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Original Paper

Mitotic Percentage Index: a New Prognostic Factor for Childhood Medulloblastoma

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We investigated the prognostic significance of a new method of mitotic figure quantitation, 'mitotic percentage index' (MPI), tumour S phase fraction (SPF) and DNA ploidy measured by flow cytometry, and various clinical prognostic factors including age, sex, tumour stage, degree of surgical resection, radiotherapy dose and adjuvant chemotherapy in 70 cases of childhood medulloblastoma diagnosed between 1968 and 1996. In univariate analysis, MPI ($P < 0.0001$), posterior fossa radiotherapy dose ($P = 0.003$), tumour stage ($P = 0.014$), craniospinal radiotherapy dose ($P = 0.019$), year of diagnosis ($P = 0.024$) and SPF ($P = 0.048$) were significantly related to survival. In multivariate analysis, including tumour c-*erb*B-2 oncogene product expression, only MPI ($P < 0.0001$), craniospinal radiotherapy dose ($P = 0.003$) and tumour stage ($P = 0.035$) retained independent prognostic significance, while age achieved significance ($P = 0.039$). A close relationship was observed between MPI and SPF (coeff = 0.8, $P < 0.0001$) and MPI and the percentage of tumour cells expressing the c-*erb*B-2 oncogene product (coeff = 0.416, $P < 0.0001$). This study has identified MPI as a new independent prognostic factor for childhood medulloblastoma. Its close relationship with tumour SPF confirms it as an accurate measure of tumour proliferation and its close relationship to expression of the c-*erb*B-2 oncogene supports a role for this growth factor receptor in the deregulation of normal mitogenic signal transduction in this malignancy. © **1997 Elsevier Science Ltd. All rights reserved.**

Key words: medulloblastoma, PNET, mitosis, DNA ploidy, c-*erb*B-2, prognosis

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INTRODUCTION

DESPITE SUBSTANTIAL improvements in the overall survival of children with cancer, the treatment of medulloblastoma remains disappointing, with most centres reporting long-term survival rates of between 50 and 60% [1, 2]. This lack of progress in therapy may be explained in part by a lack of established prognostic factors for this disease, reducing the efficient use of existing treatments and the development of new therapies for non-responders. Clinical disease features, including patient age [1, 3, 4], sex [4, 5], degree of surgical resection [3, 5] and craniospinal radiotherapy (CSI) dose [6–8] have proved unreliable in predicting the outcome of patients with medulloblastoma. Only the presence of metastases at diagnosis [3, 4, 9–11] and use of posterior fossa radiotherapy (PFR) dosage less than 50 Gy [1, 4, 5] are consistently associated with poor patient outcome. This failure of clinical factors makes the identification of new biological prognostic factors of prime importance if therapeutic advancements are to be made in the management of this malignancy.

Cellular proliferation is one of the most important features of the malignant phenotype, and its quantification

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through mitotic counting [12–14], flow cytometry [15] or immunohistochemical analysis of proliferation related antigents, e.g. Ki-67, has provided a valuable source of prognostic information for a number of human tumours. The counting of mitotic figures in standard haemotoxylin and eosin sections potentially represents the most rapid, simple and inexpensive means of assessing tumour proliferation. Early studies of its relationship to prognosis in malignancies including medulloblastoma employed the technique of 'counts per high-power field' [10, 11, 16]. This method provides a measure of mitotic frequency by simply counting the total number of mitotic figures seen in 10 randomly selected high-power microscopic fields. The results obtained from such techniques are influenced by many variables including the density of tumour cells within sample sections, the heterogeneous distribution of mitotic figures within a tumour, the number of microscope fields counted and the size of the microscope field used [17–19]. They are, therefore, unreliable, producing inaccurate, conflicting results with poor reproducibility [19]. This has led to the development of new techniques to quantify mitotic figure frequency.

One such method, the 'volume corrected mitotic index' (M/V index), proposed by Haapasalo and associates, has demonstrated mitotic frequency analysis to be a significant prognostic factor for various cancers including ovary [12], pancreas [13] and bladder [14]. Although this technique reduces error inherent in earlier mitotic count techniques caused by variation in the size of microscopic fields [19], it fails to account adequately for two major factors: the density of tumour cells within, and the heterogeneous distribution of mitotic figures throughout, sample sections [20]. The mitotic percentage index (MPI) proposed in the present study improves the assessment of tumour mitotic frequency in two ways. Firstly, it scores mitotic frequency not in relation to tumour volume or area, but as the percentage of tumour cells in mitoses thereby avoiding error resulting from variable cell size and tumour cell density. Secondly, it incorporates all available tumour material in the frequency analysis, specifically identifying areas of high mitotic activity within tumour sections for mitotic frequency analysis.

Tumour proliferation may also be quantified using flow cytometric assessment of SPF (S-phase) fraction. This technique has been shown to reflect closely other markers of tumour cell proliferation and patient survival in a number of malignancies [15]. A limited number of studies in the literature have investigated the role of SPF and DNA ploidy in the prognosis of some paediatric solid tumours including medulloblastoma and peripheral primitive neuroectodermal tumours [21–24]. Those involving medulloblastoma have, in general, employed small numbers of patients and produced conflicting results [21–23]. In 1992, Schofield and associates published their study of 55 patients with medulloblastoma, reporting a non-significant trend towards improved outcome in those with SPF < 15% and significantly improved survival of patients with aneuploid tumours [22]. Most recently, in a study of 27 DNA histograms, Tait and associates reported no prognostic significance of ploidy status of SPF in patients with medulloblastoma [23]. In contrast, larger studies of other childhood solid tumours assign a more important role to SPF in patient prognosis. For example, multivariate analysis of 59 patients with rhabdomyosarcoma by Niggili and associates revealed the independent prognostic significance of tumour SPF. In univariate analysis, 95% of patients with SPF < 14% survived five or more years versus 50% of those with SPF > 14% [24].

In the present study, we investigated the independent prognostic significance of various clinical and pathological disease features including tumour MPI, DNA ploidy and SPF in 70 cases of childhood medulloblastoma. In addition, we demonstrated a significant relationship between tumour MPI, SPF and expression of the 185 kDa transmembrane tyrosine kinase growth factor receptor c-erbB-2, an oncogene which we have recently shown to have independent prognostic significance in this disease [25].

PATIENTS AND METHODS

82 children less than 15 years of age with medulloblastoma were notified to the Northern Region Young Peoples Malignant Disease Registry between 1968 and 1996. 5 of these patients died in the peri-operative period from surgical complications. Tumour material was not available for 7 patients. The tumours from the remaining 70 cases were reviewed to confirm the histological diagnosis of medulloblastoma (RHP) and were all subsequently employed in the study. The age at diagnosis ranged from one month to 14 years with a mean of 6.05 years. 47 patients were male and 23 female, giving a sex ratio of 2.04:1.

3 patients died within one month of surgery from rapidly progressive disease. Of the other patients, 24 underwent total and 46 partial or biopsy resections of their primary tumours, respectively. Sixty-five patients also received post-operative posterior fossa and craniospinal radiotherapy. Doses of 50–55 Gy and 35 Gy to the posterior fossa and craniospinal axis, respectively, are regarded as standard treatment in the management of medulloblastoma [2]. 28 patients received posterior fossa radiation (PFR) doses equal to or greater than 50 Gy, while 43 were treated with craniospinal axis irradiation (CSI) doses equal to or greater than the standard of 35 Gy. 2 patients, aged 1 year and 1 month, respectively, received surgery and the 'baby brain' chemotherapy protocol [26].

In additon to surgery and radiotherapy, 28 patients received adjuvant chemotherapy. In 22 cases, treated prior to 1989, this consisted of 'empirical' non-trial based treatments including vincristine with or without CCNU in 20 cases, vincristine, cyclophosphamide, doxorubicin and actinomycin-D in one case, while 1 further patient received actinomycin-D and vincristine, cyclophosphamide and doxorubicin. From the late 1980s onwards, trial-based chemotherapy was used, with 1 patient receiving the SIOP II protocol and a further 5 patients being randomised to receive adjuvant chemotherapy as part of the SIOP III trial [2].

Full reviews of the surgical, radiology and tumour registry notes were made to obtain details of disease stage. Detailed intra-operative surgical descriptions of the primary tumour were available for 58 patients. In addition, prior to 1983, 4 patients had available records of CSF analysis and myelogram imaging, while all but one case presenting after 1983 ($n = 25$) had received computed tomography (CT) and myelography or CT and spinal magnetic resonance imaging (MRI). Therefore, Chang staging of the primary tumour was carried out for all cases with full surgical descriptions of the primary tumour [27]. However, metastases staging

Table 1. Chang primary tumour and metastases staging for 58 patients with full surgical notes and for 29 patients who also had neuroaxial imaging respectively

Tumour stage	Number of patients ($n = 58$)	Metastasis stage	Number of patients ($n = 29$)
T1	16	M0	20
T2	20	M1	1
T3a	5	M2	3
T3b	10	M3	5
T4	7	M4	0

could only be undertaken in 29 patients with detailed neuro-axial imaging. Reliability of the surgical notes for tumour staging was confirmed by comparing surgical and radiology reports in the 25 patients with detailed imaging. Tumour staging score by surgeon and radiologist differed in only 1 patient scored as T1 and T2, respectively. Staging data is summaried in Table 1.

All neurohistological procedures were performed using 10% formalin-fixed paraffin-embedded tumour material obtained from the patients at operation. For each case, all available tumour blocks were collected for study from the Newcastle and Middlesbrough Neuropathology archives. All microscopy was performed using an Olympus BH-2 microscope.

Mitotic percentage index

Between one and six paraffin blocks were available for each patient (mean of 3). These were used to prepare standard 5 μm haematoxylin and eosin sections for estimating the MPI. The MPI was calculated using a blinded three-step procedure.

Step 1. For each case, a high-power (×400 magnification) field by field review of all available haematoxylin and eosin sections was performed. Those tumour areas with the highest concentration of identifiable mitotic figures [18] within each section were marked (mean of five per section).

Step 2. With the aid of a square graticule, a minimum of 1000 tumour cells were counted from each of the areas identified in Step 1.

Step 3. The percentage of mitotic figures versus non-dividing tumour cells was calculated giving the mitotic percentage index.

Flow cytometric analysis

Nuclear suspensions for flow cytometry were prepared using a revision of the techniques developed by Hedley and associates [28]. Five, 50 μm thick sections were cut using a microtome from the block judged most representative of the tumour, immediately following the section used for mitotic percentage index counting. Sections were then deparaffinised in xylene and rehydrated in a graded series of alcohols to distilled water and digested in 0.5% pepsin solution (Sigma) pH 1.5 for 30 min at 37 °C with vortex mixing at 5 min intervals. The digested material was filtered and centrifuged for 5 min at 800*g*, the supernatant was aspirated and the pellet resuspended in a solution of 20 ml Triton X solution (Sigma) for 3 min at 4°C. Samples were again centrifuged for 5 min at 800*g* and the nuclei resuspended in 5 ml ribonuclease solution (Porcine ribonuclease, Sigma) and incubated at 37°C for 20 min. Centrifugation was repeated and finally the nuclei were resuspended in 3 ml propidium iodide staining solution (10 μg/ml, Sigma) and stored light free at 4°C overnight (16 h). Normal cells within sections were employed as internal diploid controls.

The fluorescence of the propidium iodide stained nuclei was measured using a Becton Dickinson FACS flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, California, U.S.A.). Prior to each sample run, the alignment of the flow cytometer was checked by using EPICS Alignment fluorospheres (EPICS Division of Coulter Corp., Hileah, Florida, U.S.A.). Immediately preceding flow cytometric analysis, the cell suspensions were refiltered. A minimum of 2×10^4 nuclei were evaluated for each tumour sample. Any samples with coefficient of variation greater than 8% were discarded and the tumour reanalysed.

Analysis of DNA histograms was performed on ungated data. Aneuploid tumours were designated on the basis of presence of two or more resolvable G0/G1 peaks. Diploid and tetraploid tumours were characterised by a single G0/G1 peak with a corresponding G2/M peak and were distinguished by measuring the size of the G2/M fraction, using the multiple broadened rectangle method [29]. Tumours with G2/M fractions of greater than 20% were judged to be tetraploid. This was confirmed by the demonstration of a corresponding octaploid G2/M peak. Samples with G2/M fractions of less than 20% on cell cycle analysis were considered as diploid. The proportion of cells in S phase was calculated using the multiple broadened rectangle model and model for dual cycling populations [29].

Immunhistochemistry

We have previously published data for c-*erb*B-2 oncogene product expression for 55 of the current study population elsewhere [25]. This population was expanded in the present study to include all 70 patients and identical immunohistochemical analysis performed using the primary antibody NCL-CB11 (Novocastra Laboratories) and the ABC technique as described previously [25].

Statistical analysis

Univariate survival analysis was performed using the Cox regression model, Kaplan–Meier survival curves and the log-rank test. This permitted the analysis of variables in both continuous and non-continuous form. Unfortunately, because of extensive missing data, metastases stage could not be included in survival analysis.

To assess their independent prognostic significance, factors were analysed using the Cox model with c-*erb*B-2 oncogene product expression. All analysis complied with the assumptions for accurate Cox multiple regression. Finally, to validate further MPI as a measure of tumour cell proliferation, correlation analysis was performed between MPI and SPF. Similar analysis was also used to assess the relationship between MPI and c-*erb*B-2 oncogene product expression.

RESULTS

Clinical disease features

Overall survival for the study population was 34% at 25 year follow-up. However, a significant difference in patient outcome was observed between cases presenting before and after 1978, respectively. 20% of patients ($n = 7/35$) diagnosed prior to 1978 were alive after 25 years versus 51%

Table 2. Cox univariate regression analysis of clinicopathological disease features for the study population. Significant factors are shown in bold

Variable	Coefficient	Standard error	Relative risk	95% confidence intervals for relative risk	*P* value
MPI	**0.568**	**0.135**	**1.76**	**1.35–2.30**	**<0.0001**
PFR	**–0.997**	**0.340**	**0.37**	**0.19–0.72**	**0.003**
T stage (T1 & 2 versus T3 & 4)	**0.890**	**0.363**	**2.43**	**1.19–4.96**	**0.014**
CSI	**–0.716**	**0.306**	**0.49**	**0.27–0.89**	**0.019**
Year of diagnosis	**–0.722**	**0.320**	**0.49**	**0.26–0.91**	**0.024**
Surgery	–0.601	0.361	0.55	0.27–1.11	0.096
Sex	–0.352	0.333	0.70	0.37–1.35	0.291
Chemotherapy	0.372	0.309	1.45	0.79–2.65	0.223
Age	–0.004	0.036	1.00	0.93–1.07	0.908

MPI, mitotic percentage index; PER, posterior fossa radiation; CSI, craniospinal axis irradiation.

(n = 18/35) of patients at 17 years, presenting after 1979 (log-rank = 5.25, P = 0.02).

With regard to sex and age (the latter analysed both as a continuous variable in the Cox regression model and as the subcategories: <2 years, >2–10 years and >10 years in log-rank analysis), no significant influence on patient survival could be demonstrated by univariate analysis (Table 2).

Patients were divided into two groups for the analysis of tumour stage to avoid excessive data sub-categorisation. 36 patients fell into group one, having either T1 or T2 primary disease, versus 22 patients in group two with T3 or T4 tumours. A significantly better prognosis was associted with lower stage disease in univariate analysis (Table 2, P = 0.014).

Radiotherapy also proved to be a significant prognostic factor in univariate analysis, with doses at or above the 'standard' for both PFR and CSI being significantly related to improved survival with P = 0.003 and P = 0.019, respectively (Table 2). However, only borderline significance was demonstrated in analysis of total versus partial surgical excision of primary tumour (Table 2, P = 0.096), while no demonstrable survival advantage was seen in patients receiving adjuvant chemotherapy (P = 0.23).

MPI

Tumour MPI was calculated for all 70 cases and ranged from 0.1 to 5.30% with a mean of 1.96% (Figure 1). The distribution of mitotic activity was random. In particular, no relationship was observed between the location of mitotic cells within sections and other histological features, e.g. necrosis, vascularisation or invading tumour edge. The distribution of mitotic activity also varied between tumour blocks from the same patient. For categorical analysis, patients were divided into three groups covering the range of MPI values calculated: 0–2%, 2–3% and >3%. The Kaplan–Meier survival curve for these three categories is shown in Figure 2. A stepwise decrease in patient survival was observed with increasing MPI. The 25 year (300 months) survival rates for the categories 0–2%, 2–3% and >3% were 46%, 33% and 0%, respectively (log-rank = 28.33, P < 0.0001). Cox univariate analysis of MPI as a cotninuous variable confirmed this significant association with patient outcome, P < 0.0001 (Table 2).

Flow cytometry

55 of the 70 patients had sufficient tumour material for flow cytometry. Of these, 45 yielded satisfactory DNA histograms for cell cycle and ploidy analysis.

The coefficient of variation ranged from 5.5% to 8.0%. Fifty-six per cent (n = 25), 27% (n = 12) and 18% (n = 8) of cases were diploid, aneuploid and tetraploid, respectively. This ploidy distribution is similar to that reported for other series of medulloblastoma [22]. SPF was calculated for all cases and ranged from 4.7% to 35% (mean = 13.5%). No

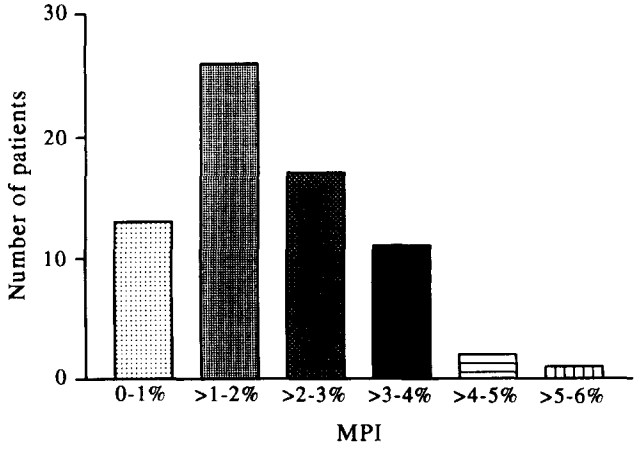

Figure 1. The frequency distribution of MPI scores for the study population.

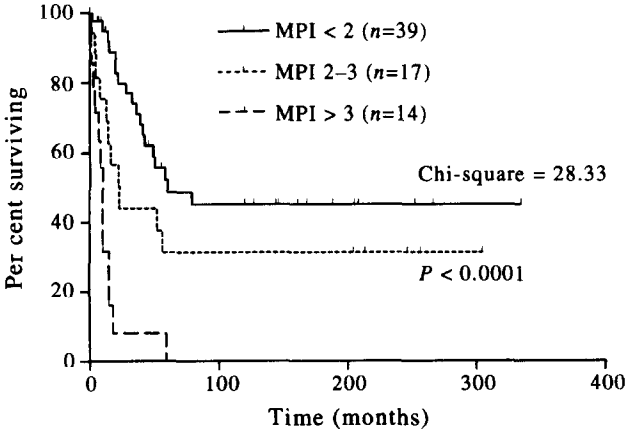

Figure 2. Kaplan–Meier survival curve demonstrating the 25 year survival rates for those patients with MPI scores: <2%, 2–3% and >3%.

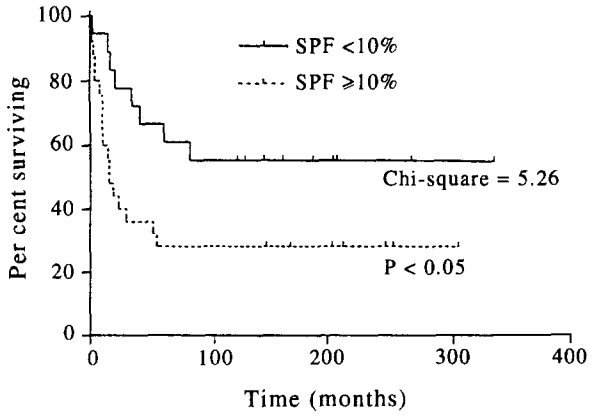

Figure 3. Kaplan–Meier survival curve demonstrating the 25 year survival rates for those patients with SPF of >10 and <10%.

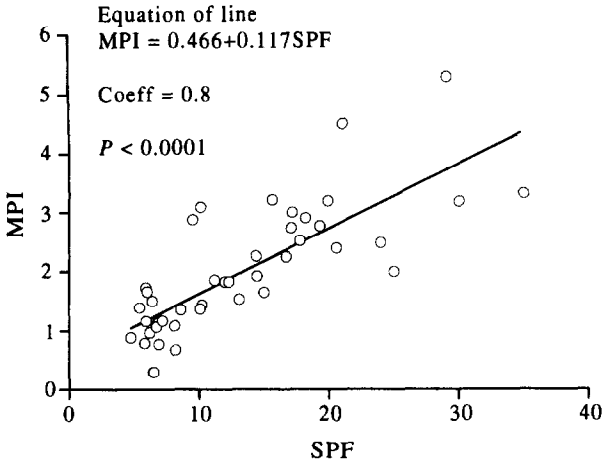

Figure 4. Regression analysis illustrating the significant relationship between tumour SPF and MPI.

significant relationship was observed between SPF and ploidy status. For univariate analysis, patients were again divided into three groups covering the broad range of SPF calculated: 0–10%, 10–20% and >20%. The 25 year survival rate for patients in the categories 10–20% and >20% was virtually identical at 21% and 26%. Therefore, all patients were further analysed as two categories, <10% and >10% SPF. A significant decrease in patient survival was observed in patients with SPF >10% (log-rank = 5.26, $P < 0.05$) (Figure 3). No significant difference in survival was observed between patients with tumours of differing ploidy status.

Multivariate analysis

In order to identify which factors had independent prognostic value, Cox multivariate survival analysis was conducted. The results are summarised in Table 3. MPI proved the most significant independent factor ($P < 0.0001$) with CSI dose and tumour stage also retaining independent prognostic value ($P = 0.003$ and $P = 0.035$, respectively). Interestingly, when all variables are accounted for in the multivariate model, age achieved significance ($P = 0.039$).

Correlation of MPI, SPF and c-erbB-2 oncogene product expression

Previous analysis of c-*erb*B-2 oncogene product expression and clinical disease features in 55 patients with this disease has revealed its independent prognostic significance [25]. However, in the current study (in which the population has been expanded to 70 cases), Cox multiple regression analysis of MPI, SPF and c-*erb*B-2 expression together resulted in MPI alone retaining significance. In order to assess whether this resulted from a close relationship between them, we conducted correlation analysis of these factors. A close relationship was demonstrated between tumour MPI and SPF, indicating it to be a true measure of tumour cell proliferation (Figure 4, $P < 0.0001$). Furthermore, overexpression of the c-*erb*B-2 growth factor receptor was also related to MPI (equation of line, MPI = 1.4 + 0.016, coeff = 0.416, $P < 0.0001$).

DISCUSSION

Medulloblastoma is the most common solid tumour of childhood and continues to present a difficult management problem. Most centres employing the conventional treatment of surgery and radiotherapy with or without chemotherapy report long-term survival rates of only 50–60% with significant iatrogenic sequelea in those achieving cure [1, 2]. Future goals in the management of this disease, therefore, include the identification of patients with a more favourable prognosis, who may be cured with less intensive treatment, and the development of new effective therapies for those patients who will not respond to existing protocols. Such progress in therapy is only likely to come from better knowledge of prognostic factors. To this end, the present study has revealed MPI as a new, rapid and inexpensive independent prognostic factor for childhood medulloblastoma ($P < 0.0001$; Table 3).

The use of mitotic figure counting for the assessment of tumour cell proliferation has been criticised because of the potential difficulty in identifying mitotic figures in fixed tumour material. This has led to the development of more sophisticated and labour-intensive techniques for the quanti-

Table 3. Table showing significant independent prognostic factors in Cox multivariate regression analysis of clinicopathological disease features for the study population

Variable	Coefficient	Standard error	Relative risk	95% confidence intervals for relative risk	*P* value
MPI	0.595	0.166	1.81	1.31–2.51	<0.0001
CSI	−1.332	0.451	0.26	0.11–0.64	0.003
T stage (T1 & 2 versus T3 & 4)	0.791	0.374	2.21	1.06–4.59	0.035
Age	0.108	0.053	1.11	1.00–1.24	0.039

MPI, mitotic percentage index; CSI, craniospinal irradiation.

tation of dividing tumour cells, e.g. immunohistochemical detection of proliferation specific antigens such as Ki-67 and assessment of SPF. The current study does not support the view that such methods are superior to mitotic counting. We have demonstrated that, providing adequate morphological criteria are employed for the identification of mitotic cells [18], and the random distribution of mitotic cells throughout sections is accounted for, accurate information regarding tumour proliferative status can be obtained. In the present study, MPI and SPF scores for individual tumours were closely related (Figure 4) with MPI proving the single most important independent prognostic factor for this series of patients with medulloblastoma.

In addition to a close relationship to SPF, this study has demonstrated a relationship between tumour cell proliferation as measured by MPI, and the percentage of tumour cells expressing the c-*erb*B-2 oncogene product (equation of line MPI = 1.4 + 0.016, coeff = 0.416, $P < 0.0001$). Based on these results, it is possible that the c-*erb*B-2 oncogene growth factor receptor may play a role in the deregulation of normal mitogenic signal transduction in this malignancy. We are currently undertaking a number of studies to investigate this hypothesis further.

CSI dose ($P = 0.003$), tumour stage ($P = 0.035$) and patient age ($P = 0.039$) also proved of independent prognostic significance in this serires of patients. Until recently, the importance of craniospinal radiotherapy dose in the treatment of patients with medulloblastoma was uncertain. However, results from the now completed SIOP II trial clearly show a reduced survival for patients receiving lower doses [2]. The present study is in keeping with these results, emphasising the need for adequate (35 Gy) CSI therapy.

Recent studies have also demonstrated the prognostic significance of disease stage as defined by the Chang TNM score in medulloblastoma [2, 4]. The current study confirmed the independent prognostic significance of the T score (Table 3). However, because of the lack of detailed neuroaxial imaging for the majority of patients, it was not possible to analyse metastasis score as a prognostic factor. It is likely that the M score is of prognostic significance in the current population and may well influence the pattern of factors demonstrating independent significance in multivariate survival analysis. This emphasises the need for routine detailed imaging on all patients with this disease to allow both accurate prediction of prognosis and efficient assessment of potentially new factors. The need for rigorous statistical analysis of potential prognostic factors is also highlighted by the analysis of 'patient age at diagnosis' for the current study population. This variable failed to show prognostic significance in univariate analysis (Table 2, $P = 0.908$), but achieved independent prognostic significance when all factors were accounted for in multivariate analysis (Table 3, $P = 0.039$). Multivariate studies of large patient populations are required if we are to confirm MPI as an important prognostic factor and identify further reliable indicators of survival for this disease.

Finally, none of the other factors analysed in this study, including tumour ploidy status, demonstrated prognostic significance. Previous studies of tumour DNA content and patient outcome in this disease have produced conflicting results [4, 21–23]. In those studies, reporting a significant relationship between survival and DNA ploidy, diploid tumours tended to carry a worse prognosis. No such relationship was observed in our study.

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