DNA PLOIDY, BROMODEOXYURIDINE LABELLING INDEX, S - PHASE FRACTION AND AgNOR COUNTS IN BRAIN TUMOURS

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Received 23 November 1999; revised version received 6 March 2000; accepted 23 February 2001

Key words: Bromodeoxyuridine labelling index, SPF, Nucleolar organizer regions (AgNOR), proliferation rate, DNA ploidy, brain tumours,

ABSTRACT

DNA ploidy and the proliferative potential in 88 brain tumours were investigated using the bromodeoxyuridine labelling index (BrdUrd LI), S – phase fraction (SPF) and an argyrophilic nucleolar organizer regions (AgNOR) technique. The study included 65 highly malignant (AIII - AIV), and 23 low-grade (AI – AII) gliomas. One fragment of the tumour was fixed in Carnoy's solution for AgNOR test, while the other fragments were used for flow cytometric determination of the labelling index, SPF and DNA ploidy. For the BrdUrdLI, tumour samples from each patient were incubated *in vitro* for one hour at 37^{o}C with BrdUrd using a high pressure oxygen method. After fixation and staining, the percentages of BrdUrd-labelled cells (BrdUrdLI) and unlabelled S-phase cells (SPF) were evaluated. The tumours showed variability in the BrdUrdLI values, SPF and AgNOR counts/cell nucleus. However, grade dependent differences in the proliferating rate were only found to exist on the basis of BrdUrdLI and AgNOR counts. The same percentage of DNA aneuploidy (56 %) was found in high-grade as well as in low-grade gliomas. A linear – regression analysis showed a significant correlation between the results of three applied methods: BrdUrdLI, SPF and AgNOR counts.

INTRODUCTION

The results of radiotherapy in malignant brain tumours are poor. Despite application of different methods of treatment such as surgery, brachytherapy, high LET radiotherapy, radiosensitizers of hypoxic cells, hyperbaric oxygen, or hypo and hyperfractionation, the mean survival time after treatment is 1 to 2 years. Many radiobiological and biological factors may influence the low radiosensitivity of malignant gliomas to ionizing radiation. This may be the reason of a significant capacity for repair of sublethal damage and low inherent radiosensitivity, as well as rapid growth rate, great number of hypoxic cells, or poor reoxygenation.

There is an increasing evidence that tumours with a high proliferative rate could benefit from an accelerated course of radiotherapy. Therefore, the knowledge of the proliferative potential is important in selecting proper treatment for an individual patient and predicting the prognosis. Also determination of the tumour growth rate may be helpful in the selection of patients with residual tumour after surgery who may benefit from additional chemotherapy or/(stereotactic) radiation therapy.

The potential doubling time (T_{pot}) which is the time required for a tumour population to double its number in the absence of cell loss [1] is considered to be the best method for describing the dynamic cytokinetic parameters of tumour cells. Although the method is useful in the clinical practice for gliomas [2, 3], its value in other tumours is still doubtful. The method requires i.v. administration of iodo - /bromodeoxyuridine (IdUrd/BrdUrd). Another method, the BrdUrd labelling index ($_{BrdUrd}LI$), which shows greater usefulness in clinical practice can be determined *in vitro* by incubation of the thymidine analoque (BrdUrd) with tissue fragments [4]. The growth rate of tumours has also been measured by estimating the S - phase fraction (SPF), or the proliferative index (PI, i.e. the percentage of cells in S + G_2/M phases of the cell cycle) using flow cytometry. Other methods include monoclonal antibodies such as Ki-67 (MIB-1 monoclonal antibody), PCNA (proliferating cell nuclear antigen) [5, 6, 7, 8], which specifically stain cycling cells and argyrophilic nucleolar organizer regions (AgNOR) technique [9]. The silver staining technique selectively stains some acidic proteins (AgNOR proteins) assocciated with ribosomal genes, which

reflect cell and nuclear activity [10]. It is a simple, and cheap method which can be applied to paraffin sections, making retrospective studies possible. However, some concern still exists as regards accuracy and reproducibility of AgNOR quantifying methods.
The current study was undertaken with the aim of comparing AgNOR counts in brain tumours with two better established methods such as: BrdUrd LI and SPF for investigating cell proliferation. As the methods refer to the same biological parameter the purpose of the study was also to establish the degree of correlation between these indices. The DNA ploidy of the examined tumours was also analysed.

MATERIAL AND METHODS

Patients

There were 65 patients with highly malignant (grade III – IV, 11), and 23 with low-grade gliomas (I – II) (Table 1). The low-grade group consisted of 13 men and 10 women, and in grade III - IV there were 36 men and 28 women. Grade I – II gliomas included younger patients (mean age 43.0 years), while in grade III – IV group the mean age was 55.0 years.

Tab.1 Correlation between histology and grade of brain tumours. Number of patients are shown in brackets.

Kernohan Grade	Histology
I	Fibrillary astrocytoma (10)
II	Astrocytoma (4) Oligodendroglioma (2) Mixed glioma (7)
III	Malignant astrocytoma (17) Malignant oligodendroglioma (11) Mixed anaplastic astrocytoma (6)
IV	Multiform glioblastoma (31)

Material

The specimens (about 0.5 cm^3) from each patient were delivered fresh from the operating room shortly after excision. One or two fragments of the tumour were fixed in Carnoy's solution for AgNOR test, while other fragments were used for the determination of the BrdUrd LI, SPF and DNA ploidy.

BrdUrd labelling index

In vitro incorporation of BrdUrd was carried out according to the high pressure oxygen method described by Steel and Bensted (1965). The BrdUrd staining procedure and flow cytometry have been described in detail elsewhere [12]. Briefly, after 1 hour of incubation with BrdUrd at 37°C, tumour fragments were fixed in 70 % ethanol. They were then minced into fragments and digested into nuclei at 37°C with 0.4 mg/ml pepsin (Sigma Chemicals, Poole, Dorset) in 0.1 M HCl for 20 min. The nuclei suspension was filtered through a 35 μm nylon mesh and centrifuged at 2000 rpm for 5 min. The pellet was resuspended in 2 M HCl for 12 min to denaturate the DNA partially. After two washes in phosphate-buffered saline (PBS), the pellet was incubated in PBS containing 0.5 % normal goat serum (NGS) (Sigma Chemicals), 0.5 % Tween 20 (Sigma Chemicals) and a mouse-derived anti-BrdUrd monoclonal antibody (DAKO). After 1 hour, the nuclei suspension was washed in PBS and suspended in PBS/NGS/Tween containing goat anti-mouse IgG FITC conjugate (DAKO) for 1 hour. After two further washes as in PBS, the suspension was stained for total DNA with PBS containing 10 μg/ml propidium iodide (PI).

Flow-cytometric data analysis

The stained preparations were analysed with a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Sunnyvale, CA, USA). Doublets and clumps were excluded from the analysis by gating on a bivariate distribution of the red peak vs integral signal. Excitation of the FITC-labelled cells and the DNA-associated PI was accomplished with an argon-ion laser tuned to 488 nm and operated at 15 mW. Ten thousand events were collected in each histogram. The BrdUrdLI was calculated as a percentage of all BrdUrd-labelled cells in a sample (without discrimination of diploid subpopulation in aneuploid tumours). The S-phase fraction was calculated with a FACScan software programme, running on a Hewlett Packard computer. The percentage of aneuploidy was estimated by evaluating the DNA index, i. e., the ratio of the modal DNA fluorescence of abnormal to normal $G_{1/0}$ cells. Aneuploidy was assessed in cases in which the normal and neoplastic cell populations gave two separate peaks. Human lymphocytes were used as a reference peak.

Ag-NOR staining technique

Tumour fragments were fixed in a Carnoy's solution for 48h at 4°C. Tissues were then routinely processed and embedded in paraffin. Sections (4μm thick) were dewaxed by successive baths in xylene and ethanol, post-fixed for 30 min in 3:1 (vol/vol) absolute ethanol - acetic acid solution and then rehydrated. AgNOR staining was carried out using a solution of 1 volume of 2 % gelatine in 1 % aqueous formic acid and two volumes of 50 % silver nitrate. Silver staining was performed at 37°C for 20 min. The silver colloid was than washed off with distilled water, the sections were dehydrated to xylene and mounted. NORs were visualized as distinct black intranuclear dots. The number of NORs per cell nucleus from about 200 cells for each slide were evaluated under the microscope with a 100x oil immersion objective lens. When large polycyclic structures (overlapping NOR) were observed, they were counted as a single AgNOR if individual AgNOR could not be identified.

Statistical analysis

The Student's *t* test was used to determine the statistical significance of differences in the BrdUrdLI, SPF and AgNORs counts (at $p < 0.05$ level). To test the relationship between tumour grade and the results of the method applied, an analysis of variance and Tukey HSD test for unequal number of patients were applied. A linear – regression analysis was performed to determine the correlation between the three applied methods ($p < 0.05$). Statistical calculations were performed using a STATISTICA ver 4.5 programme.

RESULTS

The brain tumours showed great variability in the cell kinetic parameters based on: BrdUrd LI, SPF and AgNOR counts. The BrdUrdLI ranged from 0.3 to 9.9 %, SPF - from 1.0 – 44.4 %, and AgNOR counts/cell - from 0.25 – 5.18. For high-grade gliomas (III – IV) the values for the three methods applied were the highest (fig.1 a-c). The mean BrdUrdLI for grade IV gliomas was significantly higher (2.9 %) than in grade I – II gliomas (1.0 %). The highest mean number of AgNOR counts was observed in grade IV gliomas 3.0/cell, however the lowest mean value (1.6/cell) was found in grade I – II gliomas. Only grade IV gliomas showed a significantly higher number of AgNORs/cell than grade II and other groups (I, III) ($p = 0.0002$, Fig. 1b). No significant difference between tumour grade and SPF was demonstrated.

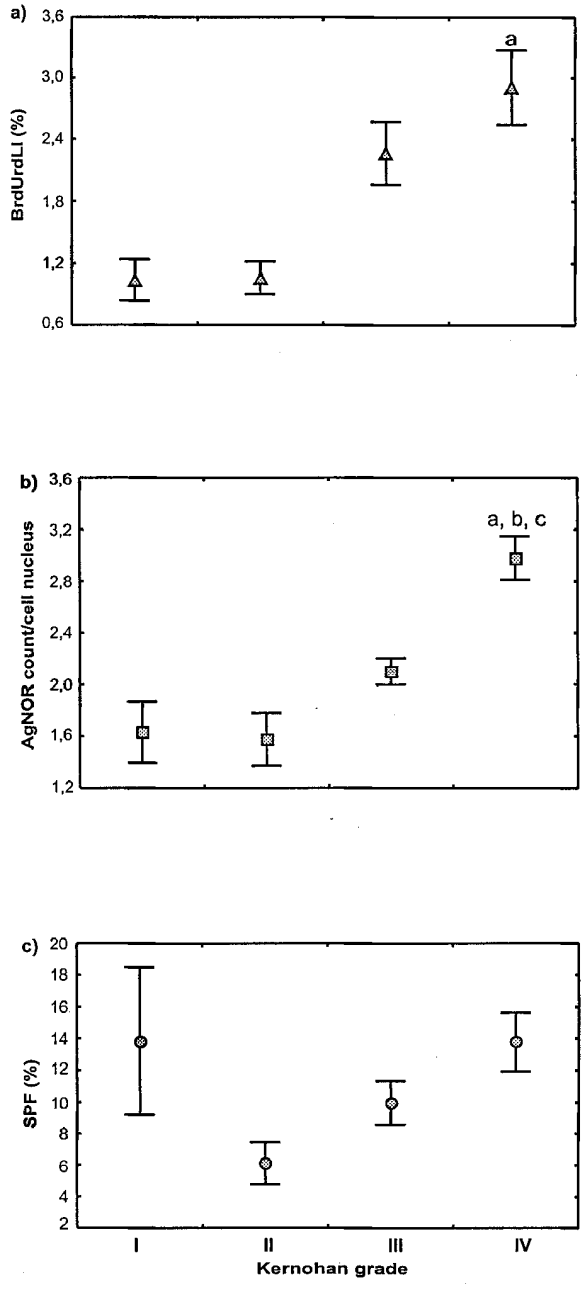

Fig. 1. Box plots showing relationship between tumour grade and **a)** BrdUrdLI, **b)** AgNOR count/cell nucleus, **c)** SPF. Each symbol represents mean value and standard error of the mean. Fig. 1 a. Statistically significant relationship between BrdUrdLI and tumour grade ($p = 0.0017$); (a) the difference between tumour grade II and IV, $p = 0.0305$. Fig. 1 b. Statistically significant relationship between AgNOR count/cell nucleus and tumour grade ($p. = 0.0000$); (a) difference between tumour grade I and IV, $p = 0.0012$; (b), difference between tumour grade II and IV, $p = 0.0002$; (c) difference between tumour grade III and IV, $p = 0.0003$. Fig. 1 c. The relationship between SPF and tumour grade.

When cell kinetic parameters were calculated for diploid and aneuploid tumours separately, significantly higher values for aneuploid tumours were observed only for BrdUrdLI and SPF (Table 2). For diploid gliomas the BrdUrdLI was only 1.68 %, while for aneuploid tumours this value amounted to - 2.57 % ($p = 0.0194$). The same was true for the S - phase fraction, which in anueploid tumours was significantly higher (15.45 %) than in diploid tumours (6.06 %, p 0.0000).

A linear – regression analysis showed a significant correlation ($p < 0.05$) between the BrdUrdLI and AgNOR counts (Fig. 2 a), BrdUrdLI and SPF (Fig. 2 b), and SPF and Agnor counts (Fig 2 c). DNA aneuploidy was found in 56 % of high – grade and low-grade gliomas. In low - grade gliomas 1 (7.6 %) of 13 aneuploid tumours was hyperdiploid (DNA index = 1.4 – 1.8) and 10 (76.9 %) were tetraploid (DNA index = 1.9 – 2.0). Five (13.9 %) of 36 high - grade aneuploid gliomas showed hyperdiploid pattern of aneuploidy, 28 (78 %) tetraploid, and 2 (5.5 %) hypertetraploid.

Tab. 2. Comparison of DNA ploidy with BrdUrdLI , SPF and AgNOR counts for brain tumours. * $p = 0.0194$, ** $p = 0.0000$.

Tumour	N	BrdUrd LI (%) *mean ± SE*	SPF (%) *mean ± SE*	AgNOR count/cell nucleus *mean ± SE*
diploid	39	1.68 ± 0.17	6.06 ± 0.60	2.06 ± 0.12
aneuploid	49	2.57 ± 0.30*	15.45 ± 1.63**	2.45 ± 0.15

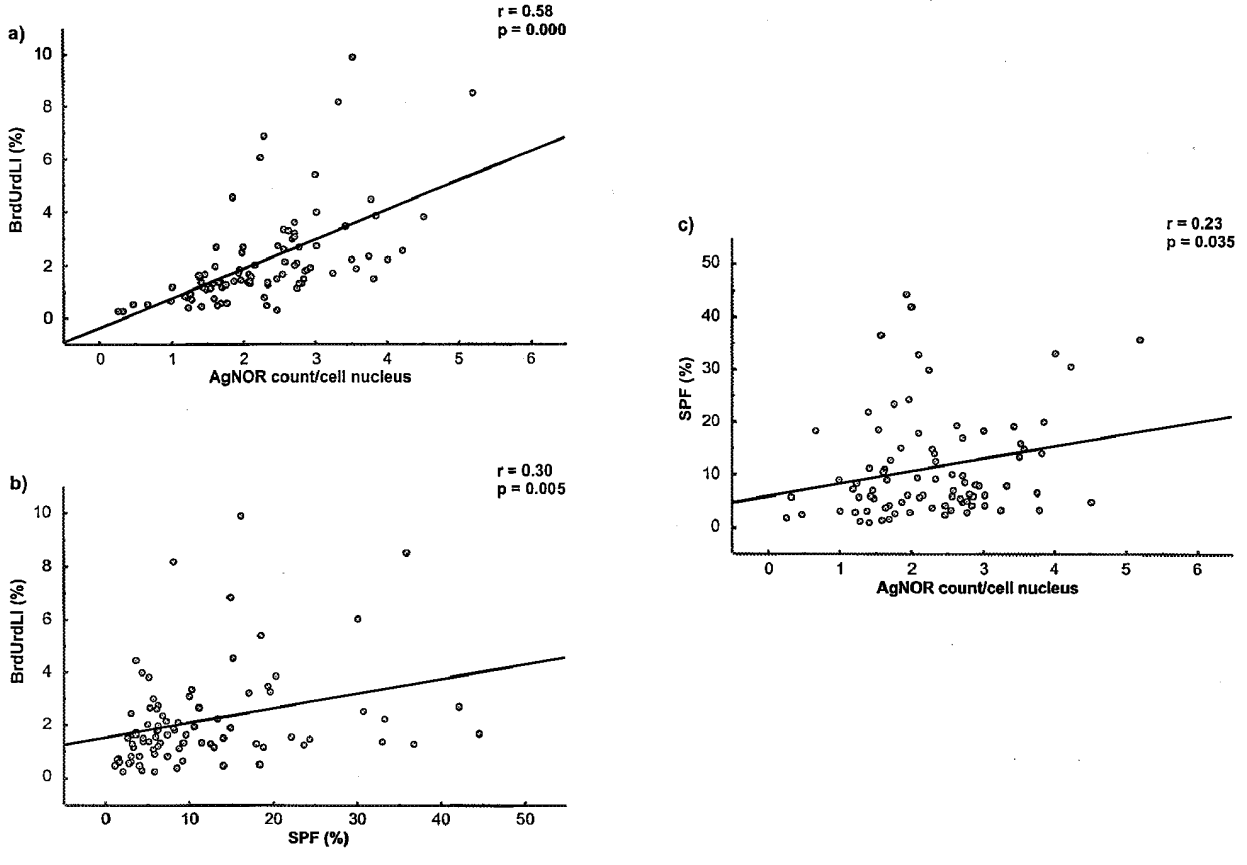

Fig. 2. A significant correlation ($p < 0.05$) between the BrdUrdLI and AgNOR count/cell nucleus (a). The BrdUrdLI and SPF (b) and AgNOR count/cell nucleus and SPF (c).

DISCUSSION

Differences in the proliferating rate between and within groups of brain tumours were determined on the basis of all three methods under evaluation: BrdUrdLI, SPF and AgNOR counts. The highest mean BrdUrdLI was observed in glioblastoma (grade IV, 2.9 %) and other high-grade gliomas (grade III, 2.3 %), while it was lowest in low-grade (I - II) gliomas (1.0 %). The present study showed LI values similar to those found in other reports [2, 3, 13, 14]. The only discrepancy was found in glioblastoma, for which some other authors have quoted higher values than those obtained by us. [15, 16]. This may be due to differences in the *in vivo* and *in vitro* incorporation procedure and to the use of immunohistochemistry rather than flow cytometry to detect the DNA precursor. The difference might be due to discrimination by the authors of diploid subpopulation in aneuploid tumours in LI counting, or to the different number of cases examined.

Several studies have been published on BrdUrdLI in brain tumours, showing significance of this parameter in patients' survival [3, 13, 15, 17], and reccurence rate [15, 18]. Some authors [3, 18] showed a significantly greater probability of survival among patients whose tumours had LI's less than 1 – 3 %. This results might confirm our data (19), which show that patients with a higher proliferative potential - LI > 1.5 % have significantly lower probability of survival, after surgery, than those with a lower proliferative potential (LI < 1.5 %). Also a strong significant correlation between low SPF < 3 % [20, 21], low Ki-67 LI (6) and longer patients' survival was shown. Thus the proliferative potential of tumour cells may be more important than the histopathological diagnosis, and should therefore be considered as an important factor in determining the prognosis of individual brain tumour patients and of selecting treatment. If more favourable outcome is due to slow growth rather than to the sensitivity to adjuvant therapies, patients with LI's of less than 1 % should be treated primarily by surgical resection. Treatment with radiation or chemotherapy might be reserved until malignant change or faster growth is observed. Therefore, a proper method of determining the tumour growth rate is necessary.

Silver staining for AgNORs is reported to be helpful in assessing tumour proliferation and its histological grade [9, 22], which is compatible with our results. Also a correlation between the number as AgNOR and survival has been demonstrate [22, 23]. In our study, the number of AgNOR in grade IV ranged from 1.2 to 5.2, and was within the same range as in other author' calculations - from 1.8 to 5.8 [8, 9]. The lowest mean value (1.0) was observed in grade I - II gliomas. The results between low and high – grade groups were statistically different. However, other authors (8) have not found any correlation between the degree of histologic differentiation or the pathologic staging and the AgNOR counts. The discrepancies between laboratories might be caused by the use of different methods of fixation of the material, different staining, or different acuracy of the method for different tumour types.

In our previous study on SCC of the cervix [12], the number of AgNORs was found to be in the same range as that in brain tumours. As BrdUrdLI revealed much higher proliferative rate for cervical tumour (range 2.7 - 30.4 %) in comparison with brain tumours (range 0.3 - 9.9 %) this should show also higher AgNOR counts. As this does not happen to be the case, there may be AgNORs overlapping (lower NORs dispersion) in cervical tumour, which could point to the inacurracy of the method and thus to the main drawback of the method.

In our study, the number of SPF and the AgNOR counts were correlated with BrdUrdLI, which confirms some other authors' studies [5, 8, 24]. However, some authors have found no correlation between BrdUrdLI and AgNOR counts (25). It may perhaps be more difficult to find a correlation for tumours with a relatively low proliferative potential. As the measurements of AgNOR counts give only an indirect indication of cell proliferative activity, and SPF is a stastic marker which does not reflect active proliferation, BrdUrdLI seems to be the most precise reflection of the proliferation potential of tumour cells. The BrdUrdLI shows the highest applicability in clinical practice.

In our study a relatively low rate of aneuploidy was found in tumours. Fourty - nine of 88 (56 %) tumours were aneuploid. Surprisingly, the same percentage was recorded for low - and high - grade gliomas. Eveng lower incidence of aneuploidy (29.1 %) in grade IV gliomas was observed by Danova et al. (3). A higher incidence of aneuploidy (57.3 - 89.0 %) in the high - grade was found by other authors [5, 20]. In high - grade gliomas tetraploid tumours were more common than in low – grade tumours. Moreover, high - grade gliomas were the only tumours to display hypertetraploidy; this might be indicative of the higher degree of malignancy of these tumours. In our study, statistically significant higher

proliferation rate was observed in the aneuploid tumours, as assessed by the BrdUrdLI and SPF, in comparison with diploid tumours. This is in agreement with other authors' findings for tumours of the same localization [3, 18, 25].

Our data showed variability in the proliferative rate, especially within the highly malignant gliomas. However, the three methods studied assessing the proliferative rate; i.e. BrdUrdLI, SPF and AgNOR counts, show correlation and seem to reflect accurate proliferative activity of the tumour cells, and it was only the SPF that was unable to reveal grade dependent differences. There still exists scarce and controversial information in the literature on the prognostic value of the AgNOR technique and SPF in gliomas, we plan, therefore, to increase the number of patients examined and compare experimental results with the clinical outcome to test the clinical prognostic value of the three methods applied.

Acknowledgments

We would like to thank Anna Cichocka M.Sc. for her valuable assistance, and Prof. Andrzej Sokołowski for statistical analysis of the data.

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