

Influence of preoperative radiotherapy on DNA ploidy in squamous cell carcinomas of the oesophagus

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Abstract

The influence of preoperative radiotherapy on the prevalence of DNA aneuploidy and the prognostic significance of tumour DNA ploidy was evaluated in 126 patients with squamous cell carcinoma of the oesophagus. Preoperative radiotherapy with 30 Gy was performed in 52 patients. DNA ploidy was analysed by flow cytometry on nuclei isolated from paraffin embedded tumour tissue. DNA aneuploidy was identified in 75 tumours (61%) and found to correlate significantly with tumour stage. The percentage of aneuploid carcinomas was significantly reduced by preoperative radiotherapy (surgery only group, 71%; radiotherapy group, 47%, $p=0.01$). Although the median survival time was slightly better in the diploid than in the aneuploid group (11.3 and 8.0 months respectively), this difference was not statistically significant. A curative tumour resection was the most important prognostic factor. Preoperative radiotherapy did not prolong survival in oesophageal cancer.

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Despite efforts at early diagnosis and advances in surgery, radiation therapy, and chemotherapy, the prognosis of squamous cell carcinoma of the oesophagus remains poor. Because chromosomal aberrations are a marker of malignancy and have been shown to correlate with changes in DNA content, DNA flow cytometry is widely used in the analysis of neoplasia.¹ Several studies on gastrointestinal cancers²⁻⁴ have shown that flow cytometric detection of an abnormal DNA content may provide additional prognostic information.

The objective of this flow cytometric study was to evaluate the impact of preoperative radiotherapy on the prevalence of DNA aneuploidy and to analyse the prognostic significance of DNA ploidy in oesophageal squamous cell cancer.

Patients and methods

PATIENTS

One hundred and twenty six patients (103 men, 23 women) with a squamous cell carcinoma of the oesophagus treated at the Department of Surgery of the Heinrich-Heine-University until December 1986 entered the study. Mean (SD) age of the patients was 56.2 (SD 9.9) years. In 86 patients, complete resection of the tumour was performed. In 52 patients, radiotherapy with 30 Gy was performed before surgery. The mean

time interval between completion of radiation treatment and surgery was 10 days. At the end of the study 119 patients had died.

All histological tumour sections were reclassified according to the 1987 updated TNF classification by one of the authors (FB). Table I shows tumour characteristics. Tumour length ranged from 0.5 to 13 cm (median, 3.0 cm).

FLOW CYTOMETRY

Formalin fixed, paraffin embedded tumour tissue was prepared and stained for flow cytometric analysis according to the slightly modified procedure described by Hedley *et al.*⁵ To decrease nuclear debris⁶ sections of 80 μ m were cut from tumour blocks stored at the Department of Pathology. Sections were deparaffinised by treatment with xylene overnight, rehydrated through a series of graded ethanol solutions and washed with distilled water.

A nuclear suspension was prepared by incubating the sections in 0.5% pepsin (Sigma, St Louis, Missouri) in 0.9% NaCl solution (pH 1.5; 37°C). After filtration through a 50 μ m nylon mesh nuclei were washed twice with phosphate buffered saline. The sediment was resuspended in a 0.1% Nonidet P 40 - trisodium citrate solution.⁷ After addition of ribonuclease A (Sigma, St Louis, Missouri; final concentration 0.1%) nuclear DNA was stained with propidium iodide (Sigma, St Louis, Missouri; 50 μ g/ml).

TABLE I Tumour characteristics of irradiated and unirradiated squamous cell carcinomas of the oesophagus

	No (%)
Tumour site:	
Upper third	17 (13.5)
Mid-third	70 (55.5)
Lower third	34 (27.0)
>One third	5 (4.0)
Grading:	
G1	12 (9.5)
G2	77 (61.1)
G3	33 (26.2)
G4	4 (3.2)
Tumour invasion:	
pT1	10 (7.9)
pT2	22 (17.5)
pT3	65 (51.6)
pT4	29 (23.0)
Lymph node metastasis:	
pN0	54 (42.9)
pN1	72 (57.1)
Distant metastasis:	
M0	107 (84.9)
M1	19 (15.1)
Tumour stage:	
I	9 (7.1)
II	47 (37.3)
III	51 (40.5)
IV	19 (15.1)

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Nuclear DNA content of at least 10 000 tumour cells in each block were measured with an EPICS C flow cytometer. One to six paraffin embedded blocks were measured for each patient (mean 2.5 blocks). Two to three sections were measured in each block. In three patients, no analysable DNA histogram could be generated because their tumours had been fixed in Bouin's solution.

Tumours were either classified as diploid or aneuploid. Samples with more than one G₀/G₁ peak in the DNA histogram were judged as aneuploid.⁸ In these tumours, the first peak on the left of the histogram was considered to represent diploid G₀/G₁ cells. For DNA aneuploid samples a DNA index was calculated as the ratio of the abnormal G₀/G₁ mean peak channel number to the diploid G₀/G₁ mean peak channel number. Histograms were classified without previous knowledge of the pathological or survival data.

We restricted our flow cytometric analysis to the determination of DNA ploidy because increased amounts of nuclear fragments after enzymatic digestion reduce the accuracy of cell cycle analysis accounting for the relatively poor correlation between proliferative activity in unfixed compared with paraffin embedded material.^{9,10} Furthermore, because of the higher coefficient of variation in paraffin embedded material cell cycle analysis is not as reliable as in fresh tissue of solid tumours. The mean (SD) coefficient of variation of the G₁ peak in this study was 5.9% (1.5%) (range 3.6% to 8.8%).

STATISTICS

Frequency tables were analysed by the χ^2 test. Survival time was defined as the period between surgery and death. Three patients were lost to follow up and have been excluded from the analysis of survival. Patients dying as a result of postoperative complications (within 30 days) were excluded from the survival analysis. Univariate survival analysis was performed with the BMDP 1L computer program by the life table method.¹¹ Data are presented as median (SEM). The significance of differences was calculated with the generalised Wilcoxon test (Breslow) and the generalised Savage test (Mantel-Cox).

The Cox proportional hazard model¹² was used in multivariate regression analyses of survival data (BMDP 2L). Variables entered into the model were sex of the patients, age of the patients (<60; >60 years), tumour site, tumour grading, tumour stage, radicality of surgery, radiotherapy, DNA ploidy, and tumour length (<3 cm; >3 cm).

Results

DNA PLOIDY

DNA diploidy was detected in 48 cancers of the oesophagus (39%) and aneuploidy in 75 tumours (61%). In four patients, more than one aneuploid peak was present. The DNA indices ranged from 1.0 to 2.64.

Tumour DNA content was not significantly

TABLE II Relation between DNA ploidy and clinical and pathological variables in 123 patients with a squamous cell carcinoma of the oesophagus

	Aneuploid (%)	Diploid (%)	p Value
Sex:			
Male	60.0	40.0	NS
Female	65.2	34.8	
Tumour site:			
Upper third	68.8	31.2	NS
Mid-third	61.4	38.6	
Lower third	54.5	45.5	
>One third	75.0	25.0	
Grading:			
G1	33.3	66.7	NS
G2	62.7	37.3	
G3	62.5	37.5	
G4	100.0	0*	
Tumour invasion:			
pT1	20.0	80.0	p=0.04
pT2	60.0	40.0	
pT3	64.1	35.9	
pT4	69.0	31.0	
Lymph node metastasis:			
pN0	50.9	49.1	p=0.07
pN1	68.6	31.4	
Distant metastasis:			
M0	60.6	39.4	NS
M1	63.2	36.8	
Tumour stage:			
I	22.2	77.8†	p=0.04
II	54.5	45.5	
III	73.1	26.9	
IV	61.1	38.9	
I+II v III+IV			p=0.04
Radiation therapy:			
Yes	47.1	52.9	p=0.01
No	70.8	29.2	
Surgery:			
Curative	61.4	38.6	NS
Non-curative	60.0	40.0	

*This group contained four patients. There was no significant difference after testing G1/2 v G3/4. †The aneuploid group in stage I contained only two patients. The analysis was therefore repeated for tumour stage I+II v III+IV.

related to gender of the patients, tumour site, or tumour differentiation (Table II). DNA ploidy significantly correlated with the invasion of the primary tumour. The percentage of aneuploid tumours rose from 20% in T1 tumours to 69% in T4 tumours. The percentage of diploid carcinomas was significantly higher in stage I and II tumours (51%) than in stage III and IV tumours (30%). The association with lymph node metastasis was of borderline statistical significance (p=0.07). DNA ploidy did not influence the result of surgery (curative v non-curative).

Prevalence of DNA aneuploidy, however, was significantly influenced by preoperative radiotherapy. The percentage of aneuploid tumours decreased from 71% in unirradiated tumours to 47% in irradiated tumours (p=0.01). Tumour diameter decreased from 4.2 (2.8) cm to 3.1 (1.6) cm after irradiation (p=0.02). Preoperative radiotherapy did not change the percentage of curative resections.

SURVIVAL

After exclusion of postoperative deaths the overall five year survival rate was 6.5%. Median survival time of the whole study population (n=101) was 9.2 (1.4) months.

In univariate survival analysis the presence of lymph node metastasis (N0: median survival, 13.8 months; N1: 7.3 months), the presence of distant metastasis (M0: 10.6 months; M1: 6.0 months), and tumour stage (I: 51.6 months; II: 11.8 months; III: 8.3 months; IV: 6.0 months) were highly significant prognostic variables for

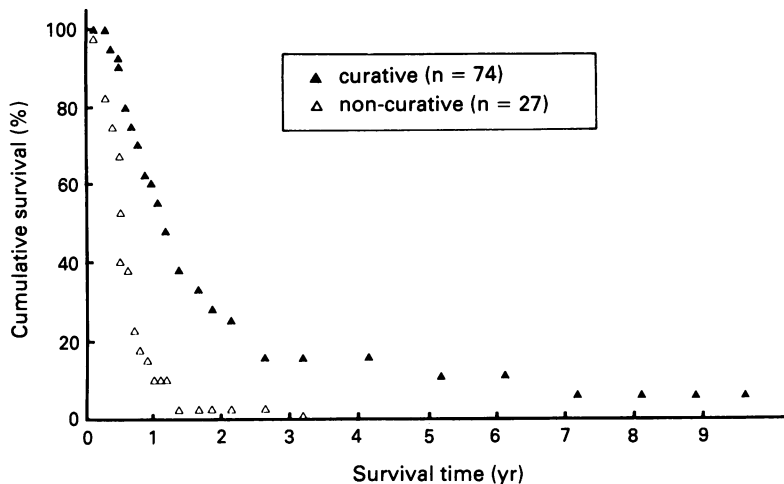


Figure 1: Survival of patients with squamous cell carcinomas of the oesophagus was significantly influenced by the result of surgery ($p < 0.0001$ by generalised Wilcoxon test and by generalised Savage test).

the whole study group. Radicality of surgery had a considerable influence on survival (curative resection: 11.8 months; non-curative: 4.6 months; Fig 1). One year after surgery, survival rate was 49% after curative resection in comparison to 11% after non-curative surgery.

Tumour grading and tumour invasion were of borderline statistical significance. Preoperative radiation treatment did not result in a significant prolongation of survival (surgery group 8.5 months; radiation plus surgery group 10.5 months). Female patients (15.0 months) tended to survive longer than male patients (8.3 months, NS). Patients with diploid tumours (median survival, 11.3 (1.2) months) survived longer than patients with aneuploid tumours (8.0 (1.3) months). This difference in survival time was not significant (Fig 2).

These univariate survival analyses were also separately performed for the group of patients with preoperative radiotherapy and the group with surgery only. Radicality of surgery remained the most important survival factor. After surgery alone, patients with diploid tumours (15.0 (12.0) months) survived longer than patients with aneuploid tumours (7.3 (0.9)

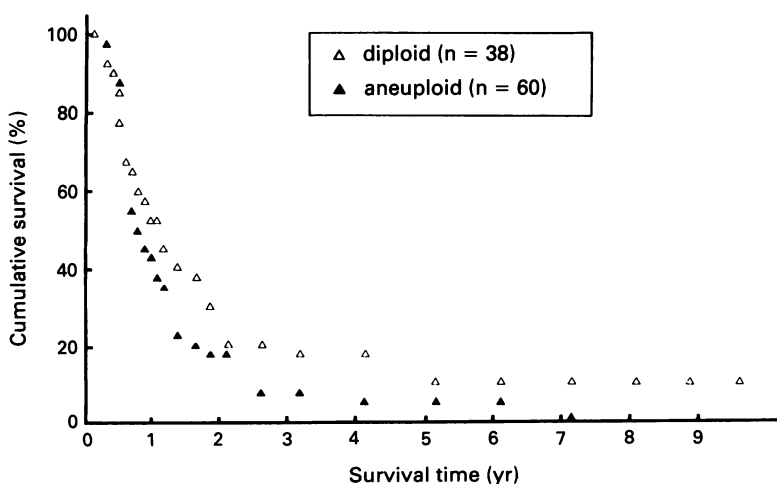


Figure 2: Survival of patients with carcinoma of the esophagus was not influenced by DNA ploidy state (life table analysis).

months; $p = 0.09$ by generalised Wilcoxon test, $p = 0.06$ by generalised Savage test). In the radiotherapy group there was a non-significant DNA diploid survival advantage (19.5 (2.0) months *v* 12.0 (2.0) months).

After a curative tumour resection, female patients (23.1 (4.3) months) survived significantly longer than male patients (11.2 (0.9) months; $p = 0.03$ by generalised Wilcoxon test, $p = 0.05$ by generalised Savage test). Preoperative radiotherapy did not prolong the median survival time. DNA ploidy did not have a significant prognostic influence on survival after a curative resection.

When the prognostic impact of the different clinicopathological and flow cytometric variables on survival was evaluated in a multivariate regression analysis, radicality of surgery emerged as the most important factor ($\chi^2 = 22.5$) followed by tumour grading ($\chi^2 = 7.1$), sex of the patients ($\chi^2 = 7.1$), and tumour stage ($\chi^2 = 5.0$). DNA ploidy did not confer independent prognostic information for survival.

Discussion

Squamous cell carcinoma of the oesophagus remains one of the deadliest malignant neoplasms because patients often present with advanced disease at the time of diagnosis. The prognosis of patients with carcinoma of the oesophagus mainly depends on pathohistological and surgical criteria.¹³⁻¹⁵ As in our study, there is a clear difference in survival between the curative and non-curative groups. In the multivariate regression analysis, radicality of surgery was the most important prognostic variable. After the development of lymph node metastases and distant metastases, median survival of patients declined significantly. Our observation that female patients carry an improved survival after a curative tumour resection is supported by data from Japan.¹³

There is considerable variability and heterogeneity in the clinical course of patients with squamous cell carcinoma of the oesophagus. Beside the TNM classification system additional variables, such as biological staging for the intrinsic malignant potential of the tumours, might be useful for determining long term survival.

Several studies have shown that flow cytometric detection of DNA aneuploidy might reflect the malignant potential of gastrointestinal cancers.²⁻⁴ Flow cytometry of archival tumour material offers the advantage that long term follow up is available. Comparative studies of fresh and paraffin embedded tumour tissue have shown that accurate determination of DNA ploidy in archival tumour tissue can be performed reliably in most tumours.^{9,10}

In our series, DNA aneuploidy was detected in 61% of all patients and in 71% of the unirradiated patients with squamous cell carcinoma of the oesophagus. Because of the known heterogeneity of DNA aneuploidy in oesophageal carcinomas¹⁶ tumour blocks were analysed until DNA aneuploidy was detected in at least one block or was excluded by measuring all available tumour blocks in one patient. This percentage is

in accordance with other flow cytometric studies in oesophageal cancer that have reported rates of aneuploidy in the range of 57% to 91%. Prevalence of DNA aneuploidy was comparable in fresh (83/112 tumours (74.1%)¹⁶⁻¹⁹ and in paraffin embedded material (77/108 tumours (71.3%).^{20,21}

The percentage of aneuploid tumours increased according to tumour invasion and the development of lymph node metastasis reflecting an increased genomic instability.¹⁷ Conflicting results concerning the relation between the degree of differentiation and abnormalities of DNA content have been reported. As in our study, Kaketani *et al* and Edwards *et al* did not find a significant correlation^{21,22} whereas this was found by three other groups.^{17,18,20}

Although a trend for an increased survival time in patients with diploid tumours was found this difference was not significant. These results are supported by the findings from English and Italian groups.^{20,22} DNA distribution patterns analysed by cytophotometry, however, have been shown to relate to survival in patients from Japan.^{23,24} Because the definitions of DNA aneuploidy differ between these flow cytometric and cytophotometric studies, the results cannot be compared directly. In these cytophotometric studies, aneuploidy was defined by the DNA distribution pattern that was largely determined by cells with DNA values beyond the 4c region. In flow cytometry, DNA aneuploidy is defined by the presence of a distinct second G₀/G₁ peak.

After preoperative radiotherapy the percentage of aneuploid tumours decreased significantly, from 71% to 47%. This result should be interpreted cautiously because serial biopsies were not available in these tumours. This result, however, most probably reflects local response to treatment with the eradication of aneuploid tumour cells in a proportion of carcinomas. This interpretation is supported by data in rectal carcinomas. Jones *et al* showed significant differences in DNA ploidy state between preoperatively irradiated and non-irradiated rectal cancers.²⁵ Similar changes were found in serial biopsies taken during radiotherapy in rectal carcinomas.²⁶

It has been suggested that aneuploid cervical tumours are more radiosensitive than diploid tumours.²⁷ In squamous cell carcinomas of the head and neck and in high grade non-Hodgkin's lymphoma aneuploid tumours are most responsive to chemotherapy.^{28,29} In oesophageal cancer, the effect of hyperthermochemo-radiotherapy is more pronounced in aneuploid than in diploid tumours.³⁰ Therefore, it can be hypothesised that DNA aneuploidy might be an indicator for response to preoperative radio or chemotherapy. This assumption, however, has still to be supported by prospective studies.

The preoperative radiotherapy did not result in a significant prolongation of survival time. This also applied to the subgroup with a curative tumour resection and is in accordance with other studies that used preoperative radiotherapy.³¹ Although the reduced percentage of aneuploid tumours can be interpreted as a local response to radiotherapy the missing effect on survival points to the fact that oesophageal cancer is often diagnosed at a locally advanced or even dis-

seminated stage. In patients who undergo surgery recurrence and death are often attributed to unsuspected early metastatic disease the presence of which has been documented in necropsies performed relatively soon after surgery.³²

In conclusion, DNA ploidy was not identified as a prognostic factor in oesophageal squamous cell carcinoma in this flow cytometric study. Survival of patients was not improved by preoperative radiotherapy, but was significantly associated with a radical tumour resection. Although the percentage of aneuploid carcinomas was significantly reduced after preoperative radiotherapy this local response did not convert into a prolongation of survival. These results support the necessity of combined modes of treatment including chemotherapy for the eradication of metastatic disease unsuspected at the time of diagnosis.

Dedicated to Professor Dr Georg Strohmeyer on the occasion of his 65th birthday.

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