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3H-Thymidine labelling index (TLI) as a marker of tumour growth heterogeneity: evaluation in human solid carcinomas

A. Becciolini, M. Balzi, M. Barbarisi, P. Faraoni, A. Biggeri* and C. S. Potten†

*Laboratory of Radiation Biology, Department of Clinical Physiopathology, University of Florence, *Department of Statistics, Viale Morgagni, Florence, Italy, and †Epithelial Biology, Paterson Institute, Christie Hospital, Manchester, UK*

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Abstract. Many studies deal with the analysis of cell kinetic, cytogenetic, biochemical and molecular cell biology parameters to identify prognostic factors relating to tumour growth but all methods use only a small part of the total tumour mass. This study is devoted to the analysis of the heterogeneity of the growth of human solid tumours assaying proliferative activity by means of 3H-thymidine labelling index (TLI) in a fixed number of samples collected in different areas of the lesion (larynx and colon cancers), or in different lesions of the same subject (breast and bladder cancers). Each sample (at the macroscopic level) was divided into small fragments (at the microscopic level) and proliferative activity was determined. The analysis of variance for hierarchical designs demonstrated that in all cases a high component of the variance is attributable to the subjects and to the fragments whereas the variance attributable to the different areas is very low. The heterogeneity of proliferative activity displays a higher focal variability among the fragments (microscopic level) compared with that among areas (macroscopic level) within subjects, provided an adequate number of fragments and cells are counted. In multiple synchronous carcinoma of the bladder the wide variability of proliferation among the single lesions demonstrated that it is necessary to analyse all the tumours in a subject because each one is characterized by a different cell growth potential.

Tumour growth has been studied over many years using a wide variety of different approaches (Steel 1977, Quinn & Wright 1990). The existence of regions within the tumour of differing proliferative activity is well documented. The reasons for these differences in proliferation are varied: (1) It may be an intrinsic aspect of cell proliferation that it varies from site to site. (2) The microenvironment within the tumour may vary as a consequence of, e.g. oxygen availability, nutrients, growth factors, local immune responses, etc. (3) Clones of cells with differing proliferative capabilities may evolve during the development of a tumour.

Correspondence: Professor A. Becciolini, Laboratory of Radiation Biology, Department of Clinical Physiopathology, University of Florence, Viale Pieraccini 6, Florence 50134, Italy.

The levels of proliferative activity are clearly one of the more important parameters affecting tumour growth and the fraction of cells replicating their DNA, the S-phase fraction (labelling index) or the fraction in mitosis (M phase, mitotic index) have been used as a representation of the growth rate. However, other factors affect the tumour doubling time (DT). The growth rate determines the fraction of cycling cells (the growth fraction) and the most important of the non-growth fraction is the number of cells that are lost from the tumour by death (cell loss via apoptosis or necrosis). Tumours that occur at the same site and with similar clinical histopathological characteristics can exhibit very different growth rates, evolution and responses to therapy.

Analyses performed on a wide cohort of patients with suitable follow-up times have demonstrated that cell kinetic parameters, especially the tritiated thymidine labelling index (TLI), have a significant prognostic value both in relation to the disease-free survival probability and overall survival length (Chauvel *et al*. 1988, Meyer & Province 1988, Tubiana 1989, Silvestrini *et al*. 1989, Becciolini *et al*. 1993, Silvestrini *et al*. 1993).

Generally, due to the inability to analyse the entire tumour, the TLI is determined from small samples of tumour tissue cut into many fragments. Therefore we believe that an accurate evaluation of the heterogeneity within the tumour should be conducted to validate the TLI approach in order to be able to proceed with the use of TLI as a routine prognostic marker of clinical value.

The present study deals with this question of proliferation heterogeneity within human primary solid tumours. Samples from different sites in the tumour from the same patient were used to estimate the variation: (1) amongst subjects, (2) amongst localization at the macroscopic level within subjects, and (3) amongst fragments (microscopic level) within the same localization.

The questions specifically being addressed were to what extent should multiple samples be obtained from different areas of a tumour when measuring TLI and, in the case of multiple simultaneous tumours in a patient, to what extent should each be individually analysed to obtain a valid estimate of TLI?

MATERIALS AND METHODS

Patients and tumours

Samples of primary tumours from different sites of sufficient size to enable multiple samples to be taken without detriment to the histopathological analysis were used for this study. Four peripheral areas and a fifth at the centre of neoplasia were collected from larynx and colon carcinomas. The multiple samples were taken at 1–2 cm distances from each other. For the larynx carcinomas six cases of epidermoid carcinomas were used from patients with a median age of 70 years (range 48–78) and the following TNM staging; T2 (one case), T3 (four cases) and T4 (one case). One-half of the patients had positive lymph nodes.

Four cases of colon cancer from patients with a median age of 64 years were studied. They were all T3 according to the TNM classification and were Dukes B (three cases) and C (one case). One of these tumours was a colloid adenocarcinoma, the others were G2 adenocarcinomas. In three of the patients the tumour was localized in the right colon and in the fourth it was in the left colon.

For the breast carconomas we could not obtain large enough samples to obtain multiple specimens. In fact the efficiency of breast cancer screening programmes have led to a reduction in the size of tumours at surgery. Additionally, the requirement for histopathological analysis and for the determination of hormone receptors further reduced the amount

of tissue available for our analysis and we were not able to undertake a similar mapping of proliferation throughout the tumour as we could for larynx and colon. We analysed two cases with multifocal tumour (several nodules in the same quadrant of the breast) and three with multicentric tumours (more modules in different quadrants of the breast). These all had the same histological classification. Patient age ranged between 40 and 60 years (median 51 years). Two cases were T1 and three were T2 and four of the tumours were invasive ductal NOS tumours and one was invasive lobular carcinoma.

For the bladder carcinoma there were three cases where simultaneous tumours localized in three different non-adjacent regions of the bladder were studied. Tumour tissue was taken at the time of transurethral resection. The patient ages ranged from 58–72 years. The tumours were all superficial transitional cell carcinomas of a pTa or pT1 classification with papillary growth and a G2 grade of differentiation. The tumours from the different sites in each patient had the same histological pattern.

3H-Thymidine labelling index

For the carcinomas in larynx and colon, samples were obtained from five different areas in each tumour, four in the periphery and one in the centre. For the multiple nodules in breast and bladder carcinomas, a single sample was taken from each tumour nodule. The samples were divided into 15–20 fragments each of about 1 mm^3. These fragments were incubated for 1 h under continuous stirring in 1 ml of a mixture containing 1% 3H-thymidine (Amersham, Little Calfont, UK, 0.37 MBq/ml, specific activity 925 GBq/mmol), 15% fetal calf serum (FCS) and 84% McCoys 5A medium. After incubation the fragments were washed in cold saline and fixed in Carnoy's fixative and embedded in polystyrene (Frangioni & Borgioli 1982). The samples were placed in the incubation medium within 1 h of surgery. Three micrometre sections containing tumour tissue were prepared for autoradiography (Ilford K5 emulsion) and stained with haematoxylin erythrosin.

This thymidine incubation and autoradiographic technique has been used for many years to study proliferative activity in both normal and tumour tissue (Balzi *et al.* 1991, Bechi *et al.* 1991, Urso *et al.* 1992, Melone *et al.* 1992, Balzi *et al.* 1993, 1995, Bechi *et al.* 1996).

Background silver grains were rare and as a consequence the recognition of labelled cells was easy. The technique is part of a national protocol and has been subjected to a quality control analysis (Silvestrini *et al.* 1991). Analysis of the slide and counting of labelled cells were performed at $\times 100$ magnification by three different operators in the peripheral area of the tumour tissue of 100 μm depth. The total number of tumour cells and labelled cells were counted and the TLI was determined. For each region of the tumour, three fragments containing tumour tissue in two or more sections cut at different depths were analysed. For each fragment at least 2000 tumour cells were counted and if a lower number of tumour cells were present in the 100 μm peripheral area, that particular count was excluded for the analysis of variance.

Statistical analysis

The study design consisted of the determination of TLI in fragments of neoplastic tissue sampled at different positions in the tumour for a given subject. For each subject or tumour there is a variation in TLI due to the localization of the sample as a consequence of local factors that can influence tumour growth. Therefore, there are three separate populations to be sampled (Figure 1). The primary aim of the analysis was to estimate the amount of variability in these three populations. We have assumed that these are separate and independent normal populations, i.e. patients or tumours, localizations within single tumours and

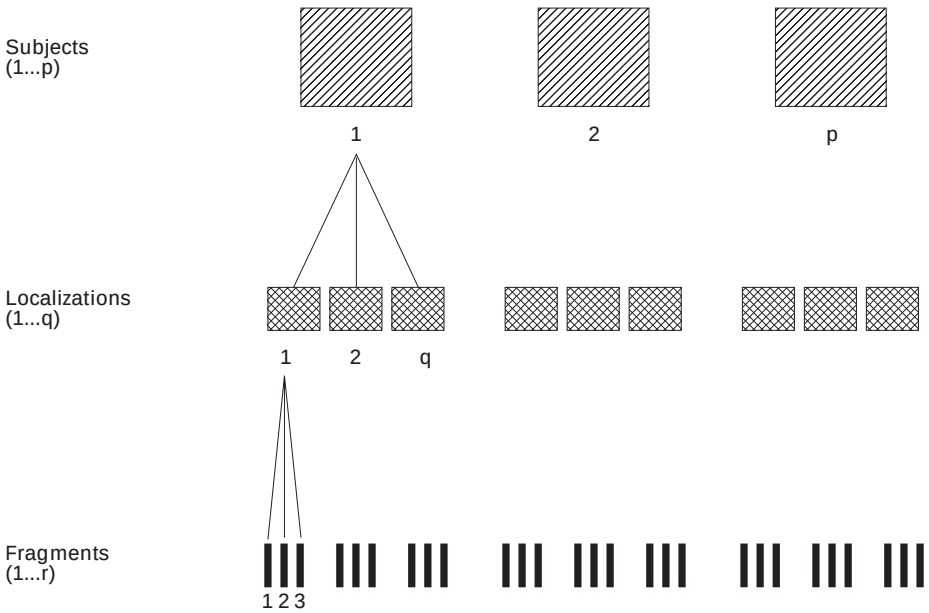

Figure 1. Plan of the analysis of the variance for hierarchical designs.

multiple fragments or measurements from a given localization. The model used is as follows:

$$y_{ijk}=\mu+\alpha_i+\beta_{j(i)}+\varepsilon_{k(ij)}$$

$i=1, \ldots, p$	subject (patient)
$j=1, \ldots, q$	localization
$k=1, \ldots, \varepsilon$	fragments

where y_{ijk} represents the TLI measurement of the kth fragment within the jth localization of the ith subject and where α_i is distributed $N(O, \sigma_\alpha^2)$, $\beta_{j(i)}$ is $N(O, \sigma_\beta^2)$ and $\varepsilon_{k(ij)}$ is $N(O, \sigma_\varepsilon^2)$.

We have p subjects sampled from a population of available subjects, q localizations sampled from each subject and r fragments sampled from each localization. An analysis of variance of hierarchical design has been applied to our data. We expected mean squares of TLI (*MS*) for each source of variation in the table of analysis of variance as shown in Table 1.

The test of the hypothesis on σ_α^2 is straightforward: $F=MS$ (between subjects)/MS (between localization) and on σ_β^2 is $F=MS$ (between localization)/MS (between fragments). Estimates

Table 1. Table of analysis of variance

Variance (mean squares)	Expected mean squares
Between subjects	$\sigma_\varepsilon^2+r\sigma_\beta^2+rq\sigma_\alpha^2$
Between localizations within subjects	$\sigma_\varepsilon^2+r\sigma_\beta^2$
Between fragments within localizations	σ_ε^2

of σ_α^2, σ_β^2 and σ_ε^2 are readily obtained from the mean squares of the table of the analysis of variance (Wetherill 1993).

This approach assumes normal distributions for the populations, additivity of effects and homoscedasticity of variance among groups. These assumptions were checked by appropriate residual analysis and sensitivity analysis (results not shown). In Figure 2 the distribution of the TLI values in all the fragments of larynx carcinoma is presented. The Kolmogorov–Smirnov test was used to evaluate the goodness of fit of the Gaussian distribution to the observed one (Wetherill 1993).

RESULTS

Larynx carcinoma

The variability in TLI was analysed in four cases and five localizations and three fragments in each localization and the results are shown in Table 2. These data show how tumours from different patients tend to have a different proliferative activity while the TLI values are fairly homogeneous within a particular tumour. The analysis of variance for hierarchical design shows that the greatest variance component can be attributed to patients even though an important element of variability can be seen amongst the fragments and within localizations (Table 3). The variability amongst different localizations within subjects in these tumours is low.

Colon cancer

A similar analysis was conducted for the colon cancer cases and the results were very similar for those for larynx carcinoma with the TLI values between patients showing the greatest

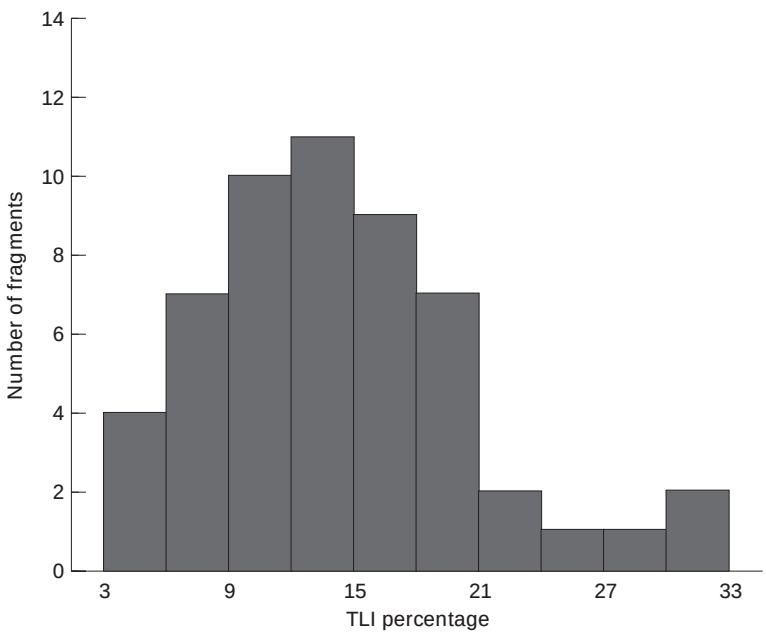

Figure 2. TLI distribution in all fragments from different localizations in six cases of larynx carcinoma.

variation. The component of variance relating to localization within tumours was much lower than the variance amongst fragments within a single site of sampling.

Breast carcinoma

The analysis of the TLI in two nodules of multifocal or multicentric tumours from five cases provided evidence that proliferative activity showed a variability among fragments of the same nodule whereas the TLI mean value for each nodule was similar in both multifocal and multicentric tumours. The analysis of variance using an hierarchical design in the five cases in which both nodules were studied showed that the component of variance amongst

Table 2. Variations of TLI in four cases of larynx carcinoma: analysis of five localizations (A–E) and three fragments (1–3)

		Localization				
Case	Fragment	A	B	C	D	E
1	1	8.48	18.46	11.9	16.57	8.23
	2	15.15	10.12	14.03	16.07	14.72
	3	15.76	13.87	7.81	10.44	8.54
	Mean	13.13	14.15	11.25	14.36	10.50
2	1	10.56	10.7	14.54	12.32	24.27
	2	13.59	12.79	12.54	8.69	14.29
	3	20.66	9.5	15.15	14.25	19.68
	Mean	14.94	11.00	14.08	11.75	19.41
4	1	9.72	4.98	10.08	19.23	5.52
	2	5.29	5.84	9.08	8.23	10.71
	3	5.8	11.9	3.15	24.73	10.33
	Mean	6.94	7.57	7.44	17.40	8.85
5	1	17.08	23.36	20.71	12.29	18.73
	2	18.21	27.03	18.89	26.22	19.46
	3	32.65	31.46	21.48	27.81	20.34
	Mean	22.65	27.28	20.36	22.11	19.51

Table 3. Analysis of the components of the variance in five localizations from four cases of larynx carcinoma

Source	d.f.	Deviance	Variance	Component of variance
Subjects	3	133.78	444.59	27.52
Localizations within subjects	16	509.13	31.82	3.72
Fragments within localizations	40	826.78	20.67	20.67
Total	59	2669.69		

d.f.=degree of freedom.

Table 4. Analysis of the components of the variance in two nodules of breast cancer with multifocal or multicentric expression from five patients

Source	d.f.	Deviance	Variance	Component of variance
Subjects	4	156.27	39.07	6.12
Localizations within subjects	5	11.69	2.34	0.00
Fragments within localizations	20	136.26	6.81	6.81
Total	29	304.22		

d.f.=degree of freedom.

fragments within nodules was equivalent to the variability amongst subjects, whereas the component attributable to the variability between nodules within a subject was very low (Table 4).

Bladder carcinoma

In each of three patients with transitional bladder carcinoma, three simultaneous tumours in non-adjacent sites of the bladder were studied. The cancers were considered as independent primary tumours. The results reported in Table 4 showed a wide variability amongst the different tumours including those from the same subject and also considerable variability between the subjects (Table 5). In contrast to the observations in other cancers, in bladder tumours, the variability amongst the fragments was smaller.

DISCUSSION

Over the last few years many parameters relating to cell growth have been analysed in tumours with the aim of providing a better biological characterization of the tumours and to

Table 5. Variations of TLI in three cases of transitional bladder carcinoma with papillary growth. Analysis of three fragments from three separate tumours from each patient

		Tumour		
Case	Fragment	1	2	3
1	1	2.13	2.48	6.57
	2	0.96	1.76	9.30
	3	4.02	2.09	11.07
	Mean	2.37	2.11	8.98
2	1	6.50	1.79	4.93
	2	2.55	1.34	4.05
	3	2.46	0.75	2.46
	Mean	3.84	1.29	3.81
3	1	10.02	8.91	15.27
	2	6.62	4.38	12.11
	3	9.84	3.17	12.18
	Mean	8.83	5.49	13.19

d.f.=degree of freedom.

Table 6. Analysis of the components of the variance in three cases with three separate transitional bladder carcinomas

Source	d.f.	Deviance	Variance	Component of variance
Subjects	2	187.29	93.64	6.83
Localizations within subjects	6	193.23	32.20	9.59
Fragments within localizations	18	61.77	3.43	3.43
Total	26	422.29		

d.f.=degree of freedom.

identify elements of value in the clinic. TLI has been shown to have prognostic value in a wide range of patients after adequate follow-up (Chauvel *et al.* 1988, Meyer & Province 1988, Silvestrini *et al.* 1989, 1993, Tubiana *et al.* 1989, Balzi *et al.* 1991, 1993a,b, 1995, Bechi *et al.* 1991, 1996, Urso *et al.* 1992, Melone *et al.* 1992). The various phases involved in TLI estimation have been carefully standardized and protocols of quality control have been introduced (Silvestrini *et al.* 1991). Under standardized conditions the determination of TLI is reproducible even if time consuming.

A large variability in proliferative activity in tumours of a particular organ collected from a wide cohort of patients in our laboratory has been seen. In thyroid carcinoma (46 cases) a mean TLI of 0.46% with a range of 0.14% to 2.6% has been observed, in breast cancer (600 cases) a mean value of 2.82% with a range of 0.07% to 24.27%, in transitional bladder carcinoma with papillary growth (157 cases) the mean TLI was 3.53% with a range of 0.13% to 26.43% and in non-papillary bladder tumours (29 cases) the range was 2.58% to 50.66%. In larynx carcinoma (70 cases) the TLI ranged from 2.78% to 32.29%, while squamous cell carcinoma of uterine cervix (72 cases) showed a range of 4.65% to 32.33% and in colorectal cancer (103 cases) the range was 1.37% to 38.43%.

Even when taking into account cancers with the same clinical histopathological T categories, lymph node status, grade of differentiation, etc., the differences in TLI were always great. The fact that the range is commonly large, means that the determination of cell kinetic parameters can be suitable for use to discriminate groups of patients with different risks of tumour progression. However, whatever the methods that are used to analyse cell proliferation, gene expression or metabolic aspects of human solid tumours, the heterogeneity of tumour growth must be taken into account. Cell growth is conditioned by, besides intrinsic factors, the microenvironmental conditions and by the eventual presence in the tumour of different clones. It is for these reasons that the cell populations making up a tumour appear potentially heterogeneous at the morphological, cytogenetic, metabolic, molecular and proliferative levels. The variability is considered to be an intrinsic property of the neoplasia and we have not conducted specific studies on the individual sources of this heterogeneity. It will be necessary for studies to verify whether the variabilities can affect any particular parameter, and whether the information that is obtained is valid for extrapolation to the whole tumour.

Other methods such as the *in vivo* administration of BrUdr to a patient allows the potential doubling time (T_{pot}) to be determined (Wilson 1991, Begg *et al.* 1992), but even this parameter does not provide information on the real growth rate because it does not take into account the cell loss factor. Recently, the intratumour heterogeneity of T_{pot} in colorectal cancer has been evaluated by means of BrUdr injection and flow cytometry (Wilson *et al.* 1993). Others have studied intratumoral variation in proliferation by using PCNA and flow

cytometry S phase estimates (Siitonen *et al.* 1993). Both approaches have revealed considerable heterogeneity in intratumoral proliferative activity, but it is worth noting that cell cycle analysis with flow cytometry is not able to discriminate healthy from neoplastic cells, moreover, the authors used a statistical analysis only with a descriptive approach.

Here we have determined the proliferative activity in different areas of tumours using TLI. The results show that in larynx and colon carcinoma there is high variability in TLI values among the fragments of the tissue, even if the mean values for the tumour appear fairly homogeneous. When multifocal and multicentric tumours of the breast are considered, the variability in TLI amongst the fragments of each nodule is high, whereas the variability between nodules is low. The histopathology of the two independent nodules is the same and this would appear to confirm the hypothesis that the tumours have a single clonal origin.

A different conclusion can be drawn when these multiple tumours of the bladder are analysed. The analysis of variance for hierarchical design shows that the component of variability among tumours within subjects is higher than among fragments within a given tumour. The results suggest that each of the three neoplastic lesions in which the TLI has been determined, in spite of having similar histopathological classification, represents a tumour with a different proliferative capacity. This may suggest that the appearance of multiple lesions in the bladder arises from a general involvement of the entire epithelium in the neoplastic process. It is worth noting that this could result in a situation where individual tumours have different levels of aggressiveness. It has previously been demonstrated that TLI values are correlated with the probability of relapse and overall survival (Balzi *et al.* 1993b) and as a consequence it will be necessary to evaluate the TLI in each bladder lesion when multiple lesions are present (Balzi 1993b).

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