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Prognostic Value of Cytokinetic Parameters in Lung Cancer After *in Vivo* Bromodeoxyuridine Labelling

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Abstract. *Background: To improve the overall survival rate in lung cancer by adjustment of treatment protocols on basis of tumour characteristics of individual patients, independent predictive parameters are required. Therefore, we evaluated the prognostic value of cytokinetic parameters such as bromodeoxyuridine (BrdU) labelling index (LI), S-phase fraction (SPF), unlabelled S-phase fraction (USPF), S-phase duration (Ts) and potential tumour doubling time (Tpot), next to more established parameters. Materials and Methods: To this end a series of 92 bronchoscopic specimens of in vivo BrdU labelled lung cancer patients, 72 presenting with non-small cell lung carcinoma (NSCLC) and 20 patients with small cell lung carcinoma (SCLC), were analysed flow cytometrically. Clinical as well as cytokinetic data were collected and related to survival times over a follow-up period of 2 to 7 years. Results: We found that Tpot was a significant independent discriminator of good and poor prognosis in NSCLC. In particular, in non-squamous NSCLC a short Tpot, short Ts and high LI predicted for shorter survival time. In squamous cell carcinoma, a high USPF may predict a shorter survival period, although the correlation was only borderline-significant. Conclusions: We conclude that these parameters may in future be of use in drawing up more adequate treatment schedules for individual lung cancer patients.*

For lung cancer it has been established that prognosis is influenced by the stage or extent of the disease at presentation [1,2], histologic tumour type [2,3], tumour size and location [3,4] and performance status [1,3]. The prognostic potential of these factors often becomes only evident from studies with large patient populations and therefore these parameters are not conclusive for the prognosis of individual cases. Since the behaviour of each individual tumour is determined by a complexity of clinical features, as well as by the intrinsic growth potential of the malignancy, survival studies aim at the integration of such parameters [5].

The growth of a tumour can be defined as the result of cell proliferation and cell loss. It is hypothesized that a high proliferative activity in a large proportion of the tumour cells is associated with a poor prognosis of patients with a malignancy [6-9]. Cell proliferation is in general measured by determining the number of cells that are actively taking part in the cell cycle, and the rate at which the various phases of the cell cycle are traversed. Several authors have published evidence on the independent prognostic value of proliferation parameters in non-small cell lung carcinoma [2,10,11], but these detect a static situation rather than reflecting active proliferative behaviour of the tumour. Dynamic kinetic parameters, such as S-phase duration (Ts) and potential doubling time (Tpot), may provide a more representative estimation of a tumour's proliferative state [12].

In the present study bronchoscopic specimens of *in vivo* BrdU labelled lung cancer patients were flow cytometrically analysed and cell cycle parameters as well as clinical parameters were related to patient survival during a follow-up period of 2 to 7 years.

Materials and Methods

Patients selected for this study were suspected for endobronchial lung carcinoma and scheduled for bronchoscopy. After informed consent the patients received 50 mg m^{-2} BrdU intravenously (Janssen Pharmaceutica, Beerse, Belgium), dissolved in 100 ml 0.9% NaCl, within a timespan of 10 minutes. Approval for the *in vivo* labelling method was given by the ethical committee of the University Hospital of Maastricht. BrdU was given approximately 4 to 5 hours prior to bronchoscopy. Biopsies were taken with a flexible bronchoscope, fixed in formalin for routine diagnosis or in cold (4°C) 70% ethanol for flow cytometric analysis. The latter samples were stored at 4°C until use. The series of 35 biopsy samples described before by Tinnemans *et al* [12] was extended with 57 cases.

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Key Words: Cell cycle parameters, flow cytometry, non-small cell lung carcinoma, small cell lung carcinoma, survival.

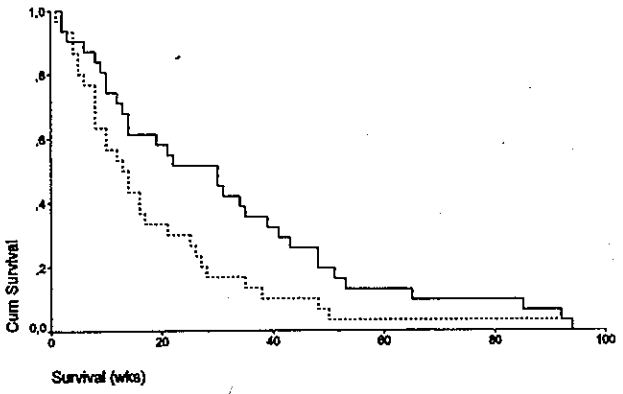

Figure 1. *Kaplan-Meier curves of patients with NSCLC, subdivided according to potential tumour doubling time (Tpot). Dotted line: Tpot shorter than the median value (n=30); solid line: Tpot longer than or equal to the median value (n=31). Logrank test p-value = 0.0403.*

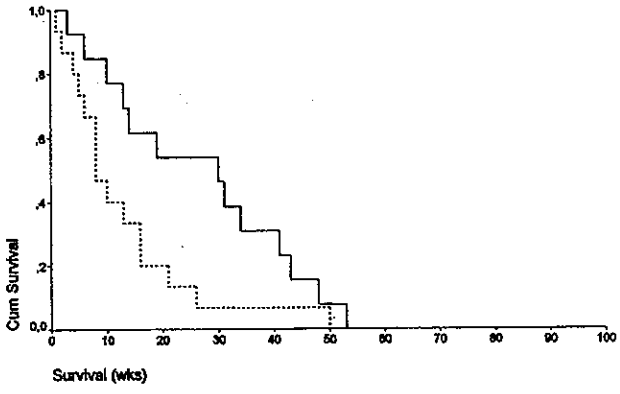

Figure 2. *Kaplan-Meier curves of patients with stage IV NSCLC, subdivided according to potential tumour doubling time (Tpot). Dotted line: Tpot shorter than the median value (n= 15); solid line: Tpot longer than or equal to the median value (n= 13). Logrank test p-value = 0.0415.*

Flow cytometric bivariate BrdU/DNA analysis. For double staining of lung cancer single cell suspensions with anti-BrdU and propidium iodide (PI) the protocol described by Schutte *et al* [13] was used. The samples were analysed using a FACSort (Becton Dickinson, Sunnyvale, CA), and evaluated with the standard Lysis and Cellfit software (Becton Dickinson). Data were gated on pulse processed PI signals to exlude doublets and larger aggregates.

The DNA index (DI) was estimated from the single parameter DNA histograms. The S-phase fraction (SPF) was determined from the DNA histogram using a rectangular fit [14]. The labelling index (LI) was defined as the percentage of BrdU positive diploid or aneuploid cells. The unlabelled S-phase fraction (USPF), the fraction of BrdU negative S-phase cells, was expressed as a percentage of the total cell population. The S-phase transit time (Ts) was determined using the method described by Begg *et al* [15] and Tinnemans *et al* [12].

Survival curves and statistical analysis. The two series of analyses were combined [12] and the two main categories of lung cancers, *i.e.* small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), were analysed separately. In order to construct survival curves, for each cytokinetic parameter the cases were classified higher/equal or lower than the median value of of their own analysis group and category. Contingency tables were constructed for each cytokinetic parameter *versus* stage of disease and *versus* tumour histology. A chi-square test was used to determine whether these parameters were related. In addition to the cytokinetic variables, also stage, gender, age (younger or older than 65 years), type of NSCLC (squamous/non-squamous) and DNA ploidy (diploid or aneuploid) were analysed. Survival curves were estimated using the Kaplan-Meier method [16]. A logrank test was used to examine the differences in survival curves.

Results

A total number of 130 patients entered the study. Seventy-one percent of the cases (n=92) gave evaluable flow cytometric data and could be included in the study. Twenty-two percent presented with SCLC and 78% with NSCLC. Aneuploidy was found in 29% of all biopsies (33% in NSCLC, 15% in SCLC). Similar to the first series of samples [12], no differences were found between SCLC and NSCLC for the mean values of LI, Ts, USPF and SPF in the second series of samples. The same holds true for Tpot, irrespective of the algorithm used.

Subset-analysis by a chi-square test on contingency tables showed that the parameters LI, Ts, Tpot, USPF and SPF were independent of clinical stage or histologic tumour type.

The group of SCLC was too small to draw definitive conclusions from the survival curves. Trends were observed for a survival benefit for patients presenting with limited disease (LD), females and patients younger than 65 years. No indications were found for a prognostic value of any of the cytokinetic parameters (data not shown).

In the group of NSCLC, stage of disease and histology (squamous *vs.* non-squamous) were found to be significant discriminators for survival. Age, gender and DNA index did not predict survival. Of the cytokinetic parameters, only a short Tpot was found to be significantly related to a shorter patient survival (Figure 1; p=0.04).

When stratified for stage of disease, the prognostic value of the Tpot was evident in stage IV (Figure 2;.p=0.04), while no significant differences were observed for this parameter in the stages I-III.

If the analysis was stratified for histology, Tpot was particularly significant to predict survival in non-squamous (adenocarcinoma and large cell lung carcinoma) NSCLC (Figure 3a; p=0.009). In this subgroup also a short Ts (Figure 3b; p=0.005) and high LI (Figure 3c; p=0.06) predicted a poor prognosis.

Furthermore, a shorter survival time was observed for patients suffering from squamous cell carcinoma with a high USPF as compared to patients presenting with squamous cell carcinoma with a low USPF, although this difference in survival rate was only borderline-significant (p=0,0529; data not shown).

Discussion

In the past, clinical and biological features of lung cancer have been analysed for their ability to discriminate between patients with good or poor prognosis and to predict response to therapy. Until now, such investigations at the cellular level have mainly concentrated on static parameters, such as ploidy and S-phase fraction. Here we extend such analyses by including data on cell proliferation dynamics.

Good correlations between dynamic cytokinetic characteristics and clinical outcome have been reported in various tumour types. In breast carcinoma and in non-small cell lung carcinoma, [^{3}H]-thymidine labelling index and percentage S-phase cells both predicted survival time [11]. The S-phase fraction (SPF) was also reported to be a significant independent prognostic factor in non- small cell lung tumours [10]. Nagashim *et al* [17] found that ependymomas with a high bromodeoxyuridine (BrdU) labelling index (LI) not only grow faster and are clinically more agressive than tumours with a low LI, but the former have also a strong tendency to recur. Benazzo *et al* [18] found a significantly higher LI and significantly shorter Ts and Tpot values in head and neck tumors with lymph node involvement, as compared to lymph node negative samples. A short potential tumour doubling time (Tpot) was also found to be significantly linked to shorter relapse- free intervals in various tumour types, while a longer Tpot predicted for longer relapse-free intervals [19].

In the underlying study, Tpot was found to be an indicator of survival in NSCLC. This was most evident in stage IV of disease and in patients with non-squamous NSCLC. In this latter group a better survival was also associated with a long Ts or low LI, although of borderline significance. This is in line with the results from the study of Silvestrini *et al* [2], who found a significantly lower overall survival in patients suffering from stage I non-squamous cell lung carcinoma with a high [^{3}H]-thymidine LI. In squamous cell carcinoma of the lung we found no prognostic value for LI, Ts or Tpot. However, patients with squamous cell carcinoma exhibiting a high USPF tended to have a worse prognosis compared to patients with squamous cell carcinoma and a low USPF (data not shown). This difference, however, was of borderline significance. In a former *in vitro* study, we have shown that a poor nutritional state, resulting in cell loss (*via* necrosis and/or apoptosis), is associated with high USPF values [20]. Together this may indicate that a high level of necrosis is associated with poor prognosis, as was also suggested by Shabab *et al* [21].

The flow cytometric detection of LI, USPF and SPF in malignancies can be hampered by the presence of non-malignant diploid cells, such as infiltrating leucocytes and fibroblasts. The presence of these cells can also influence the calculation of Ts and Tpot. The problem of admixture of non-relevant, stromal cells seems to be most prominent in diploid tumour samples [22]. It is possible that this phenomenon has also an impact on survival analyses. A solution to this problem is provided by a three color flow cytometric analysis (BrdU/DNA/cytokeratin). In this way, a detailed cytokinetic analysis can be performed on the relevant epithelial cells that are selected based on their cytokeratin expression. The feasibility of this method in fresh as well as in frozen tumour

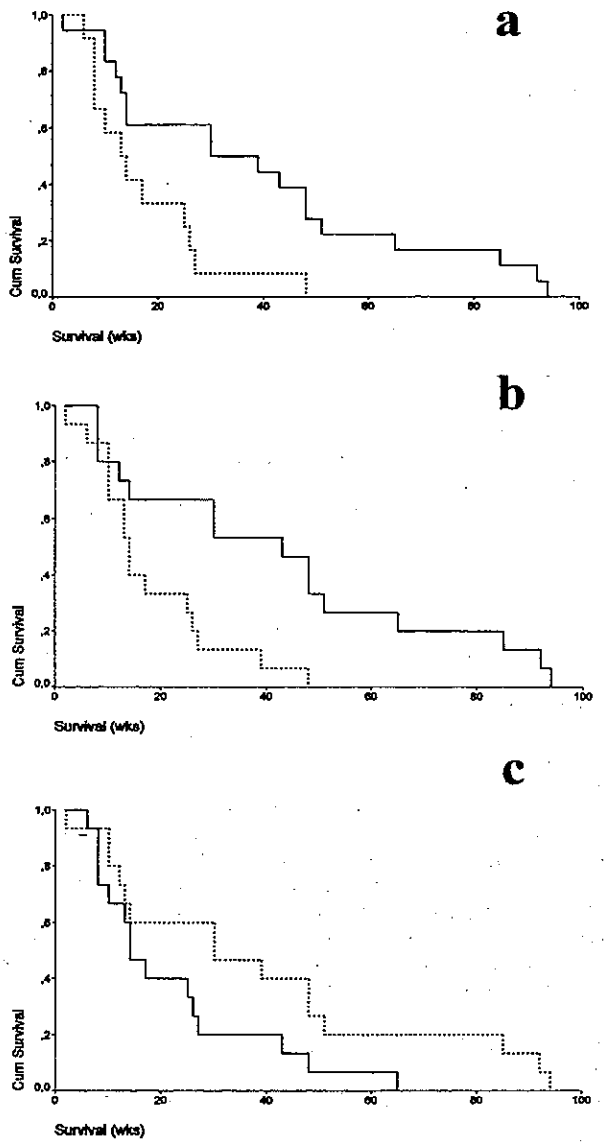

Figure 3. *Kaplan-Meier curves of patients with non-squamous NSCLC carcinoma subdivided according to (a) Tpot, (b) Ts and (c) LI.*

(a) Dotted line: Tpot shorter than the median value (n= 12); solid line: Tpot longer than or equal to the median value (n= 18). Logrank test p-value = 0.0085.

(b) Dotted line: Ts shorter than the median value (n= 15); solid line: Ts longer than or equal to the median value (n= 15). Logrank test p-value = 0.0051.

(c) Dotted line: LI smaller than the median value (n= 15); solid line: LI greater than or equal to the median value (n= 15). Logrank test p-value = 0.0597.

tissue samples has been described before by Schutte *et al* [23].

In conclusion, Tpot has predictive value in NSCLC. This prognostic significance is predominantly found in non-squamous NSCLC carcinomas. A crucial factor in determining the outcome of treatment is repopulation in surviving cells during fractionated therapy. Evidence in human tumours suggests that repopulation rates may vary between different tumours, but may be reflected by the Tpot in the untreated tumour [9] Our data support the concept that information about the Tpot can be of use in the design of treatment schedules [24].

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