Cell proliferation kinetics in human solid tumors: relation to probability of metastatic dissemination and long-term survival

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Summary

A large number of studies have investigated the relationship between the long-term survival and the percentage of tumor cells in S phase assessed by autoradiography after tritiated thymidine labelling, image cytometry, flow cytometry or labelling with an halogenated analog of thymidine, in various types of human solid tumors. The survey of the results clearly shows that the S-phase fraction (SPF) is of high prognostic significance in several types of cancers, in particular in breast cancers, non-Hodgkin lymphomas, ovarian cancers, neuroblastoma, bladder cancers and lung cancers. SPF was found of high independent significance in 10 of the 11 studies in which multivariate analyses of prognostic factors had been carried out. Proliferation appears generally to be of higher prognostic significance than ploidy. In view of the wide differences in the biological characteristics of the tumors studied, it is likely that the association between a high proliferation rate and the degree of tumor aggressiveness is a general feature of human solid tumors. However, high proliferative rate of tumor cells is probably not the cause of tumor biological aggressiveness but a variable associated with it. The extent to which cells escape from the regulatory systems which control their proliferation appears to be a good index of tumor progression.

Introduction

The introduction of adjuvant chemotherapy has given a new impetus to the search for prognostic factors. It has become essential to identify those patients with a high likelihood of occult distant metastases.

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For several decades, it has been suspected that a rapid tumor growth rate was associated with a poor prognosis. The early data have been recently analyzed in a few review articles [25,136]. In these initial studies, the estimation of the growth rate was based on the answers of the patient regarding the time course of tumor size. Despite the subjectivity and the inaccuracy of these estimations, the data suggested that in patients with breast cancer slow-growing tumors had a lower
incidence of local recurrence and distant metastases than the more rapidly growing tumors [14,19,20,78,102,105,106]. However, the data were not conclusive [63], in particular where multivariate analyses were carried out in order to assess the independent prognostic significance of growth rate. Subsequently the measurement of tumor doubling time (DT) on sequential X-rays introduced an objective and quantitative evaluation of tumor growth rate; this technique requires a long period of observation which in most patients conflicts with the early initiation of treatment, and hence it cannot be used in clinical practice. Nevertheless, a few studies showed that a long DT is associated with a good prognosis [49,67,76].

The discovery of the cell cycle by Howard and Pelt [65] and the description by Taylor et al. [129] of the thymidine labeling index (TLI) in the late 1950s provided a new approach to the study of tumor growth kinetics. A large number of clinical investigations were then undertaken which have been critically analyzed in several review papers [83,89,111,124,133]. The main findings can be summarized as follows:

1. In each histological group of human tumors the individual values of the TLI vary widely and their distribution is usually log-normal. Nevertheless, there are significant differences between the mean values of the various groups. The highest mean TLI were found in the lymphomas and the embryonal tumors, the lowest in adenocarcinomas [83,124].

2. The chemoresponsive and radioresponsive groups of tumors are those in which the mean TLI is the highest [81,83,128,137]. However, despite a large number of studies, there is no conclusive evidence as yet regarding the short-term prognostic significance of pretreatment TLI and a relationship between pretreatment TLI and the time course change of TLI under treatment [34,120]. This problem has been discussed elsewhere [33,134].

3. There is a correlation between the mean DTs and TLI, of each histological group. In a small group of breast primary tumors, a correlation was found between the individual value of TLI and DT [83,137].

4. The TLI are higher in the undifferentiated tumors located in a given site [124,133]. For example, in breast tumors the proliferation rate determined by TLI or flow cytometry [30,38,39,42,53,68,72,77,86,91,93,95,101,103,116,117,126] is higher in tumors without estrogen or progesterone receptors; it is also slightly higher in tumors with EGF receptors which are among the most aggressive ones [122].

5. A positive correlation between cell density
and TLI has been reported in human tumors [34]. This suggests that the control mechanism which regulates cell proliferation in normal tissues still operates in tumors with a low cell density and that in these tumors the cell-to-cell interactions are less disturbed than in tumors in which the cell density is high.

(6) To the best of our knowledge, the first mention of a relation between pretreatment TLI and the probability of occurrence of local recurrence or distant metastasis was made in 1975 in a study on breast cancer [135]. This was confirmed by several studies that we shall analyse below.

The measurement of TLI has two main drawbacks: (1) it is tedious and involves a multistep procedure, (2) it requires at least 10 days for processing. This is why TLI has been used only for investigation on small series of patients and has never been routinely used. The advent of new techniques has made it possible to assay the proportion of cells in S phase, or S-phase fraction (SPF), in a large number of patients and to take its result into account for their management. However, with DNA cytometry, it is much easier to measure a DNA index which is related to ploidy than to assess the SPF. Therefore, it would be important to know whether it is rewarding to make the efforts necessitated by the measurement of the SPF. To answer the question is the main goal of this paper.

To this aim, we shall review the studies which have been made on the biological significance of a pretreatment assay of the SPF in human solid tumors. We shall first briefly analyze the methods, thereafter we will review the main results and discuss their interpretation.

Methods

Thymidine labeling index (TLI)

Tritiated thymidine (³H-Th) can be administered in vivo. In experimental animals, ³H-Th is usually injected intra-peritoneally or intravenously [88,124]. In view of the long half-time of tritium, intravenous administration has only been used in a few series of patients [47,64,133,137].

In most studies, the TLI was measured in vitro by incubating fresh tumor specimens with ³H-Th. Experimental studies have shown a good consistency between TLI measured in vivo or in vitro [23]. TLI assay is simple but should be performed with a precise methodology. Two problems are critical: (1) the technique of autoradiography and film processing, and (2) the method used for background subtraction. In order to avoid large statistical fluctuations and to insure reproducibility of the assay, a large number of cells should be counted. There are a few other sources of pitfalls which should not be overlooked. Some cells can be slowly cycling and thus insufficiently labelled resulting in an underestimation of the LI. It has been shown that an uneven distribution of labelled cells in a tumor may arise from the pattern of ³H]thymidine concentration throughout the tumor. Moreover, some tumor cells may have a large endogenous nucleotide pool which cannot be flooded by a single injection of [H]thymidine [56]. These problems have been discussed in several papers [2,23,56,121].

One of the main advantages of TLI is that during the counting, normal cells can be distinguished from tumor cells, therefore non-malignant cells present in the tumor can be excluded.

Measurement of cell DNA content by image cytometry (IC)

This method was described by Caspersson et al. in 1960 [17]. Normal cells are diploid when they are quiescent or in G₁ phase of the cell cycle. Their nuclei have a 2n DNA content. In G₂ or during prophase they have a 4n DNA content, and an intermediate content in S phase. When the assay of the DNA content is sufficiently precise, it is then possible to measure the proportion of cells in S phase.

Initially the measurement was made with UV
spectrometry, later Feulgen staining was used which made the assay much easier [140]. The assay is relatively simple and accurate on smears because the cells do not overlap and the nuclei are not damaged; moreover, it is easy to select the intact tumor cells [26].

What is measured on sections is the DNA content of a section of the nucleus which is only a part of the total DNA content of this nucleus. Sophisticated algorithms based on stereological methods have been used to compute the distribution of the total DNA content of the nuclei knowing the distribution of the DNA content of the nuclear sections [12]. This correction should be made when the goal is to estimate the proportion of cells in S phase but it can introduce biases.

Image cytometry is time-consuming but has two advantages: (1) one can select under the microscope the cells in which the DNA content is measured; (2) it can be performed on paraffin-embedded sections allowing retrospective studies [107] but only when the fixation has not damaged the DNA molecules. The combination of TLI and image cytometry can identify the cells with an intermediate DNA content which are not synthesizing DNA, providing further information on the kinetics of cell proliferation in the population studied [2,48].

**Flow cytometry (FCM)**

Currently, this is the most widely used technique. The DNA is labelled with ethidium bromide, or propidium iodide, a technique which allows a precise quantitation of DNA. Flow cytometry

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>Tumor cell kinetics and follow-up studies in breast cancer.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follow-up (yrs)</td>
<td>Patient number</td>
</tr>
<tr>
<td>15</td>
<td>125</td>
</tr>
<tr>
<td>9</td>
<td>167</td>
</tr>
<tr>
<td>7.5</td>
<td>48</td>
</tr>
<tr>
<td>7</td>
<td>437</td>
</tr>
<tr>
<td>6</td>
<td>285</td>
</tr>
<tr>
<td>6</td>
<td>258</td>
</tr>
<tr>
<td>6</td>
<td>177</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
</tr>
<tr>
<td>4</td>
<td>227</td>
</tr>
<tr>
<td>4</td>
<td>52</td>
</tr>
<tr>
<td>247</td>
<td>Not specified</td>
</tr>
</tbody>
</table>

RFS = replase-free survival.
TABLE II
Tumor cell kinetics and follow-up studies in lymphomas.

<table>
<thead>
<tr>
<th>Follow-up (yrs)</th>
<th>Patient number</th>
<th>Tumor type</th>
<th>Method</th>
<th>Cut-off</th>
<th>Result</th>
<th>Observations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>&lt;90</td>
<td>NHL</td>
<td>FCM (paraffin embedded)</td>
<td>S + G2 = 20%</td>
<td>No difference in survival</td>
<td>Analysis on diploid tumors only</td>
<td>Morgan et al. [96]</td>
</tr>
<tr>
<td>6</td>
<td>64</td>
<td>Poor histology NHL</td>
<td>FCM</td>
<td>S = 10%</td>
<td>Better survival in low proliferation</td>
<td>Better survival in low proliferation</td>
<td>Young et al. [146]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LI</td>
<td>4.1%</td>
<td>(p &lt; 0.011)</td>
<td>Same result for nodular and diffuse types separately</td>
<td>Costa et al. [27]</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>NHL</td>
<td>FCM</td>
<td>S = 4%</td>
<td>Better survival in low proliferation</td>
<td>Better survival in low proliferation</td>
<td>Lenner et al. [80]</td>
</tr>
<tr>
<td>4</td>
<td>39</td>
<td>Diffuse large cell</td>
<td>FCM</td>
<td>G0 + G1 = 80%</td>
<td>Better survival in low proliferation</td>
<td>Only 6 patients in high proliferation group</td>
<td>Bauer et al. [9]</td>
</tr>
<tr>
<td>0.8</td>
<td>27</td>
<td>NHL</td>
<td>FCM</td>
<td>5%</td>
<td>Better survival in low proliferation</td>
<td>Better survival in low proliferation</td>
<td>Braylan et al. [15]</td>
</tr>
</tbody>
</table>

enables a rapid assay of a large number of cells, thus the distribution of the DNA content is known with a good statistical accuracy.

For solid tumors, one of the problems is to obtain a suitable single cell suspension since the disruption of tumors into single cells is difficult to achieve. The presence of cellular debris or microaggregates of cells may constitute a source of artefact [21,26,55]. In order to avoid it, several authors isolate the cell nuclei or extract them from tissue blocks. This technique allows retrospective studies on paraffin-embedded tissues [59]. However, when the isolated nuclei are studied, it is very difficult to distinguish a normal cell from a tumor cell. A large number of normal cells are often intermingled within the tumor; usually the percentage of S phase cells is lower in normal cells than in tumor cells, this results in an under-estimation of the proportion of cells in S phase, especially in diploid tumors [39]. Theoretically when a suspension of undamaged cells is studied, it might be possible to distinguish normal cells from tumor cells using as a discriminant cell size, membrane markers or other cell characteristics. However, these sophisticated techniques are seldom used and in most studies the results are given for a tumor population of cells which includes normal cells.

The main problem in IC or FCM is related to tumor cell ploidy. When the tumors are diploid, the determination of the proportion of cells with an intermediate DNA content is relatively simple but still requires a correction because there is an overlapping between: (1) the G1 cells and those which have just entered in S phase, and (2) those which have nearly completed the S phase and G2 cells. Softwares of varying complexities are used for these corrections and the precise determination of the percentage in S phase depends upon the accuracy of the instrument and the effectiveness of the computer program [6,66,108].

When the tumor is aneuploid, the determination of the percentage of cells in S phase becomes less precise and sometimes impossible, in particular when there is a mixture of diploid and aneuploid cells or if multiple aneuploid peaks are present. For example, in the study of Hedley et al. [60], out of 490 patients with breast cancer a valid estimation of the percentage of cells in S phase was obtained in only 60% of the patients.
TABLE III

Tumor cell kinetics and follow-up studies in other solid tumors.

<table>
<thead>
<tr>
<th>Follow-up (yrs)</th>
<th>Patient no.</th>
<th>Tumor type</th>
<th>Method</th>
<th>Cut-off</th>
<th>Result</th>
<th>Observations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>194</td>
<td>Colon</td>
<td>FCM (paraffin embedded)</td>
<td>$S = 13%$</td>
<td>Higher survival in low SPF ($p = 0.03$)</td>
<td>After stratification: significant only in Duke's C</td>
<td>Schutte et al. [114]</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>Colon and rectum</td>
<td>LI</td>
<td>–</td>
<td>No difference in survival</td>
<td></td>
<td>Bleiberg et al. [13]</td>
</tr>
<tr>
<td>3</td>
<td>84</td>
<td>Colon and rectum</td>
<td>LI</td>
<td>$17.8%$</td>
<td>No relation to survival or RFS</td>
<td>Patients treated solely with radiation</td>
<td>Meyer and Prioleau [94]</td>
</tr>
<tr>
<td>6</td>
<td>65</td>
<td>Cervix (aneuploid)</td>
<td>FCM</td>
<td>$G_1 = 60%$</td>
<td>No significant difference in survival, recurrence rate or incidence of metastases</td>
<td></td>
<td>Rutgers et al. [110]</td>
</tr>
<tr>
<td>2</td>
<td>133</td>
<td>Cervix</td>
<td>FCM</td>
<td>$S = 20%$</td>
<td>High relapse rate in high SPF ($p &lt; 0.01$)</td>
<td>Distant metastases higher in high proliferation. Also significant using 5 proliferative subgroups</td>
<td>Strang et al. [125]</td>
</tr>
<tr>
<td>1</td>
<td>54</td>
<td>Cervix</td>
<td>LI</td>
<td>–</td>
<td>Mean LI in disease-free patients and others: NS</td>
<td></td>
<td>Dixon et al. [37]</td>
</tr>
<tr>
<td>17</td>
<td>118</td>
<td>Ovary</td>
<td>FCM (paraffin embedded)</td>
<td>9.4 and 15.5%</td>
<td>RR of death: 1, 3.07, 4.82 ($p &lt; 0.0001$)</td>
<td>Also in diploid tumors (%$S$ cut-off = 9%)</td>
<td>Kallioniemi et al. [69]</td>
</tr>
<tr>
<td>3</td>
<td>48</td>
<td>Ovary</td>
<td>FCM</td>
<td>$G_1 = 85%$</td>
<td>Higher survival in low SPF ($p &lt; 0.05$)</td>
<td></td>
<td>Rutgers [109]</td>
</tr>
<tr>
<td>12</td>
<td>14</td>
<td>Medullary thyroid</td>
<td>FCM</td>
<td>–</td>
<td>$S + G_2 + M = 5.8%$ in disease-free patients and 11% in deceased patients</td>
<td></td>
<td>Schröder et al. [113]</td>
</tr>
<tr>
<td>Page</td>
<td>Platelet</td>
<td>Disease</td>
<td>Modality</td>
<td>Proliferation</td>
<td>Additional Notes</td>
<td></td>
<td></td>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>38</td>
<td>Neuroblastoma</td>
<td>FCM</td>
<td>–</td>
<td>SPF: 21.3% in survivors vs. 34.1% in deceased patients ((p = 0.004))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>350</td>
<td>Bladder</td>
<td>FCM</td>
<td>10 and 20%</td>
<td>Higher death rate in high SPF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>17</td>
<td>T3 bladder</td>
<td>LI</td>
<td>5%</td>
<td>Higher recurrence in low proliferative group ((p &lt; 0.05))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>122</td>
<td>Non-small-cell lung cancer</td>
<td>FCM</td>
<td>8%</td>
<td>Higher survival in low SPF ((p = 0.018))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>57</td>
<td>Small-cell lung cancer</td>
<td>FCM</td>
<td>21%</td>
<td>No difference in survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>48</td>
<td>Melanoma met</td>
<td>LI</td>
<td>8%</td>
<td>Better survival in low SPF ((p = 0.054))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td>Melanoma met</td>
<td>FCM</td>
<td>10%</td>
<td>Better survival in low SPF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>87</td>
<td>Head and neck</td>
<td>LI</td>
<td>15.5%</td>
<td>Better survival in low SPF ((p = 0.008))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>Oral cavity</td>
<td>LI</td>
<td>1.9 and 13.9%</td>
<td>No significant difference in survival and RFS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>31</td>
<td>Maxillofacial</td>
<td>FCM</td>
<td>–</td>
<td>Survival negatively correlated with SPF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>24</td>
<td>Gliomas</td>
<td>LI</td>
<td>5%</td>
<td>Li &gt; 5%: death within 6 months</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RFS = relapse-free survival; RR = relative risk.

References:
- Gansler et al. [50]
- Tribukait [130]
- Awwad et al. [5]
- Volm et al. [142]
- Bunn et al. [16]
- Costa et al. [29]
- Hansson et al. [57]
- Chauvel et al. [22]
- Silvestrini et al. [120]
- Müller et al. [97]
- Hoshino and Wilson [64]
Labelling with non-radioactive precursor of DNA

Bromodeoxyuridine (BrdU) is an analog of thymidine which is incorporated in DNA during S phase. Labelling with BrdU is performed in vitro or in vivo. Thereafter, labelled cells are recognized by a monoclonal antibody against BrdU [35,54, 99,115,145].

When BrdU is injected to patients prior to the biopsy, this method can give information on the duration of S phase and the potential DT (T_poll) [10,87].

Two other techniques have been used for the measurement of the growth fraction and we shall only mention them. Primer-dependent α-DNA polymerase can identify the cells which are engaged in a cell cycle independently of the cycle phase [1,112]. The Kiel group has reported that a monoclonal antibody, Ki-67, specifically stains cycling cells. This antibody reacts with a nuclear antigen which is expressed throughout the whole cycle but is absent in quiescent cells. It has been used for measuring the tumor growth fraction [51].

With all the methods, the prognostic value of SPF depends, to some extent, on the cut-off level [31]. We have given in tables the cut-off values chosen by the authors. In some articles, the authors compared the SPF in surviving or deceased patients without any reference to a cut-off level.

Results

Tables I–III assemble the data that were collected from the literature regarding breast cancer, lymphoma and other solid tumors. For breast

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Result</th>
<th>Other factors tested</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>Histological grade and LI are most important predictors of RFS and survival</td>
<td>Age, histologic type, stage, tumor size, axillary lymph node status, hormonal status</td>
<td>Tubiana and Koscielny [136]</td>
</tr>
<tr>
<td>Breast</td>
<td>LI, no. of + ve axillary nodes, nuclear grade, tumor border: predictors of RFS</td>
<td>Age, stage, grade, ER, vessel or nerve invasion, inflammatory cells</td>
<td>Meyer et al. [90]</td>
</tr>
<tr>
<td>Breast</td>
<td>LI: single predictor of RFS and overall survival</td>
<td>Size, ER</td>
<td>Silvestrini et al. [117]</td>
</tr>
<tr>
<td>Breast</td>
<td>SPF, stage, lymph node status, ER. predictors of survival</td>
<td>Size, ploidy</td>
<td>Klintenberg et al. [72]</td>
</tr>
<tr>
<td>Breast</td>
<td>Loss of significance of SPF in the presence of other factors</td>
<td>Menopausal status, size, lymph node status, grade, ER, PR</td>
<td>Hedley et al. [60]</td>
</tr>
<tr>
<td>Breast</td>
<td>LI single predictor of RFS</td>
<td>Size, grade, ER, PR</td>
<td>Courdi et al. [32]</td>
</tr>
<tr>
<td>Breast</td>
<td>SPF, lymph node status, ER. predictors of RFS</td>
<td>Age, ploidy</td>
<td>Kaufmann et al. [70]</td>
</tr>
<tr>
<td>Head and neck</td>
<td>LI, stage: predictors of survival</td>
<td>Age, grade</td>
<td>Chauvel et al. [22]</td>
</tr>
<tr>
<td>Head and neck</td>
<td>SPF associated with ploidy (risk ratio), stage, histologic type:</td>
<td>Age, grade, residual tumor, mitotic figures, type of treatment</td>
<td>Kallioniemi et al. [69]</td>
</tr>
<tr>
<td>Head and neck</td>
<td>predictors of survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>SPF, age, stage: predictors of survival</td>
<td>B symptoms, histologic type</td>
<td>Lenner et al. [80]</td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>SPF single predictor of survival</td>
<td>Age, histologic type, stage, ploidy, therapy</td>
<td>Young et al. [146]</td>
</tr>
</tbody>
</table>

ER = estrogen receptors; PR = progesterone receptors.
tumors, the 11 published series concur to underline the marked prognostic significance of the proliferation rate (Table I). This is mainly due to a significantly lower incidence of distant metastases in patients with a low SPF [136,138]. There is a strong correlation between the proliferation rate and several other well known prognostic factors such as histological grade, presence of hormone receptors, age, ploidy. This is why the most valuable studies are those in which multivariate analyses were carried out (Table IV). In 6 out of 7 multivariate analyses, SPF keeps an independent prognostic value and is generally among the 2 or 3 most powerful prognostic indicators. It should be underlined that SPF has high prognostic value in node-negative patients [32,62,70,93,117,118].

In 5 of the 6 studies on non-Hodgkin lymphoma listed in Table II, a significantly higher survival in patients with a low SPF was observed. Moreover, a correlation was reported between SPF and histologic grade [24,36]. In the two multivariate analyses performed on NHL, SPF appears to be the most important or one of the most important predictors of survival (Table IV).

In ovarian cancer, neuroblastomas, bladder cancer, lung cancer, high SPF is also a powerful indicator of poor prognosis (Tables III and IV). Volm et al. [141] reported that in patients with non-small cell lung cancer, the incidence of distant metastases detected at the time of initial treatment was significantly higher in patients with a high SPF. In head and neck cancers [22], cervix [37,110,125], colon and rectum [13,94,127], there is some prognostic impact of SPF but it appears to be smaller, probably due to a better treatment response in patients with a high SPF. In the study of Streffer et al. [127], on patients with rectal cancer, the proportion of patients with lymph node involvement or distant metastasis is 14% when SPF is below median value versus 82% when SPF is above median. In another study on colorectal tumors, it has been observed that aneuploid Duke’s C tumors had higher SPF than aneuploid Duke’s B tumors [131]. In other solid tumors, the number of patients is too small to allow any firm conclusion and further studies are warranted. It is well known that SPF is also a prognostic indicator in leukemia, but these investigations are out of the scope of this survey.

Discussion

We have discussed above the limitation of the various methods which have been used in clinical investigations for the determination of the SPF. It is necessary to stress again that significant variations in the SPF can be observed in the study of a given tumor with the same technique; intratumoral variations as high as 25% have been reported [11,79]. However, usually the average intratumoral variations in SPF are smaller [23,83], which allows valid comparison between tumors. Nevertheless, the prognostic studies which are likely to be the most significant are those carried out by an experienced group on a large number of patients, using the same technique.

A few valid comparisons between various techniques have been performed [4,15,28,75,85,92,104]. In these comparisons, several factors have to be taken into account, in particular, the precision of the technique and the biases which are associated with each of them. For example, when SPF is measured with FCM, the proportion of cells in S phase may be slightly overestimated because some of the cells with cell DNA content intermediate between G1 and G2 may be quiescent and not synthesizing DNA [40,144]. Frindel et al. [48] compared cytophotometric method and TLI in individual tumor cells. They showed that in an experimental tumor of the mouse when the tumor is young, all the cells having an intermediate DNA content between G1 and G2 may be quiescent and not synthesizing DNA [40,144]. Frindel et al. [48] compared cytophotometric method and TLI in individual tumor cells. They showed that in an experimental tumor of the mouse when the tumor is young, all the cells having an intermediate DNA content were labelled, whereas in relatively old 12-day and 14-day tumors up to 5% of the cells with an intermediate content were unlabelled which suggested that in these cells the DNA synthesis had stopped temporarily or permanently. Since this initial work, more experimental and theoretical evidence has attested that cells may arrest in phases other than
G, under nutrient deprivation or cell-crowding conditions [2]. In particular, it was demonstrated that when cultured human tumor cells are led into a state of non-proliferation by crowding and metabolite exhaustion, up to 14% of cells can be arrested in S phase [40]. Despite these various biases, there is a fair consistency between the different methods which have been used in clinical investigation [15,28,86], therefore, one can analyze together the available data.

Tables I–IV clearly show the high prognostic significance of the SPF in breast tumors, ovarian tumors and non-Hodgkin lymphomas. In view of the wide differences in the biological characteristics of these tumors, it is likely that the association between a high tumor proliferation rate and the degree of tumor aggressiveness is a general feature of human cancers. However, a few inconsistent data have been reported. Some are probably due to the small number of patients included in the study or to the heterogeneity of the group of patients. For example, in colorectal cancers, a significant impact of SPF was found only in patients with Duke’s C after stratification for histological type [115].

It should be emphasized that SPF was found to be of high independent significance in 10 of the 11 studies in which multivariate analyses of prognostic factors had been carried out (Table IV).

A possible source of inconsistency is related to treatment responsiveness. For most tumor types, response to radiotherapy or chemotherapy is greater and more rapid in tumors with a high cell turnover rate, that is with high SPF [33,37,41,58,81,98,120,125,128,133,134]. Hence, in tumor types for which local recurrence is the main source of failure, such as cervix carcinoma, a greater effectiveness of locoregional treatment may outweigh the increase in tumor aggressiveness. In this context, it is noteworthy that in the Awwad et al. [5] study, the poor impact on the local recurrence rate of a high SPF was not observed when patients were submitted to local radiotherapy. However, when SPF is high, even if the immediate response is better, the long-term outcome is generally poorer [33]. In the subgroup of patients in whom there is no clear-cut correlation between SPF and survival, there is a trend towards a higher incidence of local recurrence and distant metastasis in patients with a high SPF [125].

We have mentioned above that SPF is strongly correlated with several other indicators of poor prognosis. The correlation between ploidy and SPF deserves some discussion. It is now well recognized that ploidy as assessed by a DNA index is by itself a strong prognostic indicator [3,7,8,30,84]. On the other hand, several articles have shown that SPF is higher in aneuploid tumors than in diploid ones; for example, in the Dressler et al. [39] study carried out on 1084 patients, the median SPF was equal to 2.6% in diploid tumors and 10.3% in aneuploid tumors. These differences are too large to be due to errors in the determination of SPF in diploid tumors, or to the presence of normal cells in the tumor. Therefore, a question arises: is the prognostic significance of SPF simply due to its correlation with ploidy? Actually, SPF appears to have an independent prognostic value. When diploid tumors and aneuploid tumors are considered separately. Dressler et al. [39] found in each subgroup a correlation between SPF and hormonal receptor status or axillary node status. In Hedley et al.’s [60] breast cancer series, SPF and ploidy had independent and equal prognostic significance. In some studies [97], SPF is of prognostic significance only in diploid tumors, but this may simply result from the poor reliability of SPF assay in aneuploid tumors. Tribukait [130] reported that in bladder cancers, SPF had a stronger prognostic significance than ploidy as assessed by DNA index. In Christensson et al.’s study [24], stepwise discriminant analysis showed that SPF was a stronger discriminator between prognostic groups than DNA content in non-Hodgkin’s lymphoma. In patients with neuroblastoma [50], high SPF is significantly correlated with poor survival whereas, conversely, aneuploidy is associated with a better survival. This discrepancy is not surprising. Malignancy is related to the activation, translocation, mutation or deletion of some specific genes. The assay of cell DNA content by
cytometry has not enough sensitivity to allow the detection of gene abnormalities or chromosomal translocation when they are not associated with gross chromosomal aberrations. Further investigation is required but it can already be said that measurement of DNA index does not provide all the relevant prognostic information. It is certainly of interest to measure both SPF and DNA index, but when only one of them can be determined the current data suggest that SPF should be favored.

Before discussing the mechanisms through which proliferation rate might be related with tumor aggressiveness, one should consider another explanation, namely, that the higher incidence of relapse is only due to a more rapid growth rate of the distant metastases or of the local recurrences. Figures 1 and 2 depict the two possible models. In Fig. 1, the short-time interval between the treatment of the primary tumor and the clinical emergence of the metastasis is due to the rapid growth rate of the metastasis. Indeed, several studies have shown such a correlation between tumor-doubling time and delay between initial treatment and relapse [133]. In this model, a low SPF or a long-doubling time are associated with a long-time interval between initial treatment and death [82] but not with a better long-term outcome. In Fig. 2, a high SPF is associated with an earlier metastatic dissemination during the growth of the primary tumor. We have previously shown that the threshold volume at which the tumor initiates its first distant spread is correlated with several main prognostic indicators [73,74]. A high SPF might be one of them. This hypothesis predicts a better long-term outcome in patients with a low SPF.

A long follow-up is required for discriminating between these two hypotheses. A metastasis has to grow from one cell to one billion cells in order to reach a detectable size, this requires 30 doubling times (DT). The DT of breast cancer metastases ranges from 1 to 12 months with a mean value of 3 to 4 months. In 1984, we reported a series of 128 patients in which the 10-year survival rate was significantly higher in the subgroup of patients with a low SPF than in the intermediate and high SPF subgroups [138,139].
However, a 10-year follow-up is not sufficiently long for conclusively rejecting the model depicted in Fig. 1. Recently, we updated the results of this study and the follow-up is now equal to 15 years. The relapse-free survival (RFS) or survival of the intermediate and the high SPF subgroups do not differ anymore and are significantly lower than in the low SPF subgroups [136]. Moreover, we were able to compute the RFS and survival in three subsets of breast cancer patients: those with a short DT, an intermediate DT and a long one. There is a remarkable consistency between the results obtained with DT or SPF. After a 15-year follow-up, there is no difference between patients with a short or an intermediate DT but a distinctly higher RFS and survival in patients with a long DT. This difference remains significant at a 25-year follow-up. In some recent retrospective studies on paraffin-embedded tumors, the follow-up is also of 18 or 20 years [69,96]. Thus these long-term studies show that the better outcome of patients with a low SPF or a long DT are not simply due to the slow growth rate of the metastases and validate the model described in Fig. 2.

What are the biological mechanisms which could explain this influence of the proliferative rate on the likelihood of metastatic dissemination? Three have been hypothesized. The first one is purely mechanical. The adherence of the cell membrane is reduced during mitosis and it has been shown in vitro that increasing growth rate facilitates detachment from glass or plastic surfaces [143]. However, it should be noted that in tumors with a very slow growth rate, such as prostate or thyroid carcinomas, metastatic dissemination can occur very early during the growth of the tumor. Thus this mechanical explanation is certainly insufficient for explaining the early metastatic dissemination of tumors with a rapid proliferative rate.

A second hypothesis is related to the present views regarding the acquisition by a tumor cell of the capacity to spread and originate a metastasis. It is generally considered that this capacity is associated with specific genetic abnormalities [44]. If the occurrence of these abnormalities were simply a random event, its probability would be related to the number of cell divisions occurring in the tumor. Thus it would be higher in tumors with a high proliferative rate because in these tumors the cell loss factor is also higher [82,83], therefore a higher number of cell divisions has taken place in the tumor before it has reached a given volume. However, among human breast cancers [135], the range in the cell loss factor is indeed much less wide than the range in the threshold volume at which first metastatic dissemination occurs [73,74].

A third hypothesis is related to the concept of tumor progression. In this concept high proliferative rate is not the cause of tumor biological aggressiveness but a variable associated with it. One of the main features of malignancy is that cells escape from the mechanisms which control their proliferation in normal tissues. Among malignant tumors, even when belonging to the same histological type, there are wide variations in the extent with which tumor cells escape from these regulatory systems and therefore in the proliferative rates. A high proliferation rate is a pointer of tumor progression. Despite some divergent data [46], it is generally reported that SPF is higher in metastases than in the primary tumor [43] and the DT of metastases shorter than that of the primary tumors [18,133]. This suggests that spread was initiated by cells belonging to a subclone of rapidly proliferating cells. Moreover, the positive correlation observed between tumor cell density and SPF [34] suggests that a high proliferation rate is associated with a disturbance of cell-to-cell interactions and of cell contact inhibition. Likewise, a positive correlation is observed between high SPF and in vitro clonogenicity [45,52] and a negative correlation between the degree of tumor cell differentiation and cell proliferation rate [133]. For example, the SPF is higher in breast tumor cells without hormone receptors [30,38,39,42,53,68,72,77,86, 91,93,95,101,103,116,117,126]. High proliferation rate, early lymphatic involvement or distant metastatic spread and rapid development of chemo-resistance are not related to the same
genetic abnormalities; however, the likelihood of their occurrences might be correlated if they are associated with the degree of genetic instability. This hypothesis is consistent with the concept of tumor progression which relates the extent of tumor malignancy to the degree of genetic instability [44,100,132].

These three hypotheses are not mutually exclusive and all of them might contribute to explain the poor prognostic significance of high SPF. However, in the light of our present biological knowledge, the third one appears to be the most likely. Elucidation of the molecular defects associated with high proliferative rates and tumor aggressiveness is within our reach. This should be the goal of further research.

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