British Journal of Cancer (1996) 73, 1556-1559
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SHORT COMMUNICATION

Growth rate of lung metastases and S-phase fraction as determined by flow cytometry from the primary tumour in 25 patients with bone or soft-tissue sarcomas

C Blomqvist[1], R Huuhtanen[1], Y Pan[2], T Wiklund[1], M Tarkkanen[1], M Virolainen[1] and B Tribukait[2]

[1]*Department of Radiotherapy and Oncology, University of Helsinki, Haartmaninkatu 4, FIN-00290 Helsinki, Finland;* [2]*Department of Radiobiology, Karolinska Sjukhuset, Stockholm.*

Summary A significant correlation ($r = -0.48$) was found between the logarithm of the S-phase fraction of the primary tumour (SPF) and the logarithm of the doubling time of lung metastases (T_2). The estimated median cell loss factor was 88% (range 35-99%).

Keywords: doubling time; growth rate; flow cytometry; cell loss; sarcoma

It is well known that tumour growth rate is important for the outcome of cancer patients (Joseph *et al.*, 1971; Mattson and Holsti, 1980; Spratt and Spratt, 1964). During the last two decades proliferation measurements from tumour material has repeatedly been shown to be of prognostic value in several human cancers (Hall and Levison, 1992). Theoretically tumour growth rate is determined by the following factors: the S-phase fraction (SPF), the duration of the S-phase (T_S) and the cell loss factor (CLF) (Steel, 1967). Of these factors only SPF can be determined from tumour samples without the use of preoperative tumour labelling.

Despite the large literature on the prognostic value of proliferation measurements, the correlation between proliferation assessment from tumour material and actual tumour growth in individual cases has never been investigated. It would be of great value in clinical oncology to be able to estimate growth of metastases from proliferation measurements on primary tumour material. Tumour growth rate varies considerably even between tumours of similar histology (Blomqvist *et al.*, 1993), and it is obvious that the need for aggressive anti-neoplastic treatment is dictated by the expected clinical course of the disease without active treatment. In most cases calculation of tumour growth rate is not practical in patients owing to lack of follow-up, intervening therapeutic measures or poor measurability of tumour lesions.

A patient with tumour material available for proliferation measurements and clinically measurable lung metastases enabled us to investigate whether SPF determined by flow cytometry can be used to estimate the growth of subsequent lung metastases.

Materials and methods

Previously we included patients with lung metastases in a study on growth rate on chest radiographs. A minimum time of 14 days between two successive measurements was required. The growth of the lung metastases was calculated from serial bi-dimensional measurements from chest radiographs. Details, including reproducibility of the measurements, have been published previously (Blomqvist *et al.*, 1993). Between 1985 and 1993, 25 patients with serially measurable lung metastases from bone or soft-tissue sarcomas and tumour material from the primary tumours available for DNA measurements were found. The total number of measured metastases was 62. In patients with several measurable metastases we used the geometric mean of T_2. No patients received chemotherapy or radiation during the time of the study.

From formalin-fixed paraffin-embedded tissue samples 100 μm sections were cut with a microtome and an adjacent 5 μm slice for light microscopy. This slide was investigated to study the representativeness of the sample and estimate the proportion of tumour cells. In 21 cases more than 75% of the histological slide consisted of tumour cells, in three it was 50-75% cases (all non-diploid) and in one diploid case 25-50%. The methodology of SPF determination and its reproducibility has been published previously (Heiden *et al.*, 1990, 1991).

According to Steel (1967):

$$T_2 = T_{pot}/(1\text{-CLF}) \quad (1)$$

$$T_{pot} = \lambda T_S/SPF \quad (2)$$

from this follows:

$$T_2 = \lambda T_S/[SPF \times (1-CLF)] \quad (3)$$

$$\text{Log}(T_2) = \log[\lambda T_S/(1-CLF)] - \log(SPF) \quad (4)$$

Where T_2 clinical tumour doubling time; T_{pot}, potential doubling time; CLF, cell loss factor; λ, a constant reflecting the distribution of cells in different phases of the cell cycle (estimated to be about 0.75) (Steel, 1967); SPF, S-phase fraction and T_S, duration of SPF.

This means that theoretically there should be a negative linear correlation between log (T_2) and log (SPF) provided T_S and CLF are independent of SPF. The slope of the regression line between log T_2 and log SPF should be equal to -1, provided log $[\lambda T_S/(1-CLF)]$ is uncorrelated to SPF. This is the case when T_S and CLF are independent of SPF.

Moreover:

$$CLF = 1 - [\lambda T_S/(SPF \times T_2)] \quad (5)$$

Thus, under the assumption of the independence of T_S and SPF a rough estimate of CLF can be made by inserting an average value of T_S into formula (5). A value of 12 h for T_S was used in this study for the estimate of CLF. By using the same value for T_S in formula (2) T_{pot} can be estimated. Furthermore, (3) can be rearranged as $T_2 \times SPF = \lambda T_S/(1-CLF)$. By inserting the geometric mean of T_2 and SPF

Correspondence: C Blomqvist
Received 5 September 1995; revised 18 January 1996; accepted 18 January 1996

into this formula one obtains a value for the factor $\lambda T_S/(1-CLF)$, which can be used to estimate T_2 from SPF according to the formula $T_2=(\lambda T_S/1-CLF)/SPF$.

Linear regression of log (T_2) on log (SPF) and statistical testing of the correlation coefficient were done by the least squares method with the Statistica software on a Macintosh computer. The statistical significance of differences in SPF, T_2 and CLF between diploid and non-diploid tumours was tested by the Mann–Whitney test.

Results

Patient and tumour characteristics, SPF, ploidy, T_2 values, estimated cell loss factor and T_{pot} are shown in Table I. Nine patients had diploid tumours and 16 non-diploid tumours.

Median SPF and T_2 were 9.8% (range 1.6–19.7) and 32 days (range 6.9–1172) respectively. The geometric means were 9.3% and 36 days respectively. Median SPF was 8.4% in diploid tumours and 10.2% in non-diploid tumours ($P=0.29$). Median T_2 was 32 days in diploid and 34 days in non-diploid tumours ($P=0.77$). Median estimated T_{pot} was 3.8 days (range 1.9–23.4). The median estimated cell loss factors for all patients were 88% (range 35–99%). The median CLF was 86% in diploid and 90% in non-diploid tumours ($P=0.39$).

A scattergram of log(SPF) and log(T_2) is shown in Figure 1. There was a statistically significant negative linear correlation ($r=0.48$, $P=0.02$) between log(SPF) and log(T_2) with a fitted regression equation of $\log(T_2)=-0.83(\log SPF)+2.35$. The correlation between log(SPF) and log(T_2) was significant in the diploid cases alone ($r=-0.76$, $P=0.02$), but statistically non-significant ($r=-0.34$, $P=0.20$) in non-diploid cases. The estimated regression coefficients were however similar in the diploid $[\log(T_2)=-0.92\times[\log(SPF)]+2.34]$ and non-diploid $[\log(T_2)=-0.90\times[\log(SPF)]+2.48]$ cases.

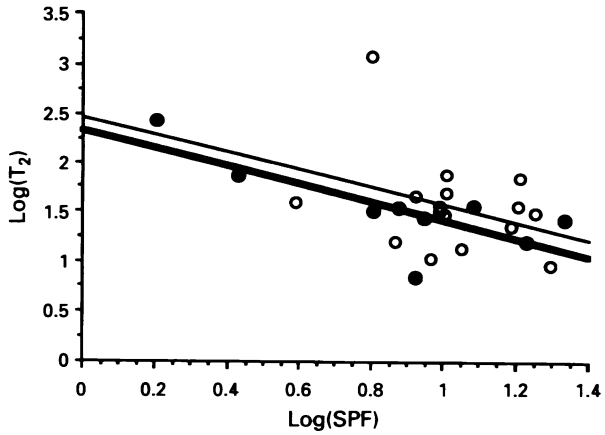

Figure 1 Correlation between the logarithm of the S-phase fraction (SPF) and log doubling time (T_2) in diploid and non-diploid sarcomas. Diploid tumours (●); non-diploid tumours (○). The thin line is the regression line for non-diploid cases and the thick line the regression line for diploid cases.

Discussion

A significant correlation between high SPF values and poor prognosis has been demonstrated in several malignancies including breast cancer, gastrointestinal and haematological malignancies (Hall and Levison, 1992). We have recently demonstrated a significant correlation between high SPF values and a poor prognosis also in soft-tissue sarcomas (Huuhtanen *et al.*, 1995). Although the impact of high SPF values on cancer prognosis probably stems from its association with tumour growth rate, there is little data available comparing clinical tumour growth and proliferation measurements.

There are several reasons for expecting the correlation between SPF and tumour growth to be far from perfect. According to Steel (1967), tumour growth is determined by SPF as described by the formula (3) $T_2=\lambda T_S/[SPF\times(1-CLF)]$. Despite lack of knowledge of CLF and T_S in the present study linear regression on log T_2 against log SPF yielded a fairly good correlation, indicating that SPF can indeed be used to obtain a rough estimate of T_2. The r^2 was

Table I Patient and tumour characteristics and tumour kinetic parameters in individual cases

Sex and age	*Histology*	*Grade*	*Site*	T_2	*SPF*	T_{pot}	*CLF*	*Ploidy*
F67	MFH	4	Calf	6.9	8.4	4.5	35	D
M42	MFH	4	Thigh	9.1	19.7	1.9	79	A
F35	LMS	4	Scapula	10.8	9.3	4.0	62	A
M66	Sarcoma NOS	4	Groin	13.7	11.2	3.3	76	A
M37	Sarcoma NOS	4	Buttock	15.6	7.4	5.1	68	A
F36	Sarcoma Ewing	4	Buttock	15.6	16.9	2.2	86	D
M18	Osteosarcoma	4	Femur	22.6	15.4	2.4	90	A
F63	Sarcoma NOS	4	Foot	26.2	21.7	1.7	93	D
M34	Sarcoma Ewing	4	Maxilla	27.8	8.9	4.2	85	D
F51	Malignant schwannoma	3	Retroperitoneum	29.2	10.1	3.8	87	A
F79	MFH	4	Thigh	30.8	18.0	2.1	93	A
M79	MFH	3	Upper arm	31.7	9.8	3.8	88	A
M29	Sarcoma NOS	4	Axilla	32.2	6.4	5.9	82	D
M54	MFH	4	Thigh	34.9	7.5	5.0	86	D
F56	LMS	3	Uterus	36.5	12.2	3.1	92	D
F62	LMS	2	Thigh	36.5	16.1	2.3	93	A
M61	Sarcoma NOS	4	Maxilla	36.6	9.8	3.8	90	A
M27	Sarcoma NOS	4	Neck	38.9	3.9	9.6	76	A
M56	Osteosarcoma	3	Femur	44.7	8.4	4.5	90	A
M21	Osteosarcoma	4	Tibia	49.5	10.2	3.7	93	A
F79	Fibrosarcoma	2	Trunk	68.7	16.3	2.3	96	A
F41	Sarcoma NOS	4	Upper arm	73.6	2.7	13.9	81	D
M55	Liposarcoma	3	Shoulder	75.0	10.2	3.7	95	A
F68	LMS	2	Retroperitoneum	276	1.6	23.4	92	D
M20	Chondrosarcoma	3	Pelvis	1172	6.3	5.9	99	A

F, female; M, male; T_2, doubling time (days); SPF, S-phase fraction (%); CLF, cell loss factor (%) estimated as $CLF = 1-[\lambda T_S/(SPF\ T_2)]$ with $\lambda = 0.75$, $T_S = 12$ h; T_{pot} potential doubling time (days) estimated as $T_{pot} = \lambda T_S/SPF$ with $\lambda = 0.75$, $T_S = 12$ h; LMS, leiomyosarcoma; MFH, malignant fibrous histiocytoma; NOS, not otherwise specified; D, diploid; A, non-diploid.

Table II Selected studies of labelling index (LI), the duration of the S-phase (T_S) and potential doubling times (T_{pot}) in human tumours

Tumour	n	Media n T_S (h)	T_S range (h)	Median SPF or LI (%)	Median T_{pot} (days)	Method	Reference
Head and neck	8	6.7	4.1–14	24.9	1.3	BRDU *in vivo*	Sakuma (1980)
Head and neck	6	9.1	6.8–12.9	11.6	4.0	IUDR *in vivo*	Begg *et al.* (1988)
Head and neck	9	10.9	5.8–18.8	4.8	4.8	BRDU *in vivo*	Wilson *et al.* (1988)
Head and neck	51	9.5*	5*–32*	11.0*	3.9*	IUDR *in vivo*	Begg *et al.* (1990)
Head and neck	82	13.7	7.3–31.5	8.0	6.2	BRDU *in vivo*	Forster *et al.* (1992)
Breast	22	7	5.5–8.5	2.1–4.0	15.3	TL *in vitro*	Silvestrini *et al.* (1974)
Breast	51	8.7	2.7–22.2	4.2	8.2	BRDU *in vivo*	Rew *et al.* (1992)
Colorectal	4	25.4	16.6–30.7	11.4	5.6	BRDU *in vivo*	Wilson *et al.* (1988)
Colorectal	100	13.1	4.0–28.6	9.0	3.9	BRDU *in vivo*	Rew *et al.* (1991)
Gastric	22	15.2	13.4–22.7	9.9	9.8	BRDU *in vivo*	Riccardi *et al.* (1988)
Glioma	10	15.3	10–22.7	6.3	13.4	BRDU *in vivo*	Riccardi *et al.* (1988)
Bladder	6	7.4	4.1–12.4	2.4	7.3	IUDR *in vivo*	Begg *et al.* (1988)
Oesophagus	4	7.0	6.4–14.2	5.7	7.1	BRDU *in vivo*	Wilson *et al.* (1988)
Lung	3	23.4	20–29.4	11.9	5.7	BRDU *in vivo*	Wilson *et al.* (1988)
Melanoma	2	8.8	8.7–8.8	5.9	5.2	BRDU *in vivo*	Wilson *et al.* (1988)
AML	54	14	6–43	28	2.0	BRDU *in vivo*	Raza *et al.* (1990)

AML, acute non-lymphocytic leukaemia; BRDU, bromodeoxyuridine; IUDR, iododeoxyuridine; TL, thymidine labelling; LI, labelling index; T_{pot}, potential doubling time. *Measured from graph.

only 0.23, indicating that other factors also have a significant impact on the doubling time, i.e. measurement and sampling errors, variations in T_S and CLF.

Labelling index (LI, which theoretically should be the same as SPF), T_S and T_{pot} of human tumours in studies that have used either radioactive or halogenated nucleotide analogues are summarised in Table II (Begg *et al.*, 1990, 1988; Forster *et al.*, 1992; Raza *et al.*, 1990; Rew *et al.*, 1992, 1991; Riccardi *et al.*, 1988; Sakuma 1980; Silvestrini *et al.*, 1974; Wilson *et al.*, 1988). The published values of T_{pot} can be compared with measured clinical doubling times (Charbit *et al.*, 1971) ranging from approximately 1 to 5 months. The discrepancy between T_{pot} and clinically determined T_2 is postulated to be caused by cell loss.

There are no previous published studies in the literature that attempt to define the extent of cell loss in human tumours by correlation of proliferation measurements and clinical doubling times in individual cases. On the basis of published values of T_{pot} and T_2 cell loss in human tumours has been estimated to be about 60–70% by Steel (1967) and 68–95% by Malaise *et al.* (1973) in tumours of different histology. In the study of Malaise *et al.* (1973) the mean value of estimated cell loss for 32 mesenchymal tumours was 68%. By substituting a plausible value of 12 h for T_S (the weighted mean of the median T_S for all tumour groups in Table II) an estimate of the cell loss factor in individual cases was made in the present study. The cell loss factor varied from 35% to 99% with a median of 88%, which is somewhat larger than the previous estimates (Malaise, *et al.*, 1973; Steel, 1967). The large cell loss factor indicates that cell death is as least as important a factor for clinical tumour growth as cell proliferation.

The estimates of CLF, especially in cases with extreme values, should be viewed with extreme caution however, because they are calculated on the basis of a number of assumptions. Firstly, there may be differences between the S-phase values in the primary tumour and its metastases as well as variation within the primary tumour itself. In a previous study on some of the patients included in this study we found that the growth rate of multiple metastases in the same patient was remarkably similar (Blomqvist *et al.*, 1993). This indicates that the growth rate in different subclones of the sarcomas in this study seems to be quite constant. One study in breast cancer reported relatively stable SPF values in primary tumours and metastases (Feichter *et al.*, 1989), whereas two studies in ovarian carcinoma both indicated considerable variation in SPF between different samples from the primary tumour, and between primary tumours and metastases in the same patient (Kaerne *et al.*, 1994; Kallioniemi 1988). Interestingly, the heterogeneity of SPF both in breast and ovarian cancer was reported to be larger in non-diploid tumours (Feichter *et al.*, 1989; Kaerne *et al.*, 1994).

Secondly, there might be a correlation between CLF or T_S and SPF. There is little data available on this issue. The slope of the regression line between log (T_2) and log(SPF) was, however, close to the theoretical value of -1, which should not be the case if either T_S or CLF were strongly correlated to SPF (for elaboration see Materials and methods).

Thirdly, patients with measurable lung metastases might be a non-random subset of sarcoma patients in general; in our department only 25 out of more than 200 sarcoma patients treated during the period of the study fulfilled the inclusion criteria.

An unexpected finding was that the correlation between SPF and clinical growth of metastases was closer in diploid than in non-diploid tumours. In fact, one would expect the opposite owing to the inevitable contamination of normal cells in the SPF estimate in diploid tumours. The difference might naturally be caused by chance in this relatively small patient sample. The regression equations between T_2 and SPF were, however, almost identical in diploid and non-diploid tumours, but the non-diploid tumours showed much larger variability. This may indicate that other factors responsible for T_2 (i.e. CLF and T_S) than SPF might be more important determinants of T_2 in non-diploid tumours. Two previous studies, one of 100 colorectal cancer cases and the other of 47 patients with head and neck carcinomas, have demonstrated significantly longer T_S times in non-diploid than in diploid tumours indicating systematic differences in T_S between diploid and non-diploid tumours (Begg *et al.*, 1990; Rew *et al.*, 1991). Interestingly, in a recent study of the patient material from which the present patients were recruited SPF was a strong predictor of metastatic development and survival in diploid tumours only (Huuhtanen *et al.*, 1995).

The estimated regression equation can be approximately reformulated in the form $T_2 = 300/SPF$, enabling a simple method of estimating the expected tumour doubling time from SPF in individual cases. In non-diploid tumours, however, this estimate is relatively inexact, since SPF explained only about 10% of the variability (variance) in T_2, whereas SPF explained about 60% of the variation in diploid tumours.

Acknowledgements
The study was supported by King Gustaf V's Jubilee foundation, by a grant donated by Zeneca Pharma to the foundation for Finnish Cancer Institute and by the Clinical Research Institute of the Helsinki University Hospital.

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