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# Correlation Between Argyrophilic Nucleolar Organizer Region Staining and Brain Tumor Classification and Grading

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## ABSTRACT

**Distinctions between benign and malignant tumors are less evident in the central nervous system than in other tissues. Since the level of cell proliferation is an important feature in tumor grading, we applied AgNOR in 50 cases of brain tumors with different grades and histological origins in order to check its efficiency in discriminating between benign and malignant cases. We found significant differences between the means of grade I (1.76) and grade IV (2.46) tumors. No significant differences were observed considering the same grading with distinct histological types or age of patients, reinforcing the efficiency of AgNOR.**

## INTRODUCTION

Nucleolar Organizer Regions (NORs) are the loops of ribosomal deoxyribonucleic acid (DNAr), which transcribe to ribosomal ribonucleic acid (RNAr) and are located on the short arms of human acrocentric chromosomes 13, 14, 15, 21, and 22. The activity of these regions is directly related to protein synthesis, therefore the number of active NORs increases according to cell activity (1, 2, 3). By using a silver colloid staining technique, the transcriptionally active NORs and the associated argyrophilic proteins can be easily visualized in fixed and paraffin-embedded tissue samples, as well as in fresh tissue preparations (3, 4). These silver-binding DNA–protein complexes are called AgNORs (5). They are nucleolar acid non-histone proteins, which are associated with ribosomal genes (6). Nucleolin (C23) and nuclephosmin (B23) are most often suggested as the main argyrophilic proteins visualized by silver staining (7). Under light microscopy, AgNOR reveals dark granules located in the inter-

phase nuclei, which are located within fibrillar centers and the adjacent dense fibrillar component of the nucleolus (4, 8). The amount of AgNOR proteins present during interphase is routinely used to evaluate nucleolar activity and cell proliferation (3, 9). Thus, AgNOR can be used to characterize human cancer cell proliferation. High levels of AgNOR proteins indicate poor prognosis and a high rate of proliferation (10).

Considering that the process of diagnosis and grading of central nervous system (CNS) tumors is very difficult because features necessary for diagnosis (such as necrosis or vascular proliferation) may be absent in small tissue samples, new methods that improve routine pathological analysis are constantly being investigated. Some of these methods focus on cell proliferation, which is one of the most fundamental biological processes, through direct or indirect analysis by cytogenetic methods, cell kinetics, Ki-76 immunohistochemistry, and flow cytometry (8, 11). The different quantitative distribution of interphase AgNORs in rapidly and slowly proliferating cells can be explained by considering the role of these structures and the AgNOR proteins in RNAr synthesis (3). A rapidly dividing cell must concentrate its ribosomal biogenesis in a shorter time than a slowly dividing cell. This can be achieved by activating a greater number of DNAr sequences for transcription. For this reason, a greater quantity of AgNOR proteins must be synthesized, which will give rise to a greater number of interphase AgNORs, the structural-functional units for RNAr synthesis (12).

*Keywords: Brain tumors, Prognostic studies, Tumor cell biology*

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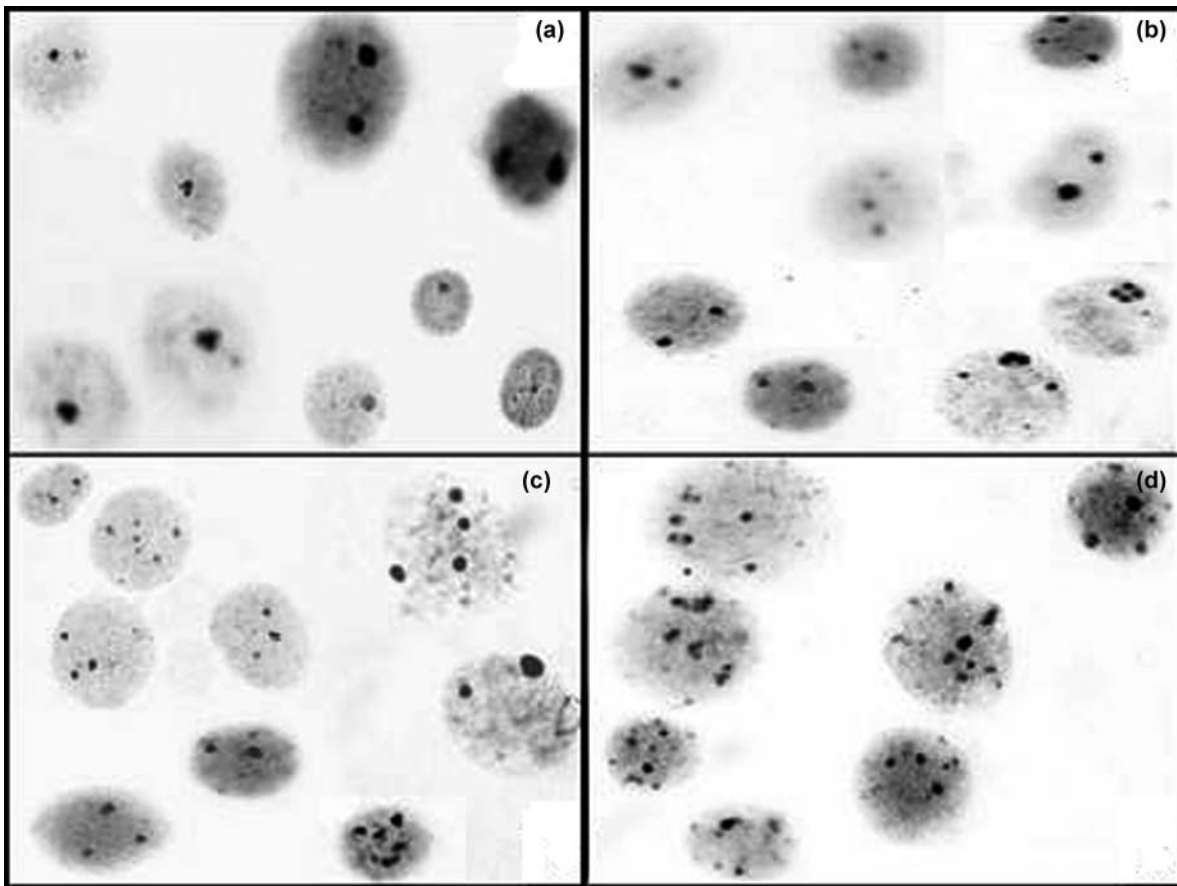
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**Figure 1.** Photomicrograph of AgNOR staining ( $\times 1,000$ ) in CNS tumors of different malignant grades. (a) Benign WHO 1 (mean 1.76 counts). (b) Benign WHO 2 (mean 1.90 counts). (c) Malignant WHO 3 (mean 2.1 counts). (d) Malignant WHO 4 (mean 2.46 counts).

Many authors have demonstrated the usefulness of AgNOR parameters for histopathology, but the number of studies dealing with AgNOR expression in CNS tumors is very small. Most of them analyzed a small group of neoplasms, and the results are often divergent (8). The aim of this study is to evaluate the relationship between the grading of CNS tumors and AgNOR number, especially between benign and malignant tumors, and determining the predictive value of AgNOR numbers for prognosis. In order to evaluate the possible influence of other factors, we also analyzed differences between distinct histological types and the age of patients.

## MATERIAL AND METHODS

We selected 50 cases of CNS tumors of different histological types and malignant grades excised from patients without a history of chemotherapy or radiotherapy treatment. These tumors were classified according to histopathological analysis, following the WHO Classification of Brain Tumors (13, 14). We considered grades I and II as benign, and III and IV as malignant tumors, following Louis *et al.* (14). Analysis considering two different groups, neuroepithelial and non-neuroepithelial tumors, was also performed. A third consideration was the age of the patients in order to analyze its influence on cell prolif-

eration. Interphase nuclei were obtained from tissue samples according to Ford and Hamerton (15), with modifications. Interphase preparations from a healthy individual were obtained from standard lymphocyte cultures (RPMI-1640 medium, 72-hr incubation). Staining of NORs was performed according to Howell and Black (16). For each case, two independent observers examined 200 intact nuclei, chosen by chance, using a Zeiss Axioplan microscope. The levels of significance of the results of AgNOR counts in different samples and between benign and malignant groups were verified by the non-parametric Mann-Whitney U test and Kruskal Wallis test, using *Biostat 5.0* package. Levels were considered significant at  $p < .05$ .

## RESULTS

Suspensions obtained from fresh tumor tissue showed clearly defined silver-stained dots and very few precipitation areas, allowing easy analysis of 200 tumor cell nuclei per case (Figure 1). Interestingly, dots were concentrated in a defined region in benign tumors, while in grades III and IV tumors they were dispersed throughout the nucleus. In benign nonrecurrent tumors, most nuclei contained only one large AgNOR dot. Interobserver variation was less than 5%.

**Table 1.** Analysis of AgNOR Numbers in Neuroepithelial Tumors of the CNS

Tumors	No. of Cases	WHO	Mean	Median	Minimum Value	Maximum Value	Standard Deviation	Standard Error
Pilocytic astrocytoma	1	1	1.7400	2.0	1.0	3.0	0.6817	0.0482
Fibrillar astrocytoma	4	2	1.9088	2.0	1.0	5.0	0.8284	0.0293
Anaplastic astrocytoma	4	3	2.1250	2.0	1.0	5.0	0.8748	0.0309
Subependymary giant cells astrocytoma	1	1	1.8350	2.0	1.0	5.0	0.9121	0.0645
Glioblastoma multiforme	7	4	2.45	2.0	1.0	7.0	1.0203	0.0273
Oligodendroglioma	1	2	1.9850	2.0	1.0	4.0	0.8112	0.0574
Anaplastic oligoastrocytoma	1	3	2.0200	2.0	1.0	4.0	0.8385	0.0593
Ependymoma	2	2	1.8825	2.0	1.0	5.0	0.7584	0.0379
Anaplastic ependymoma	2	3	2.1875	2.0	1.0	6.0	0.9949	0.0497
Medulloblastoma	6	4	2.4867	2.0	1.0	7.0	1.0078	0.0291
Central neurocytoma	4	2	1.8975	2.0	1.0	4.0	0.7601	0.0269

Normal controls provided by the lymphocytes showed on average two single silver-stained dots, coinciding with those observed in normal nervous tissue cells (8).

Table 1 shows results found in neuroepithelial tumors. Grade I tumors showed significant values when compared with others grades ( $p = .0036$  compared to grade II and  $p < .0001$  compared to grade IV). We also observed a significant difference ( $p < .0001$ ) between grades III and IV tumors. The number of AgNOR dots in astrocytic tumors was significantly higher in glioblastoma multiforme (WHO 4) than in other tumors of the same group ( $p < .0001$ ). In anaplastic astrocytomas (WHO 3), the number was lower than glioblastoma multiforme, but significantly higher ( $p < .0001$ ) when compared with benign astrocytomas (fibrillar and pilocytic astrocytomas). AgNOR counts for medulloblastomas did not differ significantly from glioblastoma multiforme ( $p = .2722$ ). Anaplastic oligodendrogliomas (WHO 2) and anaplastic oligoastrocytomas (WHO 3) did not differ significantly ( $p = .7352$ ) in spite of their different grades. On the other hand, ependymomas (WHO 2) and anaplastic ependymomas (WHO 3) showed a significant difference, with  $p = .0002$ .

Results concerning non-neuroepithelial tumors are found in Table 2. We found significant differences between meningiomas according to their graduation. Meningothelial, psammomatous, transitional, and fibrous meningiomas (WHO 1) did not differ from each other. However, when they were compared with chordoid meningiomas (WHO 2) and rhabdoid meningioma (WHO 3), the significance was higher according to the grade ( $p < .0001$ ). AgNORs counts in chordoids meningiomas (WHO 2) did not differ from germinomas (WHO 3). A higher signifi-

cance ( $p < .001$ ) was demonstrated when comparing rhabdoids meningiomas to glioblastoma multiforme and medulloblastomas, both WHO 4 tumors. The comparison of anaplastic astrocytomas, anaplastic oligoastrocytomas, and anaplastic ependymomas (WHO 3) did not show any significant differences.

Differences between benign (grades I and II) and malignant (grades III and IV) tumors are shown in Table 3: there is a significant difference of  $p = .0005$  between the means of AgNOR numbers (Figure 2). Table 3 also shows that no significant differences were found when comparing tumors by histological origins. The same was observed when comparing samples according to age ( $p < .4018$ ) distributed in three groups (Table 4). However, the mean of AgNOR dots in non-neuroepithelial tumors was lower than that for neuroepithelial tumors, although the difference did not influence distinction of grades by AgNOR as a whole.

## DISCUSSION

There is little doubt that proliferation is an important feature in characterizing the malignant phenotype and biological behavior of neoplasms. However, it appears to be the exception rather than the rule for proliferative activity to be used as a prognostic indicator in clinical decision-making. Because AgNOR numbers are intimately related to cell cycle and proliferation, it was suggested that they could be useful in analysis, indicating cell-proliferation activity levels and hence the neoplastic potential and aggressiveness of several tumors (3, 12, 17, 18). Moreover, this technique has the advantage of being easily applied to

**Table 2.** Analysis of AgNOR Numbers in Non-Neuroepithelial Tumors of the CNS

Tumour	No. of Cases	WHO	Mean	Median	Minimum Value	Maximum Value	Standard Deviation	Standard Error
Meningothelial meningioma	6	1	1.7408	2.0	1.0	5.0	0.7399	0.0214
Psammomatous meningioma	2	1	1.7200	2.0	1.0	4.0	0.7369	0.0368
Transitional meningioma	2	1	1.7175	2.0	1.0	5.0	0.7511	0.0376
Fibrous meningioma	2	1	1.7000	2.0	1.0	4.0	0.7358	0.0368
Rhabdoids meningioma	1	3	2.1500	2.0	1.0	5.0	1.0112	0.0715
Chordoids meningioma	2	2	1.8300	2.0	1.0	4.0	0.7297	0.0365
Germinoma	2	3	2.1800	2.0	1.0	5.0	0.9975	0.0499

**Table 3.** Means of AgNORs Between Benign and Malignant Tumors, Considering General Benign and Malignant, and Histological Types ( $p = .0005$ )

	Benign (Grades I and II)	Malign (Grades III and IV)
AgNOR general mean	1.7488	2.2285
AgNOR mean of nonneuroepithelial tumors	1.8748	2.2538
AgNOR mean of neuroepithelial tumors	1.5977	2.1650

paraffin-embedded tissue samples or fresh tissue preparations with the same efficiency (1, 4).

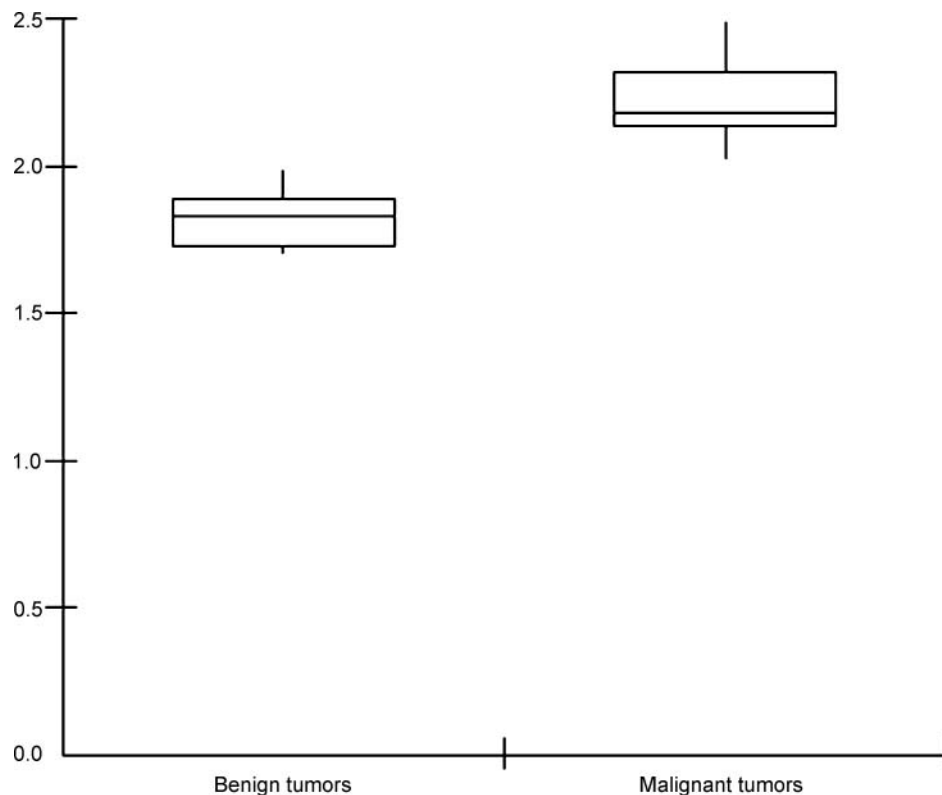
AgNOR has been applied in tumor pathology for both diagnostic and prognostic purposes. However, the lack of a standardized silver-staining protocol has led to much misinterpretation of actual structures evaluated in individual studies. For this reason, absolute AgNOR scores reported by different authors for the same types of tumors are difficult to compare and the results produced by these investigations sometimes seem to be conflicting (4). Further, although this technique is relatively cheap and easy to apply, results are very sensitive to factors such as the type of tissue preparation, silver precipitation, temperature, and the time of incubation, among others. In addition, slides should be analyzed in a short period, as the dots tend to lose definition with time (8, 10, 19, 20).

**Table 4.** Classification of Patients According to Their age

	0–25 Years	26–50 Years	>50 Years
Grade I	3	7	4
Grade II	5	6	2
Grade III	2	2	6
Grade IV	3	5	5

It has already been demonstrated that AgNOR method is useful in estimating proliferative activity in blood vessel cells of brain tumors (21). Although its importance and application are still controversial, AgNOR numbers are already used in correlation with histopathological data, differing between benign and malignant tumors. Some previous studies have demonstrated that a higher number of AgNOR is related to malignancy in intestine tumors, melanocytic lesions, colorectal adenocarcinomas, non-Hodgkin lymphomas, gastric adenocarcinomas, breast and lung tumors, parathyroid carcinoma, and lesions of the oral cavity (11, 22–26). In brain tumors, this correlation was also found in neuroblastomas, astrocytic tumors, and meningiomas (1, 21, 25).

Two studies including gliomas with different malignant grading also showed a correlation between AgNOR numbers and tumoral grade (27, 28). In the same way, in four out of five studies in astrocytomas, AgNOR numbers were higher in WHO 3 and 4 than in grades I and II tumors (5), corroborating our findings that



**Figure 2.** Box plot comparing means of AgNOR dots between benign and malignant tumors.

the mean of AgNOR numbers was also higher in glioblastoma multiforme (2.45) and medulloblastomas (2.45), both WHO 4 tumors. These values were higher than any other tumor of lesser grading, with a statistically significant difference ( $p < .0001$ ) in relation to WHO 1 tumors—pilocytic astrocytoma (1.74), subependymal giant cells astrocytoma (1.83), meningothelial meningioma (1.74), psammomatous meningioma (1.72), transitional meningioma (1.71), and fibrous meningioma (1.70). These results also corroborated the findings of Janczukowicz (8), who demonstrated statistically significant difference when comparing histological grading and AgNOR numbers in astrocytic tumors and meningiomas. In addition, Bukhari *et al.* (3) found  $p < .05$  when comparing grade IV and grade III astrocytomas.

Louis *et al.* (29) had demonstrated that AgNOR could discriminate between low- and high-grade gliomas, as observed in the present study. We found significant differences between grades I and II ( $p = .0193$ ), and between grades I and III ( $p < .001$ ) astrocytomas. Moreover, our results corroborate findings from other reports in which AgNOR numbers could differentiate between benign and malignant meningiomas ( $p < .001$ ) (30, 31).

Our results led us to conclude that, although there are still some questions raised by discrepancies found when comparing data reported by different authors, it is important to notice that most reports, including ours, have statistically significant differences that could at least discriminate low- and high-grade tumors from each other. This fact could by itself validate AgNOR as a diagnostic technique in assessing proliferation characteristics. It also reinforces the importance of its combination with histopathological analysis for a better graduation of CNS tumors, and probably in other groups of human neoplasias that having the proliferative profile of a tumor in a short period of time could bring real advantages in predicting its clinical behavior and suitable treatment. This is especially important in countries where additional modern techniques, such as molecular diagnostics, cannot be implemented due to economic problems.

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