ASTROCYTOMAS AND PROGNOSIS- FROM MORPHOLOGY TO TUMOR BIOLOGY

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ABSTRACT

Twenty-seven cases of Astrocytoma were studied to assess the role of a newly introduced proliferation marker-Proliferating Cell Nuclear Antigen (PCNA) in improving prognostic accuracy in comparison to traditional histologic methods like grading. The study revealed a direct correlation between grading and PCNA expression as determined by labelling indices (LI). A 25% PCNA LI separated low and high grade tumors. The difference between PCNA LI's of patients who were alive and those who were dead at the end of the study was statistically significant. However, in this study with limited follow-up, statistically significant relation to survival and recurrence could not be established. The study introduces a new method of assessing tumor biology that enables objectivity in prediction of tumor behaviour.

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KEY WORDS: Astrocytoma; PCNA; Proliferation markers.

Introduction

Astrocytic tumors are the most common primary intracranial neoplasms of the mature nervous system. In the new WHO classification [1] the first category comprising at least 75% of the astrocytic tumors includes the astrocytoma, anaplastic astrocytoma and glioblastoma multiforme. These define a spectrum of increasing anaplasia. The second category consists of pilocytic astrocytoma, pleomorphic xanthoastrocytoma and subependymal giant cell astrocytoma with distinctive clinicopathologic features and a more favourable prognosis.

In astrocytic tumors, as in other tumors, assessing the growth potential and degree of anaplasia is important for planning treatment modalities and also to predict survival. Clinical and pathological features such as age, functional status on presentation and histological grade can assess behaviour of these tumors. At present histological grading of astrocytomas is done by two main systems-WHO (1979) and Daumas-Duport System (also called St Anne-Mayo system) [2,3] which have shown significant correlation with patient prognosis. Accuracy and reproducibility of grading are, however, compromised by the subjective histologic criteria used, especially of mitosis [4].

The major challenge in astrocytomas remains the evaluation of an objective prognostic criterion to predict their biological behaviour. During the past dec-

ade, improvements have occurred in histopathologic criteria with introduction of immunohistochemical techniques that assess their growth potential. These detect proliferation markers such as cell cycle associated proteins Ki-67, proliferating cell nuclear antigen (PCNA), MIB-1 etc [5].

Proliferating cell nuclear antigen (PCNA) is a DNA polymerase delta auxiliary protein, which shows an increased expression in proliferating cells [5] and in many tumors including CNS tumors [6], holds promise as an independent prognostic marker. This study was undertaken to compare the conventional method of grading of astrocytomas in H & E stained sections and immunohistochemical staining of PCNA as a proliferation marker. The aim was to establish the role of PCNA as an adjunct to the existing histological grading criteria in objective assessment of the proliferative potential of these tumors and thus help in predicting the biological behaviour of the tumor and assess patient survival [6,7]. Hence the study was designed a) to study astrocytomas of the CNS with reference to the existing grading system on routine haematoxylin and eosin sections, b) to find the expression of PCNA in the astrocytomas under study by immunohistochemical staining techniques, c) to establish a relationship between morphological grade and PCNA Labeling Index and d) to correlate the outcome of grading and PCNA expression with prognosis where possible and indicate the utility of PCNA detection in these.

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Material and Methods

The present study was conducted in the Department of Pathology, AFMC, Pune from surgical pathology material of 27 patients of astrocytomas admitted and treated at the Malignant Diseases Treatment Center, Command Hospital (SC) Pune between 1987-1997. Patients diagnosed as astrocytoma were selected at random. Cases without any clinical details, all cases of mixed gliomas, and all patients who died within one week after surgery were excluded.

Clinical variables

These included age, sex, presenting signs and symptoms, tumor location, type of surgery, degree of removal of the tumor, details of post-op radiotherapy and/or chemotherapy and post op CT scan status (where done). The patients were followed up till the end of the study period and details about tumor recurrence, treatment offered and the period of survival were also collected. Detailed reports of deaths which were autopsied were also studied.

Final outcome at last follow-up was recorded as alive without disease, alive with disease and died due to disease.

Histological evaluation

All slides of the 27 cases were retrieved and reviewed for confirmation of diagnosis and study of all available material to ensure adequate representation. The sections were examined by:

(i) *Haematoxylin and Eosin stain*: The diagnosis of astrocytoma was confirmed based on standard morphological criteria [1]. Immunohistochemical staining for glial fibrillary acidic protein (GFAP) was utilised, where required, for specific diagnosis of astrocytoma especially in high-grade tumors. The tumors were graded using the Daumas-Duport system [3] into grade I (pilocytic), II, III and IV taking into consideration presence or absence of nuclear atypia, mitosis, endothelial proliferation and necrosis. In this study the number of mitosis per 10 high power fields (HPF) was also recorded in the most mitotically active area of the tumor, in addition to recording their presence or absence.

(ii) *Immunohistochemical staining*: Immunohistochemical staining using monoclonal antibodies for proliferating cell nuclear antigen (PCNA) was performed on two representative slides of each case, showing maximum tumor area with minimal necrosis. Briefly, paraffin sections, 3-5µ thick, taken on Poly-L-Lysine coated slides were brought down to water and endogenous peroxidase was destroyed by 3% H₂O₂ in methanol. Antigen retrieval was done by heating in a microwave oven. Sections were treated with blocking serum to reduce non-specific binding of secondary antibody and incubated with primary monoclonal antibody, (PCNA Ab-1, clone PC10, Neomarkers) diluted 1:100 in Tris-buffered saline, overnight in a moist chamber, at 4°C. Next day, sections were incubated sequentially with secondary antibody and streptavidin-biotin complex. Diaminobenzidine (DAB) was used as chromogen and staining monitored under the microscope. Sections were counterstained with Harris haematoxylin, cleared and mounted. A section of tonsil was put up with each batch as positive control. For negative control the primary antibody was omitted in the staining steps. Diffuse and granular staining of nuclei was taken as positive.

Counting was performed at high magnification (X 40) and a minimum of 1000 cells in different fields or minimum 10 fields were analyzed. Marginal zones, section edges, vascular components and hematogenous cells were excluded. The PCNA Labeling Index (LI) [8] was calculated as a percentage of cells showing positive staining of nuclei.

Analysis

i) Analysis of clinical features at presentation.

ii) Histological grading on H & E stained sections was correlated with PCNA-LI and both histological grading and PCNA-LI were correlated with the survival of the patients.

Statistical analyses were conducted using a computer based statistical programme "Statistica". The correlation between PCNA LI and histological grade was established with a scatter plot, finding the correlation coefficient between the two.

Probability value (p-value) was calculated using Student's t test. A p value of less than 0.05 was taken as significant.

Results

Clinical Features

The cases ranged in age from 05 to 68 years. The mean age of lower grade astrocytomas (I-II) was 31.0 years and for higher grade (III-IV) it was 50.78 years. The difference between the two was highly significant ($P=0.0003$). There were 12 females and 15 males with a male to female ratio of 1.25:1. Patients presented with localising signs and symptoms like motor and sensory deficits and non-specific neurological signs. The duration of symptoms was 145 days for Grade I-III lesions and 35.5 days for high grade lesions. The site of tumor was supratentorial (85%), infratentorial (7%), intramedullary (4%) and multiple site (4%). All patients had preoperative CT Scan for confirmation of diagnosis and tumor location and a postoperative CT scan for residual disease. Primary surgery was done in all cases and surgery for recurrence in 6 patients. Post-op radiotherapy and chemotherapy were given to the patients based on post-op residual tumor and the grade of the tumor.

Follow-up and survival

The range and mean of follow up period with the final outcome is shown in Table-1. Two cases having a survival of 5 and 9 years were excluded from calculations as they deviated markedly from the mean. During follow up, recurrence occurred in 7 patients and six of these were re-operated.

TABLE 1
Final outcome and survival

Final status	No. of patients	Survival-range (mean)
Alive without disease	06	
Alive with disease	11	04-108 months (10.87)*
Died due to the disease	07	05-60 months (09)*
Lost to follow up	03	

*One patient each, surviving 108 and 60 months respectively, excluded from calculations

Histological analysis

A total of 232 slides of the 36 specimens (including surgical specimens at first operation, operated recurrences and autopsy tissues in two patients) were retrieved and reviewed for confirmation of the diagnosis of astrocytoma. In three cases of high-grade astrocytoma immunohistochemical staining for glial fibrillary acidic protein (GFAP) was done to confirm their astrocytic origin.

Classification

There were 26 cases of ordinary astrocytoma (including one gemistocytic) and only one case of pilocytic astrocytoma.

Fig. 1 : Grades of astrocytoma

Fig. 2 : Survival of all grades

Histological grade

The number of patients in each grade by the Daumas-Duport system [3], were as shown in the figure (Fig-1). The tumor recurred in three Grade IV lesions and in three Grade II lesions. All cases recurred as Grade IV. Grade I and II tumors showed no mitosis while they numbered 5 and 12 per 10 HPF in Grade III and IV tumors respectively ($p=0.34$).

Histological grade and prognosis

Mean survival of patients in each grade is as shown in Fig-2. The difference in survival between low (Grade I and II) and high grade (grade III and IV) was not significant ($p=0.06$). However this has to be interpreted with caution keeping the low numbers of Grade I [1] and Grade III [2] cases in mind.

PCNA evaluation

Immunohistochemical staining showed nuclear staining of

Fig. 3 : A grade II astrocytoma showing moderate cellularity and mild pleomorphism with occasional nucleus staining for PCNA (arrow) (PCNA x 400)

Fig. 4 : A grade IV astrocytoma showing an area of necrosis with pseudopallisading by PCNA positive neoplastic astrocytes (PCNA x 100)

varying intensity of brown with DAB. The pattern was diffuse and granular with a few cells having large nucleolus like clumps. Positive nuclei were found to be evenly distributed in low-grade tumors (Fig-3). High-grade tumors showed a more heterogeneous spread of PCNA positive nuclei (Fig-4). Cells in mitosis also showed positivity. Normal brain did not show any PCNA staining. The infiltrating edge of the tumor showed PCNA positive nuclei interspersed with normal brain cells. Proliferating endothelial cell nuclei also showed PCNA positivity. Giant cells stained positive but gemistocytes were negative.

PCNA and histological grade

PCNA LI ranged from 0.4-72%. The H & E grade and PCNA labeling index were compared using a scatterplot (Fig-5) and a correlation coefficient of 0.84 was found between the two values, a value of 1.0 indicating perfect correlation. The relation of grade to PCNA LI is as depicted in Table-2. The difference between PCNA LI of low (grade I and II) and high (Grade III and IV) grade as well as between grade III and IV tumors was statistically significant ($p<0.001$ and $p=0.003$ respectively). The increasing expression of PCNA from low grade to high grade astrocytoma is shown in Figs. 3 and 4.

PCNA and prognosis

A PCNA LI cut-off value of 25% was taken after studying the scatterplot (Fig-5). It was noticed that majority of the values of

Fig. 5 : PCNA vs grade

TABLE 2
Grade and PCNA LI

Grade	PCNA LI Range (mean)	p value : between LI of mentioned grade	p value between LI of low/high grade
I	1.3		
II	0.4 - 27 (10.76)	I-II 0.44 (NS)	
III	12.6 - 14.6 (13.6)	II-III: 0.74 (NS)	< 0.001 (S)
IV	10.75 - 72 (45.02)	III-IV: 0.003 (S)	

Note : NS - not significant, S - Significant

PCNA LI grades I, II and III were below this value, whereas grade IV showed majority of the values above this level. The mean survival of patients with low PCNA LI (25.18 months) was higher than those with a high PCNA LI (9.15 months). However this difference in survival was not found to be statistically significant ($p=0.08$).

The PCNA LI was compared between various groups with different outcomes till last follow up. The difference between the mean PCNA LI of patients who were alive without disease (11.88%) and alive with disease (24.53%) was not significant ($p=0.24$). However, the difference between the PCNA LI of patients who were alive at the end of follow up (with or without disease) and those who died (41.18%) was significant ($p=0.03$).

PCNA and recurrence

Although it was found that the mean PCNA LI at diagnosis was higher in patients with recurrence (30.21%) compared to patients without recurrence (11.88%), the difference between these values was not significant ($p=0.14$).

Discussion

Astrocytomas, constituting 35-50% of all intracranial neoplasms form an important category of tumors of the nervous system [1]. As in all categories of malignant tumors, the emphasis in this group too is shifting from morphologic classification to objective pre-

dictions of tumor biology and growth. Currently, estimates of tumor behaviour rely on symptom duration, location and extent on part of the clinician [9] and on the classification [1] and grading systems [2,3] on part of the pathologist. In this scenario the introduction of proliferation markers holds promise in aiding the clinician in both therapeutic decisions as well as prognosticating outcome. We tried to analyse the group of tumors under study in terms of the conventional markers of prognosis referred to above with special emphasis on the proliferative potential.

Astrocytomas can affect any age group ranging from infancy to old age with a mean age ranging from 46 to 53.5 years [3,9,10], and our cases with a mean age of 41.26 years, were similar in distribution. Behaviorally, benign astrocytomas are known to occur earlier than malignant astrocytomas [10] and the same held true in our study also, the average age of lower grade astrocytomas (I-II) was 31.0 years and for higher grade (III-IV) it was 50.78 years, the difference between the two being highly significant. The reported male predominance [9] was also seen in this study.

Though neurological signs and symptoms help in localising the lesion, it is the duration of symptoms that determine tumor behaviour- being longer for low-grade tumors and shorter for high-grade tumors. Our study showed the same findings, even though the difference was not statistically significant.

Since all but one of our cases fell in the ordinary astrocytoma group no attempt was made to correlate histologic type with prognosis. There was only one case each of pilocytic and gemistocytic astrocytoma so no attempt was made to compare their survival with the others though these two categories are known to connote better outcomes [1].

Over the years a variety of grading systems evolved for use in studies of astrocytomas from Bailey and Cushing's emphasis on morphologic types, Kernohan's three-tier system, Ringertz grading, the WHO grading system and more popular currently, the Dumas-Duport system [1,3]. The usefulness has been amply demonstrated in a large series by Kim et al [10] who found the following incidence of the various grades of astrocytoma : grade I (0.4%), grade II (14.34%), grade III (13.14%), and grade IV (72.11%) in 251 patients. On the other hand Wang and Ho [11] found the incidence of the same grades to be 9.2%, 26.2%, 36.9% and 27.6% respectively. These figures highlight the varying incidence of various grades in different studies. The incidence was grade I (3.7%), grade II (44.44%), grade III (7.4%) and grade IV

(44.44%) in our study. The number of grade III tumors was low compared to other studies which could be due to difference in objective assessment of mitosis, as this is the only factor differentiating grade II and III. Coons and Pearl found significant independent correlation between survival and the field in which the first mitosis was found in grade III astrocytomas [12] suggesting that the evaluation of mitotic activity offers more prognostic information than can be obtained by noting only their presence or absence. Numbers of mitosis in our study were statistically different between grades III and IV.

The survival of patients with astrocytomas shows wide variation depending on various factors. Nakamura found a median survival of 56 months with a range of 13 to 139 months [13]. Patients with grade I-II tumors have varying reported median survival time of 57 months [14] to 108.6 months [15] in different studies. Similarly grade-III tumors had an average survival of 10 months [14] to 39.4 months [15]. Glioblastoma multiforme (Grade IV) has the most dismal outlook with average survival of 05 months in one study [14]. In our series the mean survival of grade-I to grade-IV was 13, 12.75, 9.5 and 8.5 months respectively, with a range of follow up of 04 months to 09 years. Extraordinary long survival of two patients, of 05 and 09 years, was excluded from calculations of mean survival to prevent skewing of the data.

No significant difference was found between the mean survival of individual grades and between low (grade I and II) and high (grade III and IV) grade tumors in our study. This is not in keeping with earlier studies which have shown that grade reliably predicts survival in astrocytoma patients [15,16]. The lack of significant difference in survival in the present study could be due to the short follow up available for most of the patients and the low numbers in Grade I and Grade III tumors.

Molecular factors that explore cell kinetic measurements and the percentage of cells in cell cycle are now the focus of prognostic studies in astrocytomas. They include flow cytometry [9], molecular genetic markers like p53, MDM2 and EGFR [13,17] and proliferation markers. Proliferation studies in astrocytomas gained momentum with the pioneering work of Hoshino and Wilson [18] in 1979 who used tritiated thymidine to detect cells in the cell cycle and demonstrated that labelling indices correlated histological grade. These observations were followed subsequently by use of other cell-cycle markers like Ki 67, MIB-1 and Proliferating cell nuclear antigen PCNA [19].

PCNA is a non-histone auxillary nuclear protein, an acidic polypeptide, with a molecular weight of 36 kD and is highly conserved in evolution [19,10]. PCNA is increased only slightly in most G₁ cells as compared to resting G₀ cells. In late G₁ cells PCNA expression increases and continues to increase during the DNA synthesis (S) phase of the cycle. In G₂-M phase PCNA content declines to a value intermediate between those of G₁ and S-phase cells [21]. Numerous antibodies reactive with PCNA have been generated e.g.: anti PCNA clone 19A2, and PC-10. PCNA positivity on paraffin embedded sections can be studied through immunohistochemistry using these antibodies. The utility of PCNA labelling indices (LI) has been proved in various studies of cell cycle in assessing the proliferating potential of various tissues using immunohistochemical methods and flow cytometry methods.

In our study a positive relationship between PCNA LI and histological grade with a correlation coefficient of 0.84 could be established. This relation has been seen in other series with large number of cases and utilising other proliferation markers as well [7,16,20,22,23]. The range of PCNA LI is variable, from 0.00-30.96% in some studies [20,23] to 0.1-95% in others [7,16,22]. Our range of 0.4-72% was comparable with the second group. This variability in expression may be due to expression of different epitopes by the PCNA antibodies selected in different studies [23]. A cut-off value of 25% was obtained in our study derived from a scatter-plot of PCNA LI vs Grade to separate low from high grade tumors. In other series this has ranged from 6-50% [24,25].

Technical considerations observed important in our study on PCNA labelling included selection of blocks containing maximum representative tissue with minimal necrosis, counting a minimum of 10 HPF, control of staining intensity to prevent variability in observations and exclusion of positively staining nuclei of proliferating endothelial cells in the counting procedure. In addition, good fixation and accurate counting [26] need to be ensured. One or more of these factors have been pointed out as important in obtaining standardised results [7].

In an attempt to separate out prognostic categories on the basis of PCNA LI we assessed the different grades of tumors and observed significant difference in values between low (I+II) and high (III+IV) grade tumors and between grade III and IV tumors. However no significant difference was found between grade II and III which could have affected treatment. Such overlap of values have been seen by others as well [27].

In predicting the outcome we could establish a statistically significant difference of PCNA LI for those who were alive (with or without disease) and those who had died at the end of the study period ($p=0.03$). However, difference in survival between low PCNA LI group (25.18 months) and high PCNA LI group (9.15 months) was not significant. While a few studies say PCNA loses significance when compared in multivariate analyses to age, grade and type [23,25] several studies have shown a significant association with prognosis [16,22].

Prediction of recurrences could be a useful asset to the treating team and a few studies have found a high LI in cases that recurred [28]. We observed this trend in our six cases but could not establish significance because of the overall short follow-up of all cases. In childhood gliomas which are conventionally low grade and believed to be a good prognostic group no difference has been found in PCNA results as compared to adults [29]. In our study only two cases were included hence could not be analysed.

In conclusion, it can be said that PCNA immunostaining is simple, applicable to paraffin-embedded material and objective in interpretation. It correlates well with grading and can be introduced easily in routine surgical pathology reporting on astrocytomas. It serves to improve prognostic accuracy and understanding of tumor biological behaviour being a predictor of increased aggressiveness, recurrence and reduced survival. Recent works on a large series of cases [17,30] have established convincingly the need to look beyond classifications and grading towards molecular markers of cell biology to make pathology reports on such tumors prognostically viable in the next millennium.

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