Value Of Proliferative Cell Nuclear Antigen Immunohistochemistry And The AgNOR Method For Preoperative Histological Grading Of Glial Tumors

Glial Tümörlerin Preoperatif Histolojik Derecelendirmesinde Proliferatif Hücre Nükleer Antijeni İmmünositokimyası ve AgNOR Yönteminin Yeri

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Abstract: Extended short forms again here – same in the key words so please modify according to your journal's style the aim of this study was to determine whether or not assessing cell kinetics by argyrophilic nucleolar organizer region counting, and using immunohistochemistry to detect proliferative cell nuclear antigen are valuable methods for predicting the biological behavior of glial tumors. Specimens were obtained from 17 patients with glial tumors using stereotactic procedures, and smears of the tissue were made. For each smear, we investigated for antibodies to proliferative cell nuclear antigen (PC10, DAKO) using the streptavidine peroxidase method, and also counted the number of argyrophilic nucleolar organizer regions using a specific staining technique to identify these. Of the 17 tumors, 3 were grade I pilocytic astrocytomas, 3 were grade II fibrillary astrocytomas, 1 was a grade II oligodendroglioma, 4 were grade III anaplastic astrocytomas, and 6 were grade IV glioblastomas. We analyzed each case based on independent assessment of the proliferative cell nuclear antigen labeling index, argyrophilic nucleolar organizer region count, histological type, and histological grade, and then compared the results statistically. Although the proliferative cell nuclear antigen labeling index and argyrophilic nucleolar organizer region count both increased in parallel with histological grade, we found that the only statistical difference between low-grade (grades I and II) and high-grade glial tumors (grades III and IV) was the groups' argyrophilic nucleolar organizer region counts. The findings indicated that the proliferative cell nuclear antigen labeling index is not a good prognostic indicator in the preoperative histological evaluation of glial tumors, and that argyrophilic nucleolar organizer region counting can be used to differentiate benign tumors (grades I-II) from malignant ones (grades III-IV).

Key Words: AgNOR method, glial tumors, proliferative cell nuclear antigen, immunohistochemistry, stereotactic biopsy

Özet: Bu çalışmanın amacı, hücre kinetiğini değerlendirme yöntemlerinden biri olan AgNOR sayımının ve proliferatif hücre nükleer antijeni (PCNA) immünositokimyasının, glial tümörlerin biyolojik davranışlarını öngörmede yeri olup olmadığını saptamaktır. Stereotaktik işlem yoluyla glial tümörlü 17 hastadan materyelleri elde ettik ve dokulardan yaymalar hazırladık. Her bir yaymayı, streptavidin-peroksidaz yöntemiyle antiproliferatif hücre nükleer antijeni (PC10, DAKO) ve AgNOR sayma yöntemini uygulayarak, inceledik 17 tümörün, üçü derece I pilositik astrositoma, üçü derece II fibriler astrositoma, biri derece II oligodendroglioma, dördü derece III anaplastik astrositoma ve altısı derece Iv glioblastoma idi. Her bir vaka için, PCNA bağlanma indeksi, AgNOR sayımı, histolojik tip ve histolojik dereceyi ayrı ayrı analiz ettik ve sonuçları istatistiksel olarak kıyasladık. PCNA bağlanma oranları ve AgNOR sayımları histolojik dereceye paralel artış gösterse de, istatistiksel olarak farklılığı sadece, AgNOR sayımı açısından, düşük dereceli (derece I ve II) ve yüksek dereceli (derece III ve Iv) glial tümör grupları arasında tespit ettik. Sonuç olarak, glial tümörlerin histolojik derecelendirmesinde PCNA oranının bir ölçüt olarak alınamayacağına, AgNOR metodunun ise selim tümörleri (derece I-II) malign (derece III-Iv) olanlardan ayırmakta kullanılabileceğine karar verdik.

Anahtar Kelimeler: AgNOR, glial tümörler, prliferatif hücre nükleer antijeni, immünositokimya, stereotaktik biyopsi

INTRODUCTION

Many features are used to predict clinical prognosis in patients with glial tumors, including histological type of tumor, histological grade, anatomic sites involved, tumor size, encroachment on the ventricular system, extension across the midline and invasion of the contralateral cerebral hemisphere, extension to the contralateral side of the tentorium cerebelli, patient age, Karnovsky score and others. Two main features used to predict the biological behavior of glial tumors are histological type and grade (16), but the behavior of a tumor does not always match its histological grade, a label that is based solely on morphology (9). Possible explanations for this include the remarkable histological heterogeneity of glial tumors, the fact that the assigned histological grade (especially that of small biopsy samples) may not represent the whole tumor, and the progression of tumor malignancy that occurs over time (32,31). In efforts to improve our ability to prognosticate and to overcome the limitations of traditional histopathological evaluation, research has focused on adapting knowledge and advances in cell kinetics and molecular genetics for use in diagnostic neuropathology (8).

Although an objective, cost-effective, easily applicable and reliable method has yet to be developed, it has been suggested that of tumor proliferation rates can be used to determine histological grade and predict biological behavior (24,27,38). Options for measuring such rates include bromodeoxyuridine (BrdU), Ki-67 and proliferative cell nuclear antigen (PCNA) labeling. The use of BrdU requires intraoperative injection of this halogenated pyrimidine, whereas monoclonal antibodies Ki-67 and PCNA can be applied to frozen sections and to paraffin-embedded tissue.

PCNA is a 36 kD nuclear protein that is an auxiliary protein of DNA polymerase delta, and takes part in DNA synthesis and repair (43). It is detectable in interphase, begins to rise in the middle of G1 phase, is threefold higher at the G1/S transition than in interphase, and remains elevated during G2 phase and mitosis (43,49). It follows that the proportion of cells marked by anti-PCNA reflects the proliferation rate of a tumor (29).

Another means of assessing proliferative activity in tumors is the argyrophilic nucleolar organizer region (AgNOR) method, which is a fairly simple technique based on the detection of colloidal silver. The process involves staining an acidic phosphoprotein of nonhistonic structure that exists in the nucleus together with DNA coils that carry RNA genes. The number and shapes of AgNORs can be assessed under light microscopy, and the quantity of these figures rises in parallel with increased proliferative and metabolic activity (13).

MATERIAL and METHOD

Material for histopathological evaluation was obtained through image-guided stereotactic biopsy (Leksell stereotactic system, Elekta AB, Stockholm, Sweden) of 17 patients with glial tumors. Five to eight biopsies of each tumor were obtained, and smears from these were prepared and stained with hematoxylin and eosin. Histopathological evaluation was done according to the World Health Organization's 1993 classification of tumors of the central nervous system. The series used in the study included three pilocytic astrocytomas (grade I), three fibrillary astrocytomas and one oligodendroglioma (grade II), four anaplastic astrocytomas (grade III) and six glioblastomas (grade IV).

For PCNA assessment, anti-PCNA (PC10, DAKO) was applied to smears fixed in concentrated ethyl alcohol using streptavidine peroxidase. Diaminobenzidine tetrahydrochloride was used as a chromogen, and Mayer's hematoxylin was used for counterstaining.

For AgNOR staining, the first phase involved submersing the slides in a solution of one part ethyl alcohol and two parts concentrated acetic acid for 3 minutes. This was followed by a wash in deionized water. Next, the specimens were bathed in working solution and shielded from light for 20 minutes, after which they were again washed with deionized water. The working solution was made up of two parts solution A and one part solution B, with solution A containing 50 g silver nitrate in 100 ml distilled water and solution B containing 2 g of gelatin and 1 ml of formic acid in 100 ml distilled water. In the final phase, the slides were placed in 5% sodium thiosulphate, washed in distilled water for 1 minute, dehydrated in absolute ethyl alcohol, cleared in xylene, and mounted with mounting medium (Canada balsam).

The nuclei that stained in the anti-PCNA and AgNOR methods were counted under the light microscope using 10x40 and 10x100 fields of

magnification, with 20 different sections of the slide being counted for each case (approximately 2,000 cells). The staining in all cells marked with anti-PCNA was limited to the nucleus, was diffuse or granular in character, and was of varying density. Density was not taken into account in our assessment, so any nuclei that were labeled with diffuse or granular staining were considered positive for PCNA (Figure 1). Regarding AgNOR staining, since nucleolar and prenucleolar AgNOR spots cannot be counted individually, each group as a whole was accepted as one spot (Figure 2). For each case, the percentage of PCNA-labeled cells (as PCNA labeling index) and the average AgNOR count were determined separately.

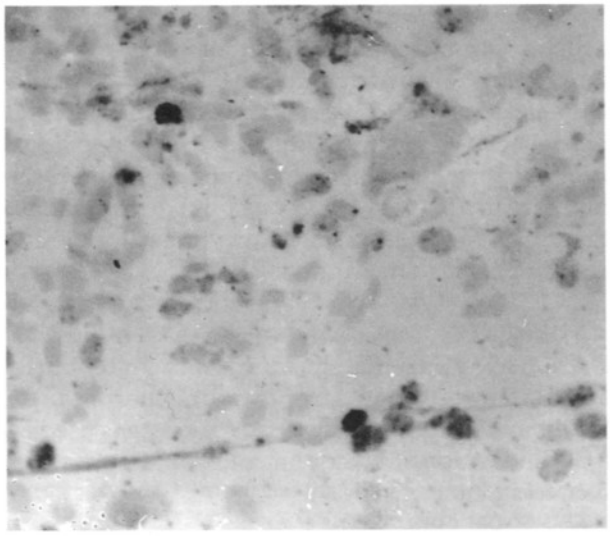

Figure 1: PCNA immunohistochemical staining in a smear from one glioblastoma case. Approximately 25% of the malignant cells are stained. (400 X)

The data obtained were grouped according to the specimens' histological grades. The results were evaluated using nonparametric variance analysis (Kruskal Wallis test). We used the Mann Whitney U-test to make comparisons among all groups, with a significance limit of 0.008, and used the student's t-test to compare benign (grade I and II) and malignant (grade III and IV) glial tumor groups, with a significance limit of 0.05.

RESULTS

Our findings showed that the PCNA labeling index and AgNOR count rise in parallel with histological grade (Table I). However we found that the largest ranges overlapped to various degrees for both PCNA labeling index and AgNOR counts.

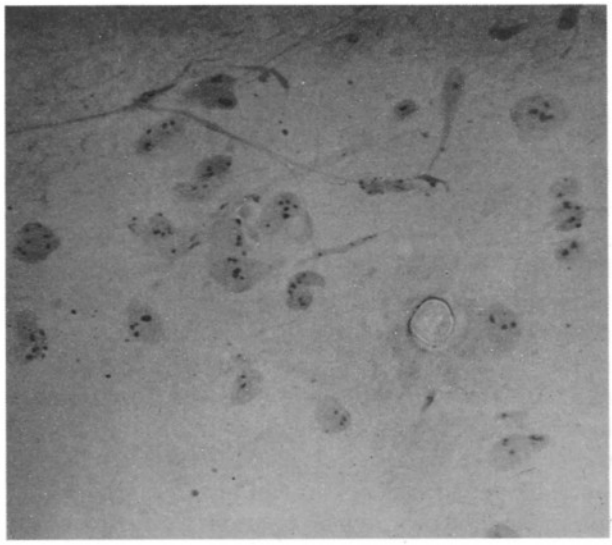

Figure 2: AgNOR preparation of a smear from one glioblastoma case that had two to seven (mean, five) spots in the nuclei of the malignant cells. (400 X)

Table I: Histological type, grade, PCNA labeling index and AgNOR count for the 17 cases.

Grade AgNOR count
Abbreviations list – PCNA, AgNOR

When specimens were compared according to histological grade, there were no significant differences in AgNOR counts and in PCNA labeling indices among the four groups (p:0.0219, Mann Whitney U-test). When the tumors were paired as low-grade (I and II) versus high-grade (III and IV) and analyzed for differences in PCNA labeling indices and AgNOR counts, only the AgNOR counts were significantly different (p:0.004, student's t-test).

DISCUSSION

Studies of PCNA labeling indices for glial tumors according to histological grade have revealed extremely wide ranges with considerable overlapping. The reported figures are 0.00-8.3% for grade I (23,34,37), 0.00-32.7% for grade II (1,19,23,37),

0.04-86.3% for grade III (1,19,23,34,37,40) and 2.7-86.3% for grade IV (1,19,23,37,40). Similarly, the mean indices calculated for each histological grade by different laboratories have varied widely. The inconsistency of these PCNA results has made it impossible to establish limits that separate cases into groups that reflect histological grades or biological behavior.

Another point of interest is that some researchers have suggested that PCNA labeling indices for astrocytoma and glioblastoma far exceed 50% (23,37,40). Although kinetic studies on glioblastomas have found the proliferation rate for almost all these tumors to be below 50% (16), there is still question as to what the reported high PCNA labeling indices truly reflect.

There are also other questions surrounding the accuracy of this index. Experimental investigations involving rats with PCNA in their CHOK1 cell series have shown that up to 10% of the cells leaving the cell cycle and entering G0 are positive for PCNA (6). This indicates that approximately 10% of the cells that stain for anti-PCNA at interphase, which, to date, have contributed to the proliferation rate figure, should actually be considered normal.

A study that compared BrdU and PCNA findings showed that the PCNA labeling index was 2.2 times greater than the BrdU labeling index ± 0.8 (27). Such high values for PCNA in this and many other studies may reflect the differences between the methods which used, and may not represent the true cell proliferation rate. The authors of this particular study stated that the PCNA labeling index does not reflect histological grade and is not a reliable value. They focused on problems inherent in the technique investigators have used to derive this figure (19,21,46).

In order to eliminate problems associated with this method, various groups have examined different types of fixative, different fixation times, and the effects that microwave rays have on tissue fixed with different fixatives. Investigators have found that tissues fixed with 10% buffered formalin yields proliferation rate results closest to those obtained with Ki-67 (12,48). Some authors have suggested that the length of the fixation period does not affect results (42), while others have bracketed the most appropriate fixation time between 6 and 30 hours (44). Casasco et al. suggested that the structure of PCNA in tissues fixed in formalin deteriorates at an increasing rate after 3 hours, and stated that alcohol-based fixatives should be used (10). It has also been proposed that a mixture of 4% paraformaldehyde and 5% glutaraldehyde is suitable fixative for PCNA immunohistochemistry, that digestion be accomplished using 0.001% pepsin (14), that methanol be used for fixation (7,29), and that fixation be accomplished with periodonate-paraformaldehyde (33).

With regard to fixing specimens, the best results have been obtained using 15 minutes of acetone fixation followed by 15 minutes of methanol fixation (4). After optimal fixation has been achieved and before the immunohistochemical procedure, some authors have proposed that tissue sections should be irradiated in microwave oven (at 600 W to maximum power). The various suggested ways of accomplishing this include irradiating in distilled water for 2x5 minutes (15), in 0.05 M glycine-HCl buffer (pH 3.6) containing 0.01% EDTA for 2x5 minutes (18), in 1% zinc sulphate for 7.5 minutes (41), and in citrate buffer solution (pH 6) for 10 minutes (20,45).

Clearly, the variety of methods listed for all these steps shows there is still no standardized technique for anti-PCNA labeling. Still, despite the problems associated with determining PCNA properties, the literature discusses the merits and possible applications of the technique. Korkolopoulou and colleagues suggested that the PCNA labeling index could be used separate from histological grade (24). Also, Vigliani et al. proposed that labeling indices of greater than 5% allow the identification of subgroups of benign tumors that will begin to exhibit anaplastic behavior but which, to date, have not been recognized in advance (47). Kim and associates claimed that recurrence can be predicted for glioma groups of each grade by looking at the PCNA labeling indices of tumor cells at the resection border (22). Miyake et al. found that up to 5% of the cells in the reparative tissue followed brain injury, were labeled with PCNA (30). Although this rate was calculated on the third day of wound healing, it is still significant because it indicates high levels of PCNA positivity in areas of gliosis. Other researchers have reported similar findings (28,39).

Some studies have revealed that PCNA immunopositivity rate of up to 4.5% in slow virus infections of the central nervous system might occur (2,5,26). Thus, there is suspicion about the 5% PCNA positivity limit. The foundation of these authors'

views is the hypothesis that anti-PCNA labeling in the nontumoral brain tissue should be ignored only if it is extremely low. The error in this hypothesis has been clearly proven by other studies.

Only a few studies in the literature discuss the use of PCNA labeling for smears (17,35). One of these reports (35) compared results with those from Ki-67 experiments, and found PCNA labeling to be sufficient for determining proliferation rate.

With regard to AgNOR count, although some studies state that this method can be used to determine the histological grade and biological behavior of glial tumors (2,11,34), others have claimed just the opposite (3,11,25,34,36).

In conclusion, we believe that PCNA immunohistochemistry is of no definitive value for predicting the biological behavior of glial tumors, since the wide ranges obtained make it impossible to assign tumor grade. Our opinion is that the AgNOR method can be used as an auxiliary method for predicting tumor behavior, in support of morphological data.

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