ROUND TABLE 91ST NATIONAL CONGRESS S.I.O.

Prognostic relevance of cell proliferation in major salivary gland carcinomas

Rilevanza prognostica della proliferazione cellulare nei carcinomi delle ghiandole salivari maggiori

M. VACCHI SUZZI, A. ALESSI[1], C. BERTARELLI[1], A.CANCELLIERI[1], L. PROCACCIO[2], D. DALL'OLIO, P. LAUDADIO

ENT Unit; [1] Pathology Unit; [2] Laboratory Analysis Unit. Maggiore Hospital, Bologna, Italy

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Parole chiave

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Summary

Several proliferation markers, such as DNA ploidy, Ki67, MiB1 and proliferating cell nuclear antigen have been shown to correlate with clinical course and prognosis in several epithelial tumours and lymphomas. In the present study, the prognostic relevance of these markers was evaluated in major salivary gland carcinomas. A sample of 36 cases out of 85 patients submitted to surgery for major salivary gland carcinomas at our institution between 1987 and 1997 were studied. The sample comprised 8 adenoid-cystic carcinomas, 6 ductal carcinomas, 11 mucoepidermoid carcinomas and 11 acinic cell carcinomas. Follow-up ranged from 1 to 12 years (mean 6.2). In some patients, DNA ploidy (euploid or aneuploid) was studied by flow cytometry. In others, proliferation activity was studied by means of monoclonal antibody MiB1, identifying cells in the proliferative cycle. In some patients, both techniques were used. Follow-up was related to these indices, TNM and stage. Even if ploidy suggested a favourable outcome in diploid cancer (13 favourable *vs.* 2 unfavourable) and poor outcome in aneuploid cancer (4 favourable *vs.* 5 unfavourable), the difference was not statistically significant with $p = 0.06$ in Fisher's exact test. Instead, the proliferative tumour cell fraction, evaluated by MiB1, was statistically correlated with prognosis. Comparing survival curves by Log rank Test it yielded $p = 0.007$ using an MiB1 cut-off of 5. Applying a cut-off of 20 yielded $p = 0.001$. Of particular interest were MiB1 values in acinic cell carcinomas for which grading is challenging and lacks consensus. In our group of acinic cell carcinomas, survival correlated with values of MiB1 > or < 15 with $p = 0.009$ in Log rank test. In conclusion, despite a trend towards correlation between ploidy and prognosis, the present study yielded $p = 0.06$, whereas the proliferative fraction assessed by MiB1 was significantly correlated with outcomes. Indeed, "growth fraction" in acinic cell carcinomas may stratify different classes of risk.

Riassunto

È stato dimostrato che diversi markers di proliferazione cellulare, come ploidia del DNA, Ki67, MiB1, PCNA, si possono correlare con il decorso clinico e la prognosi in diversi tumori epiteliali e nei linfomi. Abbiamo valutato il loro valore prognostico nei carcinomi delle ghiandole salivari maggiori, raccogliendo un campione di 36 casi tra 85 pazienti sottoposti ad intervento per carcinoma delle ghiandole salivari maggiori presso la nostra Unità Operativa tra il 1987 ed il 1997. Il campione era costituito da 8 carcinomi adenoidocistici, 6 carcinomi duttali, 11 carcinomi mucoepidermoidi ed 11 carcinomi a cellule aciniche. Il follow-up variava tra 1 e 12 anni, con un valore medio di 6,2 anni. In alcuni di questi pazienti abbiamo studiato il contenuto in DNA come ploidia (euploidia od aneuploidia) per mezzo della citometria di flusso. In alcuni abbiamo studiato l'Attività Proliferativa per mezzo dell'anticorpo monoclonale MiB1 rivolto verso le cellule in ciclo proliferativo; in alcuni pazienti sono state applicate entrambe le tecniche. Abbiamo correlato il follow-up con questi indici, con il TNM, con lo stadio. Sebbene la ploidia suggerisca un esito favorevole nei tumori diploidi (13 favorevoli contro 2 sfavorevoli) e sfavorevole nei tumori aneuploidi (4 favorevoli contro 5 sfavorevoli), non si è raggiunta la significatività statistica con $p = 0,06$ al Test Esatto di Fisher. Al contrario la Frazione Proliferativa delle cellule tumorali valutata attraverso il MiB1 ha manifestato di correlarsi con la prognosi. Il confronto delle curve di sopravvivenza per mezzo del Log rank Test dimostra $p = 0,007$ ponendo il "cut off" del MiB1 a 5. Scegliendo come cut off 20 si ottiene $p = 0,001$. Di particolare interesse sono i valori di MiB1 nel Carcinoma a Cellule Aciniche. In questo carcinoma, il grading è di difficile determinazione e non vi è consenso al proposito. Nel nostro gruppo di Carcinomi a Cellule Aciniche la sopravvivenza si correla con valori di MiB1 maggiori o minori di 15 con $p = 0,009$ nel Log rank test. In conclusione, la correlazione tra ploidia e prognosi, sebbene suggerisca una associazione, dimostra $p = 0,06$; la valutazione della Frazione Proliferativa per mezzo dell'anticorpo monoclonale MiB1 nel nostro studio dimostra una correlazione significativa con l'esito. Particolarmente nel Carcinoma a Cellule Aciniche la Frazione Proliferativa potrebbe stratificare differenti classi di rischio.

Introduction

Malignant tumours of the major salivary glands are classified into histological types and subtypes broadly reflecting their natural history correlated with prognosis. This is unanimously accepted for mucoepidermoid and adenoid cystic carcinomas but there is no consensus on the grading of acinic cell carcinomas [1-3], and the histological features of these tumours do not correlate with the clinical course. Furthermore, ductal carcinomas currently lack a subclassification into histotypes predicting patient outcome; only tumours size > 3 cm seem to carry a worse prognosis than smaller lesions [4].

Several proliferation markers are correlated with the clinical course and prognosis in different tumours[5]: Ki67, MiB1 and % S by flow cytometry have a prognostic value in breast carcinoma, DNA ploidy is one of the most significant prognostic indicators in ovarian tumours, and Ki67 is correlated with the clinical course in non-Hodgkin's lymphoma. These markers also have prognostic relevance in head and neck tumours [6] and salivary gland carcinomas [7-11].

The present report deals with personal findings on the prognostic relevance of cell proliferation in malignant tumours of the major salivary glands.

Materials and methods

A total of 85 patients with malignant tumours of the major salivary glands underwent surgical treatment at the Otolaryngology Division of Maggiore Hospital, Bologna between 1987 and 1997. Depending on the availability of sufficient paraffin-embedded material, 36 patients were selected comprising 8 adenoid cystic carcinomas, 6 ductal carcinomas, 11 mucoepidermoid carcinomas and 11 acinic cell carcinomas.

The TNM classification, 5th edition of the UICC, 1997 was adopted. Since the study covered a considerable period of time, these cases were not reclassified according to the 6th edition of 2002.

Patients underwent radical tumour resection sparing the facial nerve when possible. Surgery was associated with neck dissection in 9 patients and adjunct radiotherapy in 12. Follow-up consisted of outpatient visits, telephone calls and postal questionnaires.

Cell cycle recall

Cell proliferation was divided into different stages: from rest phase G0, the stimulated cell enters the first phase G1 in which it prepares for DNA synthesis; it then moves to phase S when DNA synthesis occurs with duplication of the genome; phase S is followed by a phase of apparent inactivity, phase G2, in which the cell prepares for the subsequent mitosis phase M in which the chromatids separate to generate daughter cells. Only phase M can be identified morphologically, whereas cells in the interphase G1-S-G2 are revealed by immunohistochemical techniques using monoclonal antibodies to antigens expressed during the proliferative cycle.

Tests performed

Ploidy, proliferative activity and tumour stage were compared with clinical course.

Measurement of DNA content

DNA content in the nuclei of tumour cells was measured by means of flow cytometry. Monocell suspensions suitable for flow cytometry were prepared from the paraffin-embedded surgical specimens available for each patient according to the procedure described by Hedley [12 13]. For each suspension, ploidy was assessed as a DNA-index, i.e., the DNA content of the tumour cell genome. A population of normal quiescent cells has a 'diploid' genome of 23 pairs of normal chromosomes expressed by a DNA-index of 1. Quantitative chromosome changes in tumour cells determine an 'aneuploid' genome expressed by a DNA-index different from 1 (< 0.96 or > 1.04) [14]. The team members performing the flow cytometry procedure were aware of the tumour histotype but were unaware of patients' clinical and follow-up data.

Immunohistochemical assessment of proliferative activity

The MiB1 monoclonal antibody was used for the Ki 67 antigen expressed in the cell nucleus throughout the G1-S-G2 proliferation cycle. This antibody was used on 4 µm sections of material fixed in formalin and embedded in paraffin counting the number of positive stained cells out of at least 1000 cells at 40X magnification expressed in percentage terms as the "growth fraction". Again, those performing the cell count were unaware of patients' clinical staging and follow-up data.

Statistical analysis

Fisher's exact test for small numbers was used to calculate the significance of difference in behaviour in relation to ploidy or "growth fraction" expressed by MiB1. Survival curves were calculated according to the Kaplan-Meier method defining time 0 as the time of first treatment. The Log rank test was used to evaluate the difference between survival curves stratified for the variable of interest. Tumour recurrence, presence of disease at the last follow up (LWD) or death of disease was taken as the "final event".

Results

A total of 36 patients were enrolled in the study, 20 males (56%) and 16 females (44%). Mean age was 59.9 years (range 27-88) with a median age of 61 years at the time of diagnosis. The distribution of data for the entire cohort, divided into histological type, is summarised in Table I. None of the acinic cell carcinomas presented lymph node metastases at onset, whereas metastases were present in 67% of ductal carcinomas.

DNA ploidy was determined in 31 patients in all four histotypes considered. The cell proliferation fraction with MiB1 was determined in 16 patients: 6 with the ductal histotype and 10 with acinic cell carcinoma. Overall, only 28 cases were suitable for statistical analysis as data were incomplete in 7 patients and one patient with ductal carcinoma died a week after

Table I. Patients enrolled in study.

Patient	Sex/Age	TNM	Stage	Ploidy	MiB1	Follow-up
Adenoid cystic carcinoma						
P.A.	F/45	T2N2bM0	4	A		Dod 3 yrs
F.L.	F/66	T2N0	1	A		Ned 12 yrs
M.M.	M/65	T4N0	4	D		Dod 4 yrs
B.O.	M/76	T3N1	3	D		?
F.N.	F/73	rT4N0M1	4	D		?
P.M.	F/62	T4Nx	4	A		Ned 9 yrs
P.D.	F/49	T1N0M0	1	D		Ned 8 yrs
M.M.	F/27	T1N0M0	1	? (%S 2.8)		Ned 4 yrs
Ductal carcinoma						
M.G.	M/53	rT4N2bM0	4	A	45%	Dod 13 mths
C.A.	M/50	T3N1M0	4	A	35%	Doc 7 days
B.A.	M/61	T2N0M0	1	A	70%-20%*	Ned 6 yrs
B.P.	M/74	T4N1	4		10%	Lwd 5 yrs
Q.R.	M/85	T4N0M0	4		30%	Lwd 4 yrs
M.B.	M/74	T4N2b	4		40%	Dod 3 mths
Mucoepidermoid carcinoma						
R.T.	F/42	T1N0M0	1	D		?
T.G.	F/61	T2N0	2	D		?
L.F.	M/80	T4N1	4	A		Dod 7 yrs
A.D.	M/81	T4	4	D		Doc 8 mths
B.A.	F/69	T4N2b	4	D		?
B.C.	F/61	T1N0	1	D		Ned 10 yrs
M.N.	M/38	TxN1M0	3	D		Ned 8 yrs
B.G.	F/53	T1N0M0	1	D		Ned 7 yrs
F.V.	F/41	T2N0M0	2	D		Ned 6 yrs
C.M.	M/70	T2N0M0	2	A		?
R.E.	M/64	rT2N0M0	2	D		Ned 3 yrs
Acinic cell carcinoma						
M.S.	F/65	T4N0M0	4		50%	Dod 7 yrs
B.S.	M/46	T4N0M0	4	D	2%	Dod 12 yrs
U.S.	M/66	T1-2N0M0	1	D		Ned 10 yrs
M.G.	F/61	T1N0M0	1	A	5%	Dod 10 yrs
B.I.	M/51	T1N0M0	1	D	2%	Ned 8 yrs
T.N.	F/88	rT2M0	2	D	1%	Ned 7 yrs
S.G.	M/54	T1N0M0	1	D	4%	Ned 8 yrs
A.F.	M/38	T2N0M0	2	D	15%	Ned 6 yrs
V.S.	F/67	pT4N0M0	4	A	40%	Dod 4 yrs
F.R.	M/44	T2N0M0	2	A	3%	Ned 6 yrs
P.E.	M/49	T1N0M0	1	D	4%	Ned 6 yrs

D: diploid; A: aneuploid; Ned: no evidence of disease; Lwd: living with disease; Dod: died of disease; Doc: died of other causes; *: values obtained in two tumour areas with different degrees of differentiation; ?: lost to follow-up.

Table II. Ploidy and outcome.

	Favourable outcome	Poor outcome	Total
Diploid	13	2	15
Aneuploid	4	5	9
Total	17	7	24

Fisher exact test: p = 0.061; Favourable outcome: Ned, Doc. Poor outcome: recurrence, Lwd, Dod.

discharge from causes unrelated to surgery and was, therefore, excluded (Table I).

Nuclear DNA ploidy could be correlated with clinical follow-up in only 24 patients with the following tumours: 10 acinic cell carcinoma, 7 mucoepidermoid carcinoma, 5 adenoid cystic carcinoma, 2 ductal carcinoma. Ploidy showed a trend towards a favourable outcome for diploid tumours (disease-free patients or patients who died from unrelated causes) and an unfavourable clinical course for aneuploid tumours (patients living with disease or presenting recurrence or patients who died of the disease). Albeit, the correlation was not statistically significant (Table II), with Fisher exact test p = 0.061 and Log Rank test p = 0.063 (Fig. 1).

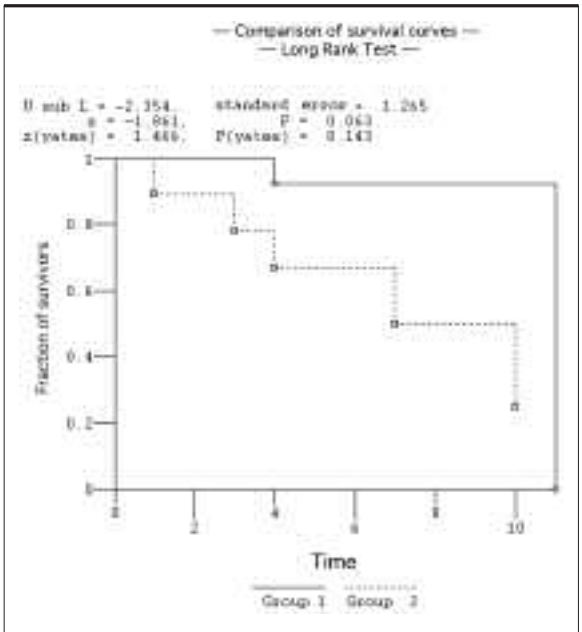

Fig. 1. Ploidy. Group 1: diploid; group 2: aneuploid. N.B. In all figures tumour recurrence or presence of disease at the last follow-up (Lwd) or died of disease (Dod) was taken as the "final event".

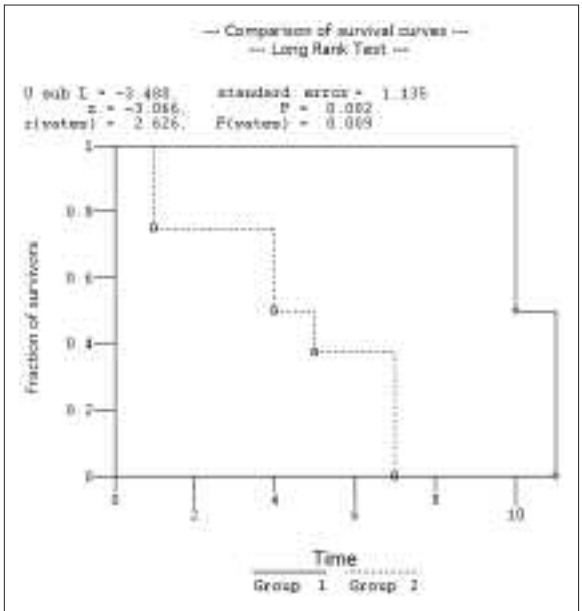

Fig. 2. Ductal and acinic carcinomas. Group 1: MiB1 ≤ 5; group 2: MiB1 > 5. "Final event", as defined in Figure 1.

Cell proliferation correlated with prognosis. Dividing patients into two groups on the basis of the MiB1 value with a cut-off of 5 (Fig. 2) and correlating the MiB1 value with favourable or poor outcome yield-

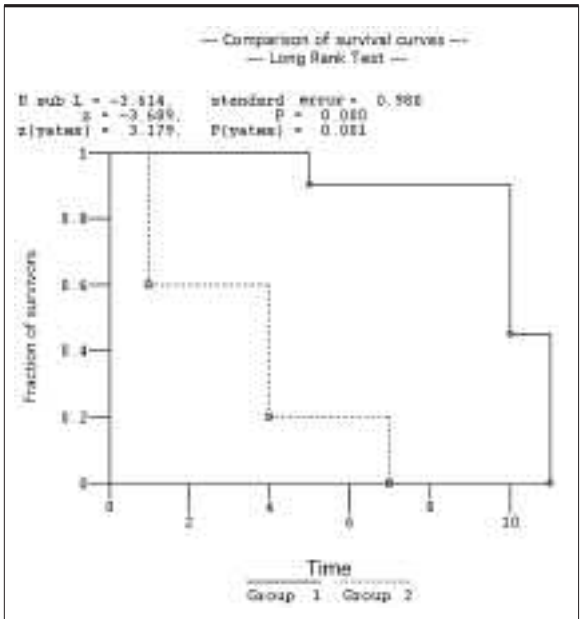

Fig. 3. Ductal and acinic carcinomas; group 1: MiB1 ≤ 20; group 2: MiB1 > 20. "Final event", as defined in Figure 1.

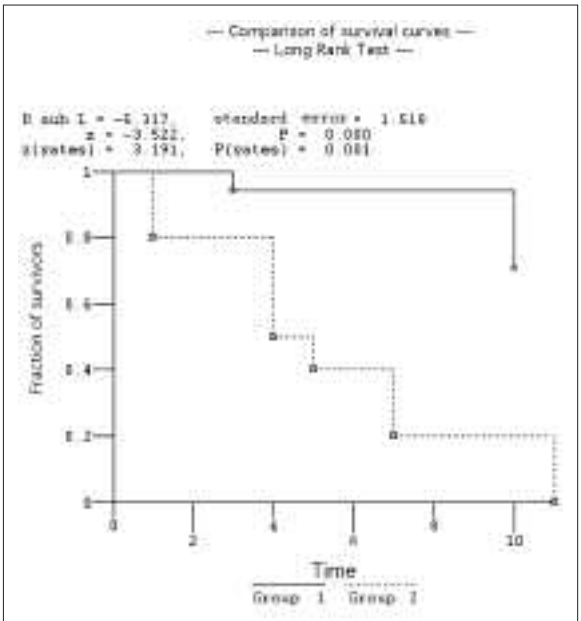

Fig. 4. Favourable/unfavourable outcome in relation to "T". Group 1: T1 + T2; group 2: T3 + T4. "Final event", as defined in Figure 1.

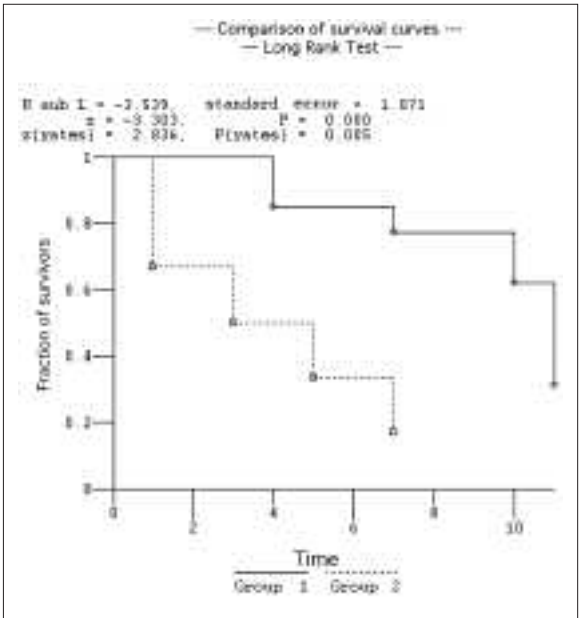

Fig. 5. Favourable/unfavourable outcome in relation to "N". Group 1: N0; group 2: N+ . "Final event", as defined in Figure 1.

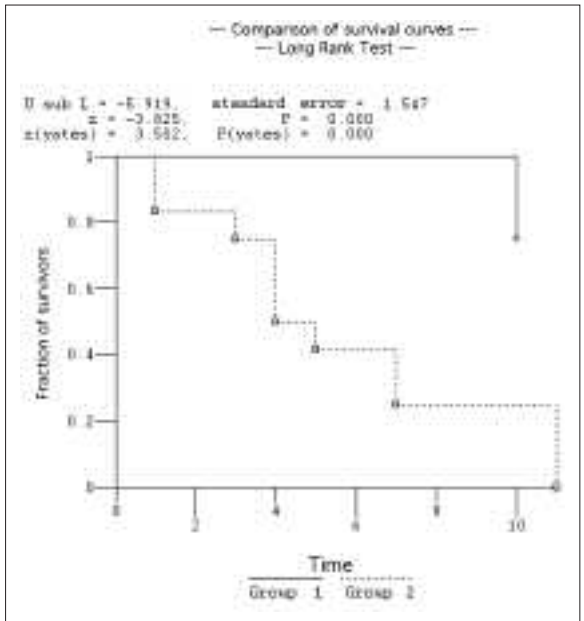

Fig. 6. Favourable/unfavourable outcome in relation to Stage. Group 1: stage 1-2; group 2: stage 3-4. "Final event", as defined in Figure 1.

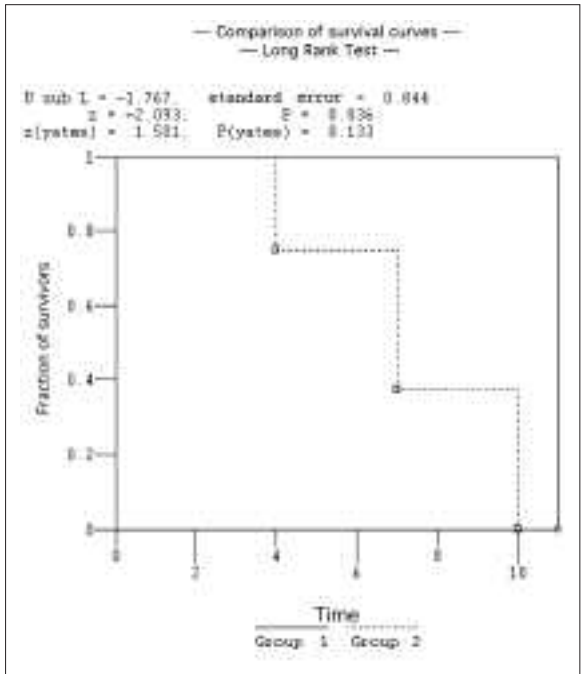

Fig. 7. Acinic carcinomas (n. 10 patients). Group 1 MiB1 < 5; group 2: MiB1 ≥ 5. Tumour recurrence or presence of disease at the last follow-up (Lwd) or death of disease (Dod) was taken as the "final event".

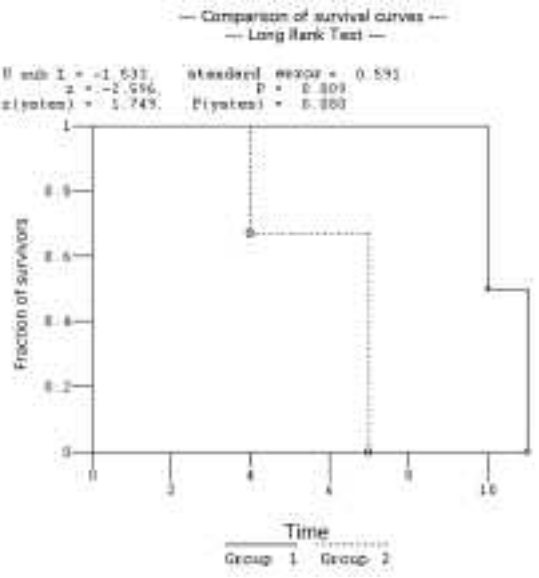

Fig. 8. Acinic carcinomas (n: 10 patients). Group 1 MiB1 < 15; group 2: MiB1 ≥ 15. Tumour recurrence or presence of disease at the last follow-up (Lwd) or death of disease (Dod) was taken as the "final event".

ed a statistically significant difference (p = 0.002, Log rank test); the same result was obtained with a cut-off value of 20 (p < 0.001, Log rank test) (Fig. 3). A high predictive value was confirmed for T, N and Stage (Figs. 4 - 6, respectively).
Interestingly, in the patients with acinic cell carcinoma, a comparison of survival curves for MiB1 using a cut-off of 5 was significant with p = 0.03 (Fig. 7), with a cut-off of 15, p = 0.009 (Fig. 8).

Discussion

Cell proliferation assessed by monoclonal antibody MiB1 proved an independent prognostic indicator in the present cohort of acinic and ductal salivary carcinomas. This is a particularly interesting finding in acinic cell carcinomas since this histotype lacks a unanimously accepted morphological grading, predictive of tumour aggressiveness.

Acinic cell carcinoma

This tumour is deemed the least aggressive salivary carcinoma with ten-year survival rates of 85% [15] or 68% [16]. Prognosis of the clinical course is difficult, on the basis of histomorphological criteria [1 17 18]. In 1990, Batsakis et al. [15] proposed a three tier grading of aggressiveness for acinic cell carcinomas, but acknowledged that the grading was relatively unproven and empirical. A mainly solid histological tumour aspect was correlated with a worse prognosis in one study [19], but the finding was subsequently not confirmed by these same Authors [2]. Acinic carcinomas arising in a lymphoid-cell rich stroma appear to carry a good prognosis [20].
Skalova et al. [18] reported a significant correlation between prognosis and growth fraction assessed by the MiB1 monoclonal antibody in 30 cases of acinic cell carcinoma; tumours with MiB1 indices < 5% had a more favourable outcome than those with MiB1 indices > 5% (p = 0.001). Hellquist et al. [21] also found that the MiB1 index with a cut-off of 10 was an independent prognostic factor in 16 salivary gland acinic cell carcinomas. Our findings confirm that determination of tumour growth fraction using the MiB1 antibody will define different classes of risk.

Ductal carcinoma

Kleinsasser et al. first described this tumour in 1968 under the name "salivary duct carcinoma" (Speichelgangcarcinome). However, this histotype was not included as a separate entity in the first WHO classification of salivary gland tumours drawn up by Thackray and Sobin in 1972 and was listed among the not otherwise specified adenocarcinomas. The tumour was finally classified as a "salivary duct carcinoma" (SDC) in the second edition compiled by Seifert et al. [22].
Although these tumours are uncommon, an increasing number of reports have appeared in the literature as well as larger case series (sometimes revised diagnoses) suggesting that SDC are not as rare as was previously held.
SDC are highly malignant tumours which tend to give rise to regional lymph node and distant metastases resulting in the death of most patients within three years of the first treatment. The tumour has a male prevalence with a male/female ratio from 2.5:1 to 6:1 in the different series. Mean age at onset is ~ 60 years and the tumour is seldom encountered before 50 years of age. Factors negatively influencing prognosis include: size > 3 cm and the presence of lymph node metastases. Recent efforts to identify the histological features of SDC having a favourable outcome aimed to establish a definition of "low grade SDC" [23-25]. In agreement with other reports in the literature [1 26 27], the SDC in our cohort showed a marked male prevalence, advanced mean age (66 years), most commonly location in the parotid gland (83%), lymph node metastases present at onset in a large percentage of patients (66%) and a high mortality rate. Cell proliferation activity assessed by MiB1 was invariably high in our patients, between 20% and 70%.

Cut-off

Although many studies report the prognostic significance of Ki67/MiB1 in salivary carcinomas, there is

still no consensus on the cut-off best stratifying different risk classes. Nordgård et al. [11] set the cut-off at 4% in a study on adenoid cystic carcinomas; Skalova et al. [18] set it at 5% for acinic cell carcinomas but found a more significant stratification for mucoepidermoid carcinomas with a cut-off of 10% [9]. In our series, combining acinic cell and ductal carcinomas, the best significance value was obtained using a cut-off of 20 ($p < 0.001$, Log rank test); analysing acinic carcinomas separately, we obtained the most significant value with a cut-off of 15 ($p = 0.009$, Log rank test).

Ploidy

Although the DNA ploidy of tumour cells in our cohort tended to show a favourable outcome for diploid tumours and a poor outcome for aneuploid lesions, the correlation was not statistically significant with $p = 0.06$. Furthermore, the correlation was not significant in the group of acinic cell carcinomas, with $p = 0.18$. El-Naggar et al. [3], in a series of 15 patients with acinic cell carcinoma and a follow-up of at least 10 years, found 8 patients with aneuploid tumours of whom 4 died of the disease and a fifth has metastases whereas none of the 7 patients with diploid tumours died of the disease. They concluded that DNA content in flow cytometry yields valid prognostic information.

Timon et al. [19] reached different conclusions in a personal study of 45 patients, claiming that DNA ploidy and AgNOR were not statistically significant prognostic indicators in their series. However, the most significant values in their study were obtained comparing survival curves discriminated in terms of the %S value indicating the number of cells in only one of the four stages of cell proliferation.
In our opinion, the prognostic value of DNA ploidy and %S in salivary gland carcinomas has yet to be established, but may prove valid. It is, however, a time-consuming complex procedure which may not offer advantages over other methods.

Conclusions

We failed to establish a correlation between ploidy and prognosis obtaining values of $p = 0.06$ with Fisher's exact test and the Log rank test. Instead, tumour cell proliferation as assessed by MiB1 antibody may serve as an independent prognostic index also for acinic cell carcinomas for which the growth fraction may stratify different classes of risk. More widespread use of the MiB1 index in major salivary gland carcinomas should be encouraged to establish its prognostic value.

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■ Address for correspondence: Dr. M. Vacchi Suzzi, Divisione ORL, Ospedale Maggiore, Largo Nigrisoli 2, 40133 Bologna, Italy..