The Journal of Laryngology and Otology
March 1996, Vol. **110**, pp. 243–248

Predictive value of flow cytometric analysis in DNA contents in patients with locally advanced head and neck carcinoma

GENE WONG, M.D., F.R.C.P.C.*, CHRISTINE STIDLEY, PH.D.†, LYNN DRESSLER, M.A.‡, MICHAEL CASTILLO, M.D.**, LIDA CROOKS, M.D.††, SUSAN BARTOW, M.D.††

Abstract

A restrospective study was performed on 61 eligible patients with stage III and IV (AJC/UICC Staging System) squamous carcinomas of the head and neck region who were treated with definitive radiotherapy with, or without, surgery. DNA contents were measured by flow cytometric analysis of archival paraffin blocks and were correlated with clinicopathological findings, tumour response and patient survival. Comparison of variables including treatment modality was performed for identification of significant prognostic factors. There were 28 diploid, 27 aneuploid tumours and the remaining six were questionable. All patients were followed-up for at least two years or until death.

Aneuploid tumours had a significantly higher S-phase fraction (percentage S-phase) ($p<0.001$). Neither ploidy nor percentage S-phase were found to have predictive value in tumour response or patient survival within the power of a sample size of 61. Twenty of the 27 (74 per cent) aneuploid tumours had a complete response (CR) whereas 19 out of 28 (68 per cent) diploid tumours achieved CR. Five-year survival by the Kaplan-Meier method was 33 per cent for both aneuploid and diploid tumours. However, nodal stage (N stage) was found to have significant predictive value in both tumour response and patient survival. The complete response for stage N_0 patients was 96 per cent, N_1 patients 61 per cent, N_2 patients 60 per cent and 43 per cent for N_3 patients ($p<0.002$). Similarly, the five year survival for the N_0 and N_3 groups of patients was 53 per cent and 29 per cent respectively ($p<0.05$).

Key words: Flow cytometry; Carcinoma, squamous cell; Head and neck neoplasms; Radiotherapy

Introduction

Head and neck carcinomas account for three per cent of all malignancies (Cancer Facts and Figures, 1995). Patients with advanced stage disease (Stage III and IV) have poor local-regional control and five-year survival despite standard treatments with radiation and/or surgery. Chemotherapy has been tested many times in non-randomized studies, but its value is still questionable because of selection and other biases, inadequate follow-up and a tendency to publish only positive results (Tannock, 1989). Other investigational treatments such as dose-intensive chemotherapy, hyper-accelerated fractionation irradiation or radio-sensitizers carry a higher risk of toxicity. There is an immediate need to develop a predictive assay in such patients so that more aggressive treatment can be directed to high risk patients and individual treatment can be optimized.

Clinicopathological parameters (e.g. tumour grade, stage, nature of invading margins) have been studied as possible prognostic factors. However, because they are semi-quantitative and subject to inter-observer variation, they cannot provide consistent predictive value to assist in the patient's management (Boyd and Reade, 1988). Flow cytometric analysis (FCM) of cellular DNA content now provides a rapid and quantitative assessment of tumour ploidy and proliferative activity. It has been recognized as a useful determinant of survival in a number of tumour sites (Ewers *et al.*, 1984; Balijham *et al.*, 1985; Look *et al.*, 1988).

There are a number of reports on the prognostic significance of FCM in head and neck carcinomas. However, most of them did not account for other confounding prognostic variables nor the inhomogenicity of treatment in the analysis. Unfortunately, the studies that controlled for confounders showed conflicting results thus making conclusions difficult (Goldsmith *et al.*, 1987; Kerasley *et al.*, 1991; Rua *et al.*, 1991). The objective of this study was to assess the predictive value of FCM in patients with locally

From the Department of Radiation Oncology*, the Tumour Registry†, and Tumor Facility‡, Cancer Center, the School of Medicine** and the Department of Pathology††, University of New Mexico, Albuquerque, USA.
Supported by grant RAC# C-1040 for the School of Medicine, University of New Mexico.
Accepted for publication: 5 November 1995.

advanced head and neck carcinomas treated with radiation. Variables including ploidy, S-phase fraction, treatment modality, disease stage and site, gender, age and tumour grade were assessed as potential predictors for tumour response and patient survival.

Materials and methods

Selection of patients

This was a retrospective study of patients with head and neck carcinomas diagnosed between 1979 and 1989. They were eligible for the study if they met the following criteria: pathologically confirmed squamous cell carcinoma of the head and neck region, except nasopharynx; locally advanced Stage III and IV disease (according to the American Joint Committee on Staging Classification) with no distant metastasis; treatment with definitive radiation (over 4500 cGy) with or without surgery; no previous or current chemotherapy; retrievable archival paraffin blocks, and no concurrent cancer. Sixty-one patients were eligible. Archival paraffin blocks were retrieved and reviewed by the pathologists. Representative sections of the tumour were then sent to the tumour facility for DNA content analysis. Three patients received radiation doses of less than 4500 cGy (2700 cGy, 3420 cGy and 3940 cGy respectively) because of progressive disease and rapid deterioration during treatment. They were included in the study because the inadequate treatment was a result of progressive disease and was felt not to induce bias in the analysis. The age of patients ranged from 39 to 82 years old and the median was 63. There were 45 males (74 per cent) and 16 females (26 per cent). The characteristics of the carcinomas are as shown in Table I.

Treatment technique

All 61 patients received radiation treatments from a 6 Mev linear accelerator and 49 of them had surgery for the primary tumour and/or lymphatics also. The primary tumour and the lymphatics at risk were treated with doses ranging from 2700 cGy to 9500 cGy with a median dose of 5940 cGy. The reason for giving the 9500 cGy to one patient was to compensate for the multiple interruptions during treatment. The daily fraction was 180–200 cGy and treatments were given five days per week. Patients who received implants or chemotherapy were excluded from this study. Patients were followed-up three months post-irradiation for evaluation of response and then regularly until death or loss to follow-up.

Paraffin block preparation

Available haematoxylin and eosin stained glass slides were retrieved from the files and reviewed for each case evaluated. The diagnosis of invasive squamous cell carcinoma was confirmed and each case was graded using a modification of the Broder System. Well-differentiated carcinomas (grade 1) were those composed predominantly of mature squamous cells, with abundant keratin pearl formation. Mitotic activity was minimal. The nuclei were uniform. The cell displayed obvious intercellular bridges in some areas. Moderately differentiated carcinomas (grade 2) were those composed of cells with less abundant cytoplasm. The cell borders were less distinct and the nuclei were more pleomorphic. Mitotic figures were more numerous. Poorly-differentiated carcinomas (grade 3) were composed of sheets and nests of small primitive cells. Cytoplasm was scant. The nuclei were hyperchromatic or vesicular. Mitoses were numerous. Keratinization was minimal to absent.

TABLE I

CHARACTERISTICS OF THE HEAD AND NECK CARCINOMAS IN THE STUDIED POPULATION

Characteristic	Group (Total n = 61)	Frequency	%
Tumour stage	2	10	16.4
	3	30	49.2
	4	20	32.8
	TX*	1	1.6
Nodal stage	0	26	42.6
	1	13	21.3
	2	15	24.6
	3	7	11.4
Site of disease	Oral cavity	6	9.8
	Oropharynx	11	18
	Hypopharynx	7	11.5
	Nasal cavity	3	4.9
	Larynx	33	54.1
	Branchial cyst	1	1.6
Grade	1	7	11.7
	2	42	70.0
	3	11	18.3
	Missing	1	
Surgery	Yes	49	80.3
	No	12	19.7

*TX – Tumour stage cannot be assessed.

https://doi.org/10.1017/S0022215100133316 Published online by Cambridge University Press

TABLE II
RELATION OF TUMOUR GRADE TO TUMOUR RESPONSE AND PATIENT SURVIVAL

Variable	Group	CR* n (%)	NR⁺ n (%)	*p*-value from: Fisher's exact test
Grade	1	6 (86)	1 (14)	
	2	31 (74)	11 (26)	0.66
	3	7 (64)	4 (36)	
Variable	Group	n	Median survival (months)	*p*-value from log-rank test
Grade	1	7	37.9	
	2	42	35.4	0.91
	3	11	12.7	

*CR = Complete response.
⁺NR = No response.

Representative sections of the primary tumour were sent for DNA content analysis. Samples of uninvolved lymph nodes were sent to be used as controls for identifying the diploid peak.

Flow cytometry

DNA flow cytometry was performed on formalin-fixed, paraffin-embedded material as previously described (Dressler and Bartow, 1992). Briefly, four 50 micron sections were deparaffinized in xylene and rehydrated in an alcohol series. Following digestion in pepsin-saline, samples were centrifuged through a double-layered sucrose cushion to minimize contamination by debris. Released nuclei were stained in a Tris fluorochrome buffer containing propidium iodide, RNase and NP-40 (Dressler and Bartow, 1992).

Samples were run on a Coulter EPICS 753 flow cytometer using the 488 nM argon laser. Single parameter, 256 channel integrated red fluorescence histograms were collected on a minimum of 20,000 events. Doublet contribution was minimized by bit map gating on peak versus integrated red fluorescence. Cell cycle analysis was performed using models previously described (Dressler *et al.*, 1987). The MODFIT software for cell cycle analysis was used for all analyses (Verity Software, Maine), incorporating the 'cut nuclei' debris component to estimate debris contribution. An interpretation of DNA diploid was given to histograms that contained a single symmetric population in the DNA diploid region with a G_0/G_1 coefficient of variation (cv) less than, or equal to, eight per cent. The use of an external DNA diploid control block (uninvolved lymph node) identified the diploid region (Dressler and Bartow, 1992). An interpretation of DNA aneuploid was given to histograms which showed two distinct G_0/G_1 populations. The abnormal peak had to comprise at least five per cent of the total events, to be given an interpretation of DNA aneuploid (most DNA aneuploid tumours had >10 per cent aneuploidy). Cell cycle analysis (S-phase fraction) was reported only in DNA aneuploid tumours that contained at least 10 per cent events in the aneuploid G_0/G_1 peak and whose (cv) was less than, or equal to, eight per cent to allow for accuracy and reproducibility of S-phase fraction. A histogram was interpreted as 'questionable ploidy' if it could not be defined according to the ploidy status using criteria stated above. This category largely consisted of single peak tumours whose G_0/G_1 (cv) was greater than eight per cent or where resolution could not identify distinct peaks from shoulders.

The median S-phase value was used as the cut-off point to define 'high' versus 'low' S-phase values in this study.

Outcome measures

Response to treatment was defined as complete if there was no clinically detectable disease at three months after radiation treatment. The rest was defined as no response (less than a complete response). Overall survival was computed from the day of pathological diagnosis to that of the last day of follow-up or death. Patients lost to follow-up were censored at the last day of follow-up. All patients were followed for at least two years or until death.

Statistical analysis

Patients were grouped based on flow cytometry results for S-phase and ploidy status. For S-phase, patients were grouped as low (less than, or equal to, the median) or high (above the median). The other grouping was diploid versus aneuploid. For the analysis of response, Fisher's exact test was used (Fleiss, 1981). Survival distributions were estimated using the Kaplan-Meier method with differences in

TABLE III
TUMOUR RESPONSE RATES IN DIFFERENT N STAGES

Variable	Group	Response CR n (%)	NR n (%)	*p*-value from: Fisher's exact test
N-stage*	0	25 (96)	1 (4)	
	1	8 (61)	5 (39)	
	2	9 (60)	6 (40)	
	3	3 (43)	4 (57)	0.002

*N stage = Nodal Stage American Joint Committee.

https://doi.org/10.1017/S0022215100133316 Published online by Cambridge University Press

TABLE IV
RELATIONSHIP BETWEEN PLOIDY AND S-PHASE FRACTION

Variable group	Ploidy Aneuploid n	Diploid n	p-value, from: Fisher's exact test	X^2 test
S-phase fraction < = 11.15	2	21		
> 11.15	17	7	<0.001	<0.001

the distributions assessed by the log rank test (Lee, 1992). Cox proportional hazards models were used to assess the significance of the prognostic variables on survival (Lee, 1992). All analyses were performed using the statistical computing package SAS (SAS Institute Inc, 1989 and 1991). The following confounding variables were included in the models: age, gender, T-stage, N-stage, tumour grade, ploidy, percentage S-phase and treatment modality.

Results

Histopathology

All tumours were squamous cell carcinoma. The distribution of T-stage and N-stage are shown in Table I. There were seven (12 per cent) grade 1, 42 (70 per cent) grade 2 and 11 (18 per cent) grade 3 tumours. There was a trend showing grade 3 tumours had poorer response rate and survival though it was not statistically significant (Table II). While neither tumour grade nor T-stage were found to have significant predictive value in both tumour response and patient survival, N-stage was. The higher the N-stage, the worse was the complete response rate and median survival. The complete response rate for N_0 patients was 96 per cent as compared to 43 per cent in N_3 patients ($p = 0.002$) as shown in Table III. The five year survival for N_0 patients was 53 per cent as contrasted to 29 per cent in N_3 patients ($p = 0.049$). The survival distribution of each nodal subgroup is shown in Figure 1.

Flow cytometry

Out of 61 tumour specimens, 28 were diploid (46 per cent), 27 were aneuploid (44 per cent) and six

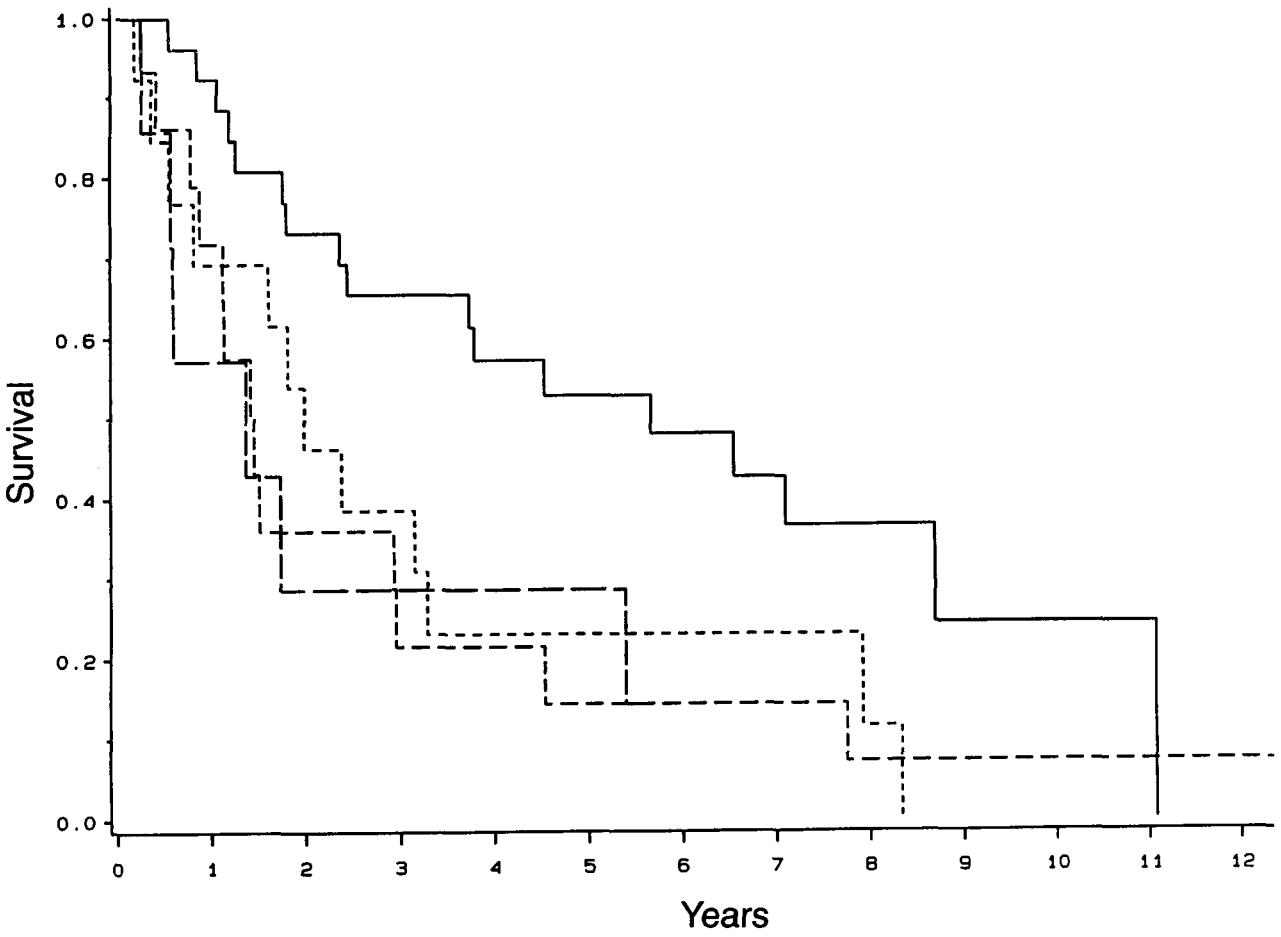

FIG. 1
Survival by nodal (N) stage.
——— $N_0 = 26$; - - - - - - $N_1 = 13$; – – – – $N_2 = 15$; — — — $N_3 = 7$.
Log-rank test: $p = 0.049$.

https://doi.org/10.1017/S0022215100133316 Published online by Cambridge University Press

TABLE V
DIFFERENCES BETWEEN LEVELS OF PLOIDY AND PERCENTAGE S-PHASE WITH RESPECT TO RESPONSE AND SURVIVAL

Variable	Group	Response CR n (%)	NR n (%)	p-value from: Fisher's exact test
% S-phase	< = 11.15	16 (69)	7 (31)	1.000
	> 11.15	16 (67)	8 (33)	
Ploidy	Aneuploid	20 (74)	7 (26)	0.77
	Diploid	19 (68)	9 (32)	
Variable	Group	5-Year Kaplan Meier Survival		p-value from: log-rank test
% S-phase	< = 11.15	39%		0.46
	< 11.15	29%		
Ploidy	Aneuploid	33%		0.88
	Diploid	33%		

were questionable. The S-phase fraction (percentage S-phase) was obtained in 47 tumours (77 per cent) and the remaining was uninterpretable. The range was 1.23–34.23 with a median of 11.15. The median was used to separate high and low percentage S-phase in this study. Out of the 47 tumours with interpretable results, 23 tumours were in the high percentage S-phase and the remaining 24 were in the low percentage S-phase. There was a strong and significant association between aneuploidy and high percentage S-phase ($p = 0.001$) as in Table IV.

Clinical outcome analysis

Five-year overall survival of all 61 patients was 35 per cent (male 26 per cent; female 56 per cent; $p = 0.0463$). There were 45 patients with complete response (74 per cent) and 16 (26 per cent) with no response (see definition for response under the paragraph of outcome measure). Twenty-five (56 per cent) of the 45 complete responders relapsed with a mean time to relapse of 49.8 months and a range of 5.3–151.8 months.

There was no statistically significant difference in the response pattern between aneuploid and diploid tumours. Twenty (74 per cent) out of 27 aneuploid tumours and 19 (68 per cent) out of 28 diploid tumours achieved complete response. Similar results were obtained with percentage S-phase. The complete response rate for low and high percentage S-phase was 69 per cent and 67 per cent respectively. In survival analysis, there was again no significant predictive value with ploidy or percentage S-phase. The five year survival for low and high percentage S-phase was 39 per cent and 29 per cent respectively ($p = 0.46$); survival was 33 per cent, identical for both aneuploid and diploid tumour groups (Table V).

Treatment variables were compared with respect to clinical outcome. There was no significant difference observed whether patients received surgery as part of their treatment, or not, although there was a trend suggesting the addition of surgery may achieve a higher complete response rate and longer median survival (Table VI).

Survival analysis using the Cox proportional hazards model of clinical outcome with different prognostic variables was performed. Ploidy and percentage S-phase, grade of tumour, T-stage, surgical treatment and age were found to have no statistically significant association with response or survival. The only two significant independent factors identified were gender and N-stage. The risk ratio of N_{1-3} as compared to N_0 patients in survival was found to be 2.72 ($p = 0.001$). The death rate among males was estimated to be 2.6 times higher than that of the females ($p<0.01$).

Discussion

The result of standard treatment of advanced stage squamous cell carcinoma of the head and neck region is unsatisfactory. The need to develop a predictive assay to select poor prognosis patients for aggressive treatment becomes more and more urgent. Several studies have been performed to assess the value of DNA content analysis in predicting the clinical outcome of these patients in order to individualize treatment. However, the result is conflicting and multivariate analyses were not performed in some studies (Goldsmith *et al.*, 1987; Tytor *et al.*, 1987; Farrer *et al.*, 1989; Kerasley *et al.*, 1991; Rua *et al.*, 1991; Bundgaard *et al.*, 1992). One study showed reversed outcome in that patients with aneuploid tumours had a better outcome than diploid (Goldsmith *et al.*, 1987).

In this study, only patients with Stage III and IV squamous cell carcinoma of the head and neck (excluding nasopharynx) intended for cure were included. All of them had radiation treatment and none received chemotherapy which delineated a more homogenous group of patients than other studies. Prognostic variables, such as treatment

TABLE VI
TREATMENT MODALITIES AND CLINICAL OUTCOME

Variable	Response CR n (%)	NR n (%)	Median survival (months)
Radiation + Surgery	38 (78)	11 (22)	35.3
Radiation	7 (58)	5 (42)	10.1
	($p = 0.18$)		($p = 0.28$)

https://doi.org/10.1017/S0022215100133316 Published online by Cambridge University Press

modality, were included in models to adjust for confounders. There was no association between tumour grade, percentage S-phase and ploidy as contrasted to the results of two previous studies on larynx and tongue (Hemmer *et al.*, 1990; Rua *et al.*, 1991). However, the importance of N-stage as a prognostic variable as suggested by other studies (Goldsmith *et al.*, 1987; Tytor *et al.*, 1987; Rua *et al.*, 1991) was confirmed (Table IV).

No data was available on the relationship between tumour response and DNA contents prior to this study. All previous studies compared ploidy and percentage S-phase with disease-free survival but not tumour response. Both univariate and multivariate analysis of the variables in this study did not show any difference in tumour response or patient survival between either ploidy or percentage S-phase groups (Table V).

Within the limitations of a retrospective analysis with small sample size, we found no predictive value for tumour ploidy or percentage S-phase in patients with advanced stage squamous cell carcinoma of the head and neck treated by radiation. Despite the effort to include all eligible patients over a period of 11 years and after reviewing more than 600 charts, only 61 patients were found to be eligible. The number of patients studied is small which reduces the statistical power for detecting small differences. For example, given that there were 28 diploids and 27 aneuploids with a response rate of approximately 70 per cent in this study, the minimum difference in response rates between the two groups that could have been statistically significant is 23 per cent, which is much larger than the observed difference of six per cent. Thus, more effort should be spent to find a predictive assay in this group of patients for customizing treatments.

Acknowledgements

Many thanks to Erma L. Pohl for her secretarial support and Dr George Browman for his advice on this project.

References

Balijham, G., Schulte, B., Reynders, M., Wiggers, T., Arends, J., Volvoics, L., Bosman, F. (1985) Flow cytometric determination of ploidy level and life cycle analysis on 297 paraffin-embedded colorectal carcinoma specimens. *Proceedings of American Society of Clinical Oncology* **4:** 22.

Boyd, N. M., Reade, P. C. (1988) Differences between preneoplastic cells, neoplastic cells and their normal counterparts. *Journal of Oral Pathology* **17:** 257–265.

Bundgaard, T., Sorensen, F. B., Gaihede, M., Sogaard, H., Overgaad, J. (1992) Stereologic, histopathologic, flow cytometric and clinical parameters in the prognostic evaluation of 74 patients with intraoral squamous-cell carcinoma. *Cancer* **70:** 1–13.

Cancer Facts and Figures, 1995, p 6. Published by American Cancer Society Inc.

Dressler, L. G., Bartow, S. A. (1992) DNA flow cytometry in solid tumours: Practical aspects and clinical applications. *Seminars in Diagnostic Pathology* **6:** 55–82.

Dressler, L. G., Seamer, L., Owens, M., Clark, G. M., McGuire, W. L. (1987) Evaluation of a modeling system for S-phase estimation in breast cancer by flow cytometry. *Cancer Research* **47:** 5294–5302.

Ewers, S. B., Langstrom, E., Baltorp, B., Killander, D. (1984) Flow cytometric DNA analysis in primary breast carcinomas and clinical pathological correlations. *Cytometry* **5:** 408–419.

Farrar, W. B., Sickle-Santanello, B. J., Keyhani-Rofagha, S., DeCenzo, J. F., O'Toole, R. V. (1989) Follow-up on flow cytometric DNA analysis of squamous-cell carcinoma of the tongue. *American Journal of Surgery* **157:** 377–380.

Fleiss, J. L. (1981) *Statistical Methods for Rates and Proportions.* Wiley, New York, pp 24–26.

Goldsmith, M. M., Cresson, D. H., Arnold, L. A., Postma, D. S., Askin, F. B., Pillsburg, H. C. (1987) Part 1: DNA flow cytometry as a prognostic indicator in head-and-neck cancer. *Otolaryngology – Head and Neck Surgery* **96:** 307–318.

Hemmer, J., Schon, E., Kreidler, J., Haase, S. (1990) Prognostic implications of DNA ploidy in squamous-cell carcinomas of the tongue assessed by flow cytometry. *Journal of Cancer Research and Clinical Oncology* **116:** 838–886.

Kerasley, J. H., Bryson, G., Battistutta, D., Collins, R. J. (1991) Prognostic importance of cellular DNA content in head-and-neck squamous-cell cancers. A comparison of retrospective and prospective series. *International Journal of Cancer* **47:** 31–37.

Lee, E. T. *Statistical 'methods for Survival Data Analysis.* (1992) 2nd Edition, John Wiley, New York, pp 67–78, 109–112, 250–263.

Look, A. T., Douglas, E. C., Meyer, W. H. (1988) Clinical importance of near-diploid tumour stem lines in patients with osteosarcoma of an extremity. *New England Journal of Medicine* **318:** 1567–1572.

Rua, S., Comino, A., Fruttero, A., Cera, G., Semeria, C., Lanzillota, L., Boffetta, P. (1991) Relationship between histological features, DNA flow cytometry, and clinical behavior of squamous-cell carcinoma of the larynx. *Cancer* **67:** 141–149.

SAS Institute Inc. (1989) SAS/STAT^R User's Guide, Version 6, 4th Edition, SAS Institute Inc., Cary, North Carolina, USA. pp 997–1025.

SAS Institute Inc. (1991) SAS^R Technical Report, p 229. SAS/STAT Software: Changes and Enhancements, Released 6.07, SAS Institute Inc., Cary, North Carolina, USA, pp 433–479.

Tannock, I. F. (1989) Combined modality treatment with radiotherapy and chemotherapy. *Radiotherapy and Oncology* **16:** 83–101.

Tytor, M., Franzen, G., Olofsson, J., Brunk, U., Nordenskjold, B. (1987) DNA content malignancy grading and prognosis in T_1 and T_2 oral cavity carcinomas. *British Journal of Cancer* **56:** 647–652.

Address for correspondence:
Gene Wong, M.D., F.R.C.P.C.,
Department of Radiation Oncology,
St Joseph Cancer Center,
601 Dr Martin Luther King Jr Avenue,
Albuquerque, NM 87102,
USA.