA, Woolgar JA (2003). The prognostic implications of the surgical margin in oral squamous cell carcinoma. *Int J Oral Maxillofac Surg* **32:** 30–34.

Suzzi MV, Alessi A, Bertarelli C *et al* (2005). Prognostic relevance of cell proliferation in major salivary gland carcinomas. *Acta Otorhinolaryngol Ital* **25:** 161–168.

Tytor M, Gemryd P, Wingren S *et al* (1993). Heterogeneity of salivary gland tumors studied by flow cytometry. *Head Neck* **15:** 514–521.

Vander Poorten VLM, Balm AJ, Hilgers FJ *et al* (1999). The development of a prognostic score for patients with parotid carcinoma. *Cancer* **85:** 2057–2067.

Vargas PA, Torres-Rendon A, Speight PM (2007). DNA ploidy analysis salivary gland tumours by image cytometry. *J Oral Pathol Med* **36:** 371–376.