Pergamon

European Journal of Cancer Vol. 30A, No. 12, pp. 1787–1791, 1994

0959-8049/94 $7.00+0.00

0959-8049(94)00252-5

In vivo Cell Kinetic Measurements in Human Oesophageal Cancer: What can be Learned From Multiple Biopsies?

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The importance of intratumour variability of cell kinetics was studied in 60 patients with cancer of the oesophagus. Five biopsies per tumour were taken. The labelling index, S-phase duration and potential doubling time (T_{pot}) were measured using flow cytometry. The mean T_{pot} value was 5.56 ± 4.43 days (± 1S.D.) for adenocarcinomas and 4.40 ± 2.45 days (± 1S.D.) for squamous cell carcinomas. These values were statistically significantly different. Although intratumour variation in T_{pot} measurements occurred, the intertumour variability was more important ($P < 0.00001$). This feature permits classification of tumours into slow and fast proliferating groups, leaving an intermediate group of tumours that could not be unequivocally categorised. The relative distribution of tumours into these three categories depends on the intratumour and intertumour variability of T_{pot}, and on the cut-off values used. Increasing the number of biopsies from one to five reduces the number of non-classifiable tumours.

Key words: cell kinetics, flow cytometry, oesophageal neoplasms
Eur J Cancer, Vol. 30A, No. 12, pp. 1787–1791, 1994

INTRODUCTION

THE FINAL outcome of protracted treatment modalities, such as radiotherapy and chemotherapy, will be influenced by the proliferation characteristics of both tumours and normal tissues. This is well known for normal tissues and is becoming clearer for neoplasms, based on preliminary clinical data [1] and theoretical considerations [2]. As familiarity with unconventional radiotherapy [3–6] and chemotherapy (intensification schedules) schedules grows, it will become increasingly important to know the proliferative characteristics of an individual tumour, in optimising treatment. Indeed, not all patients will benefit from such intensified treatments [2] which cause greater acute and sometimes also more severe late toxicity [7, 8]. Moreover, proliferation kinetics may not only be used as a decision factor in treatment strategy, but also a predictor of natural history and prognosis.

Proliferative parameters widely used in experimental settings are the labelling index (LI) and the potential doubling time (T_{pot}). T_{pot} is defined as the time that would be required by a tumour to double in cell number in the absence of cell loss [9].

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Revised 13 June 1994; accepted 24 June 1994.

T_{pot} can be obtained *in vivo* by flow cytometric analysis of a tissue biopsy obtained several hours after a single infusion of 5-iodo-2′-deoxyuridine (IUdR), a thymidine analogue which can be detected using a specific monoclonal antibody, thus avoiding radioactive isotopes [9, 10]. Clinical experience with the measurement of T_{pot} and its practical applicability is limited. Some of the problems emerging are the intratumour variability of T_{pot} [11] and the definition of relevant cut-off values on which to base treatment decisions.

In the present study, the proliferation kinetic parameters of oesophageal carcinoma were investigated as a first stage of a more systematic evaluation of the practical problems encountered in their clinical application.

MATERIALS AND METHODS

Patients

From February 1991 to March 1992, 60 patients treated with radical surgery for carcinoma of the oesophagus or cardia were included in this study.

Pathology

27 of the 60 patients had squamous cell carcinomas, 32 suffered from adenocarcinomas of the distal part of the oesophagus and cardia and 1 patient had signet ring carcinoma. The distribution of patients by pathological T stage is shown in Table 1. None of the patients had received pre-operative therapy.

Biopsy

Immediately after resection, before fixation, three to five biopsies were taken from the tumour as follows: when the

Table 1. Distribution of tumours according to T stage

	T1	T2	T3	T4
Adenocarcinomas	2	2	27	1
Signet ring carcinoma				1
Squamous cell carcinomas	5	5	17	0

tumour was not circular, one central biopsy and two to four at the periphery (left, right, cranial and caudal biopsies); and when the tumour was circular, three at the centre and two at the cranial and caudal periphery. Of the 60 tumours studied, a total of 288 samples were analysed. In addition, 30 biopsies were taken from two tumours each. Biopsies were fixed in cold 70% ethanol and stored at 4°C in the dark until flow cytometric analysis.

Cell kinetics

IUdR labelling. IUdR was obtained as a freeze dried powder in 200-mg vials from the Pharmacy of the University Hospital of Leuven (40 patients) or from the National Cancer Institute Bethesda (20 patients). The drug was dissolved in 10 ml sterile water immediately before use, and administered 6–10 h before surgery as an intravenous (i.v.) infusion in 150 ml of 5% glucose, during a maximum of 15 min, at a dose of 100 mg/m^2 [1].

Staining. Each ethanol-fixed biopsy, weighing approximately 0.05 g, was first incubated in a pepsin solution (0.4 mg/ml) for 60 min at 37°C, followed by acid denaturation with 1.5 ml 2 N HCl for 20 min at 37°C, incubation with a mouse anti-human anti-BrdU purified monoclonal antibody (30 min, room temperature, Becton Dickinson, Erembodegem, Belgium); incubation with an FITC-labelled goat anti-mouse IgG (30 min, room temperature, dilution 1/50, Sigma Chemical Co., Bornem, Belgium); followed finally by staining total DNA with a 10 μg/ml propidium/RNase solution (Sigma) [11].

Flow cytometry. Flow cytometry was carried out using a FACSstarPlus flow cytometer (Becton Dickinson). The exciting light was 488 nm, and the emission filters were 515–545 nm band pass (BP) filter (green; IUdR) and 619–641 nm BP (red; DNA). Instrument alignment and standardisation was performed with Calibrite beads (Becton Dickinson) and DNA QC particles (Becton Dickinson). Ten thousand cells were acquired per sample. The green fluorescence signal was displayed on a log scale, the red signal on a linear scale. The settings on the FACSstarPlus were 400 V on the photomultiplier for green and 508 V on the photomultiplier for red. Compensations were seldom necessary and were made on the basis of vertical lines of labelled cells on the green/red fluorescence cytograms above the G_1 position.

Cell kinetic analysis. DNA analysis was performed by using Cellfit software (Becton Dickinson). After gating out the doublets on width versus area, the Polynomial and SOBR model were utilised.

The method of determining the length of the S-phase (T_s) from a relative movement (RM) analysis has been described previously [9]. Briefly, after gating out the doublets on width versus area, the analysis was carried out by placing computer-drawn windows around the different populations and assuming that the RM was 0.5 at time 0. The mean red fluorescence of the labelled cells relative to the red fluorescence values for G1 (F_{G1}) and G2 was first determined. T_s could then be calculated knowing the time elapsed between IUdR administration and the taking of the biopsy, t. The two equations used for calculating T_s were: (1) $RM = (F_L - F_{G1})/F_{G1}$ and (2) $T_s = (RM - 0.5)t/0.5$; where F_L is the mean red fluorescence of IUdR labelled cells. The labelling index (LI) was determined as the percentage of green labelled cells, after correcting for the labelled cells which had divided. The potential doubling time (T_{pot}) was calculated as the ratio T_s/LI, assuming that the age distribution factor, lambda, was unity [12].

Labelling in the tumour was adequate for the above analysis in all 347 samples. Sometimes staining had to be repeated until the population of green fluorescent cells was sufficiently separated from the bulk of cells so that the windows could be placed around the labelled and unlabelled populations.

Statistical methods

The intratumour variability versus the intertumour variability for the different kinetic parameters was assessed with a one-way analysis of variance (ANOVA). The differences in kinetic measurements between central and peripheral biopsies were studied using Student's t-tests and confidence intervals. Normality of the T_{pot} distributions was verified with both the Shapiro-Wilks and the Lilliefors tests. Confidence intervals (CI, 95%) were calculated from the five biopsies of each tumour.

RESULTS

Assay variability

To test the assay's reproducibility, five biopsies of one tumour were digested separately to a suspension of nuclei. Each suspension was divided over five tubes and all 25 tubes were stained and analysed separately on a blind basis by one person. Analysis of variance showed that the assay variability was significantly smaller than the interbiopsy variability observed in the present study (cfr. infra tumour kinetic parameters) ($P < 0.00001$).

Tumour kinetic parameters

On DNA analysis, 85% of the biopsies were aneuploid. In six tumours, all five biopsies were diploid. The proliferation parameters measured for the latter were statistically significantly slower than those of the aneuploid tumours. On histological examination, the 30 biopsies of the diploid tumours contained at least 50% tumour cells; in the biopsies of two tumours nearly 100% of the cells were malignant.

The mean values and standard deviations of T_{pot}, LI and T_s are presented in Table 2. The mean value for T_{pot} for the whole group was 4.93 ± 3.64 days, for the adenocarcinomas, 5.56 ± 4.43 days, and for the squamous cell carcinomas, 4.40 ± 2.45 days. The T_{pot} of adenocarcinomas was statistically significantly slower than that of squamous cell carcinomas ($P < 0.01$).

Figure 1a and b presents the individual T_{pot} measurements for squamous cell carcinomas and adenocarcinomas, ranked

Table 2. Mean T_{pot}, T_s and LI values ± 1 S.D. for all tumours, adenocarcinomas and squamous cell carcinomas

	T_{pot}(:days)	T_s	LI
All	4.93±3.64	16.21±7.27	0.17±0.08
Adenocarcinomas	5.56±4.43	15.76±6.71	0.16±0.09
Squamous cell carcinomas	4.40±2.45	17.16±7.98	0.19±0.07

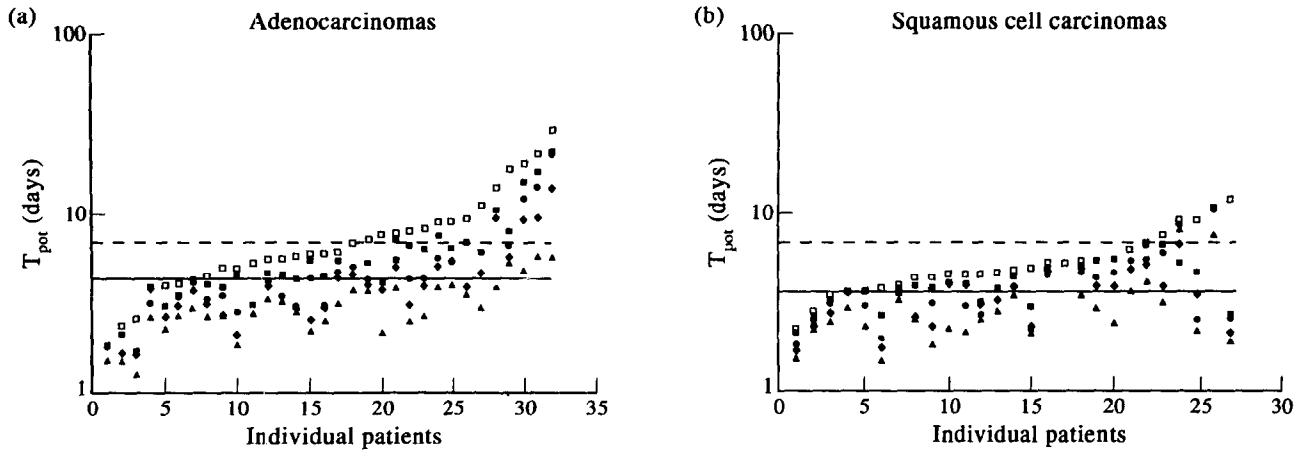

Figure 1. Separate plots of all T_{pot} values for adenocarcinomas and squamous cell carcinomas, ranked according to their highest value. Cut-off values shown as horizontal solid lines (——, median; ---, 7 days).

according to their highest value. The coefficient of variation as a measure of intratumour variability, for the squamous cell carcinomas varied between 7 and 39% and for the adenocarcinomas between 16 and 51%.

Using the median of means (4.336 days for the adenocarcinomas and 3.696 days for the squamous cell carcinomas) as a reference, 55 (34%) of the T_{pot} measurements were below and 107 (66%) above this value for adenocarcinomas; for squamous cell carcinomas, the numbers are 62 (49%) and 64 (51%), respectively. All but eight tumours have at least one T_{pot} measurement below the median of means. When an arbitrary value of 7 days was used as the cut-off level, numbers below and above were 128 (81.5%) and 29 (18.5%), respectively, for adenocarcinomas, and 108 (89%) and 13 (11%), respectively, for squamous cell carcinomas. All tumours, except one squamous cell carcinoma, have at least one T_{pot} value shorter than 7 days.

In Figure 2a and b, the mean T_{pot} values ± 95% CI, calculated for the individual biopsies of each tumour, are shown for adenocarcinomas and squamous cell carcinomas. The CI for seven (21%) adenocarcinomas and six (22%) squamous cell carcinomas were below the median, while the CI of 21 adenocarcinomas and 21 squamous cell carcinomas were below 7 days. In 22 adenocarcinomas and in 16 squamous cell carcinomas the CI spanned the median, so these tumours could not be classified as slow or fast. With 7 days as a cut off, only 11 (33%) adenocarcinomas and five (19%) squamous cell carcinomas were not classifiable (Table 3).

Intratumour variability

In one squamous cell carcinoma and one adenocarcinoma, 30 biopsies were taken to assess the distribution of T_{pot} within a tumour. For the squamous cell carcinoma, the mean value for T_{pot} was 4.19 days with a S.D. of 0.95 days, a minimum value of 2.00 days and a maximum value of 6.38 days (Figure 3a); in the adenocarcinoma, the mean T_{pot} value was 5.20 days with a S.D. of 1.66 days, a minimum value of 2.07 days and a maximum value of 9.45 days (Figure 3b). No significant departure from normality ($P > 0.2$) was observed, making the statistical methods used applicable.

The mean values of T_{pot}, LI and T_s for peripheral and central biopsies were not significantly different. This is true not only when considering the whole sample, but also when the larger tumours were studied separately. There was no significant difference between central and peripheral biopsies in the T3–T4 group (46 cases), nor in the group of tumours with a length of more than 5 cm (41 cases).

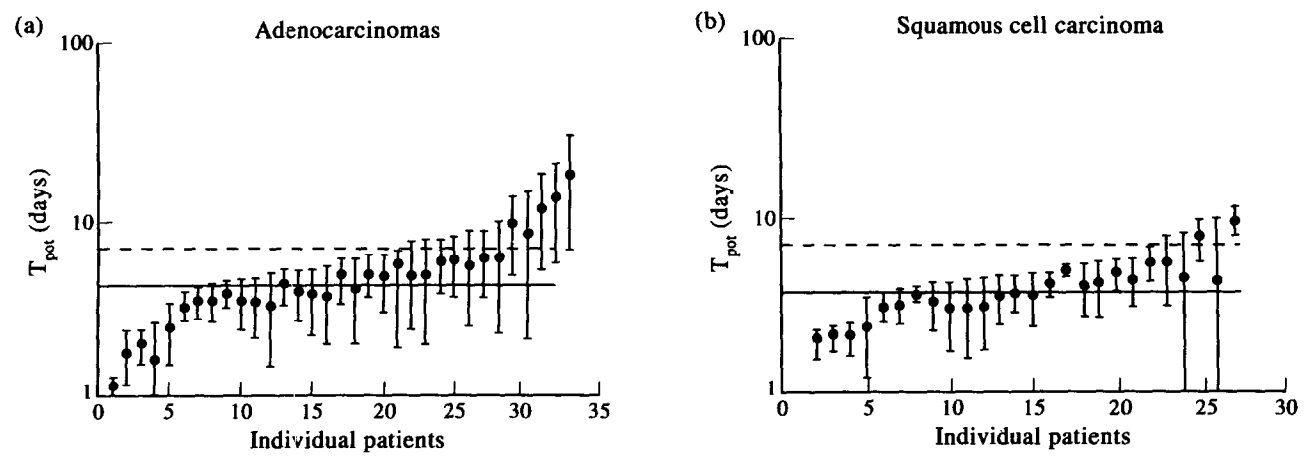

Figure 2. T_{pot} confidence intervals for adenocarcinomas and squamous cell carcinomas, ranked according to their means. Cut-off values shown as horizontal solid lines (——, median; ---, 7 days).

Table 3. Distribution of tumours (all and aneuploids only) according to different cut-off values: 7 days and the median of means

	All		Adenocarcinomas		Squamous cell carcinomas	
	All	Aneuploid	All	Aneuploid	All	Aneuploid
7-day cut-off						
Fast	42	40	21	21	21	19
Intermediate	16	14	11	9	5	5
Slow	2	0	1	0	1	0
Median of means*						
Fast	13	12	7	6	6	5
Intermediate	39	38	22	22	16	14
Slow	8	4	4	2	5	5

*Median values: all, 4.083; adenocarcinomas, 4.336; squamous cell carcinomas, 3.696.

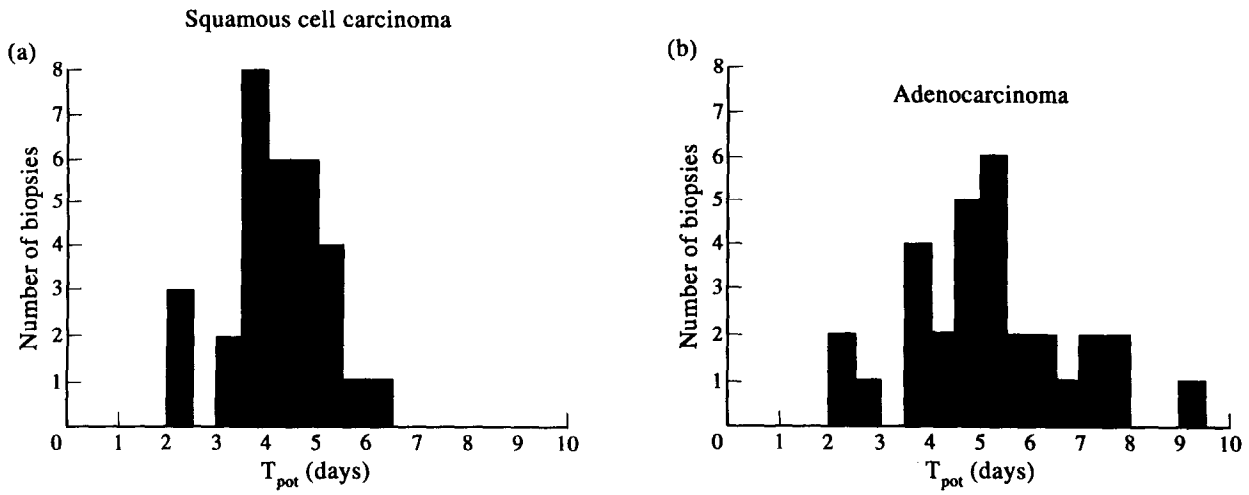

Figure 3. Distribution of T_{pot} values in one squamous cell carcinoma and one adenocarcinoma (30 biopsies per tumour).

Intertumour versus intratumour variability

For adenocarcinomas, as well as for squamous cell carcinomas, the intratumour variability was significantly smaller than the intertumour variability (analysis of variance). For adenocarcinomas, the *P*-value was 0.002, for squamous cell carcinomas, $P < 0.00001$.

DISCUSSION

The results presented indicate that oesophageal cancer is a fast proliferating tumour. There was a statistically significant difference in T_{pot} between adenocarcinomas and squamous cell carcinomas, the adenocarcinomas being more slowly proliferative (Figure 1a and b). However, for both histological types, the intertumour range was limited and, with few exceptions, T_{pot} values were small. Assay variability was negligible in comparison with interbiopsy variability, proving that the intratumour variability was real and not simply due to experimental imprecision. Moreover, intratumour variability was not negligible, with a coefficient of variation ranging between 7 and 39% for squamous cell carcinomas and between 16 and 51% for adenocarcinomas. The intratumour variation was not dependent on the site of the biopsies. Differences in cell viability, oxygen status and vascularity can cause differences in proliferation kinetics between different tumour areas. Theoretically, proliferation is expected to slow down in the more central parts, because of hypo-oxygenation. However, no difference between central and peripheral biopsies was found, not even in the largest tumours, whether growing in depth or spreading superficially. This independence of T_{pot} from biopsy site has also been found in head and neck, breast and bladder cancer [11, 13]. The existing intratumour spread of T_{pot} values is significantly smaller than the observed intertumour variability. Therefore, T_{pot} can be considered as a genuine tumour property.

The possibility of incorporating tumour growth kinetics in the overall management of malignant disease, as an indicator of prognosis and as a co-factor for treatment decision making, has generated increasing interest in recent years. The present study highlights two important problems for the clinical use of T_{pot}. The first problem is the intratumour variability of T_{pot} and related to this, the representativeness of a tumour biopsy. The question is then how many biopsies are required for a reasonable estimate of tumour proliferation characteristics. The second problem is the definition of slow and fast proliferation. When applied to the clinical situation, these two problems are intertwined.

To define slowly and quickly proliferative tumours, different options can be taken. Fowler [2] calculated that for tumours with a T_{pot} less than 7 days, overall treatment times shorter than the conventional 6–7 weeks would be advantageous. This value can thus be used as a basis to select patients for accelerated

treatment. Alternatively, in the EORTC head and neck study [1], using only one biopsy per tumour, the median T_{pot} value of all tumours was taken as the cut off.

For each tumour in the present study, the 95% CI was calculated from samples of three, four and five biopsies. Obviously, the larger the sample size, the smaller the CI. A tumour was considered as correctly classified if the entire 95% CI fell below the chosen cut-off value. Two possible cut-off values were applied for the following calculations. With 7 days as the cut off, 42 tumours were classified as fast, two as slowly proliferating while 16 could not be classified because their CI includes 7 days (Table 3). Assuming that five biopsies give a reliable estimation of the proliferative activity of a tumour, the probability of correctly classifying the fast proliferating tumours as fast when less than five biopsies were taken, was also calculated. This probability is 85% with one biopsy, 94% with two biopsies and 97% with three biopsies. Following Begg's proposal, using the median of the means as the cut off, only 13 tumours were classifiable as quickly proliferating, eight as slowly proliferating, while 39 were not classifiable (Table 3). The probability of correctly classifying the quickly proliferating tumours as fast with less than five biopsies is only 73% with one biopsy, 86% with two biopsies and 92% with three biopsies. Thus, the number of biopsies required for a reliable idea of the tumour's proliferation rate depends not only on the intrinsic intratumour variability, but also on the definition of the cut-off value used.

Using the median as the cut-off value, only a minority of tumours will unequivocally be defined as fast or slow, leaving the majority in an undefined gray zone (Table 3). The relative proportion in each group depends on the magnitude of intertumour and intratumour variability. Furthermore, when an arbitrary cut-off value is used, the relative distribution within the three groups depends not only on the range of T_{pot} values, but also on the cut-off values as such. The influence of the latter becomes more important when the cut-off value differs more from the median.

This study shows that measurement of human tumour cell kinetics is feasible in a clinical setting, using *in vivo* labelling with low doses IUdR. Immediate- or short-term adverse reactions due to the IUdR administration were not observed. The technique is fast, reliable and reproducible in other laboratories [14, 15]. Oesophageal tumours, both adenocarcinomas and squamous cell carcinomas, are fast proliferating neoplasms, with a small intratumour and a limited intertumour variability of T_{pot}.

Cell kinetic studies, such as the present, are needed to assess, within a given tumour type, the intertumour variability of T_{pot}. If intertumour variability is large enough, correlations with prognosis could be investigated prospectively. Such correlations are a prerequisite for defining relevant T_{pot} values that can contribute to treatment decision making, such as accelerated radiotherapy and neoadjuvant chemotherapy.

Meanwhile, until prospective studies are available, preliminary work, such as the present study, can help in the design of new clinical studies. They are needed to determine whether the range of T_{pot} values is wide enough to permit discrimination between slow and fast growing tumours and what T_{pot} value should be used as the cut off for this classification. Moreover, they will permit the prediction of the relative distribution of patients in the different treatment groups according to the definition of the cut-off value used.

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Acknowledgements—This study was supported by "Het Belgisch Werk tegen Kanker". We would like to express our thanks to M. Ramaekers and G. Pottie for technical assistance, to A.J. Van der Kogel Ph.D., P. Spaas M. D. and W. Landuyt for useful comments.