Diagnostic value of silver nitrate staining for nucleolar organizer regions in cerebral astrocytic tumors

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Abstract

Aim: to compare the AgNOR's mean number with the histological type and grade of cerebral astrocytic tumors. 16 primary cerebral astrocytic tumors (4 diffuse astrocytomas, 4 anaplastic astrocytomas and 8 glioblastomas) stereotactic biopsied in the Department of Neurosurgery, Clinical Hospital „Prof. Dr. N. Oblu" Iaşi, and histopathologically conventional diagnosed in Department of Neuropathology of the same hospital, were retrospectively identified. Tumor specimens were submitted to a combined staining technique: one – step silver nitrate method for AgNOR protein sites (modified after Ploton et al, 1986) counterstained with periodic acid-Schiff staining for basement membrane of vascular components. The mean AgNOR values (mAgNOR) for tumoral and vascular nuclei were determined. The average values of mean AgNORs/nucleus (mAgNOR/nucleus) presented a linear increase with increasing grade of malignancy from 1.96 for diffuse astrocytoma (GII), 2.34 for anaplastic astrocytoma (GIII), to 3.18 for glioblastoma multiforme (GIV). mAgNOR/tumoral nucleus also showed a linear correlation with the histological tumor grade: 2.27 for diffuse astrocytomas, 2.78 for anaplastic astrocytomas, and 3.35 for glioblastomas multiforme. A distinct difference between the mean values of AgNORs/vascular nucleus was expressed: 1.52 for diffuse astrocytoma (GII), 1.90 for anaplastic astrocytoma (GIII), and 3.18 for glioblastoma multiforme (GIV). There were some overlaps between GIII and GIV astrocytic tumors regarding the mAgNOR/tumoral nucleus: maximum value in anaplastic astrocytomas (GIII) was 2.91 and minimum value in small cells glioblastomas (GIV) was 2.47. Differentiation could be achieved with mAgNORs/vascular nucleus as no extreme value overlapped: maximum value in anaplastic astrocytomas (GIII) was 1.99 and minimum value in small cells glioblastomas (GIV) was 2.54. The malignancy grade of an astrocytic tumor can accurately be establish both on histological features of the conventional stained sample and on the average number, the shape and the distribution of AgNORs within tumoral and vascular nuclei, as AgNORs determinations supplement the histological information in small biopsies.

Keywords: astrocytic tumor, nucleor organizer region, tumoral grade

In the practice of neurooncology it is extremely important to know the histological grade of a brain tumor and particularly of an astrocytic neoplasia. Objective tumor growth potential is achieved today by immunohistochemical

methods, but there are many other cell cycle-associated molecules that are potential targets for histochemistry assessment of cell proliferation.

The metaphase Nucleolar Organizer Regions (NORs) are chromosomal segments or loops (rDNA) containing ribosomal genes associated with proteins. These genes are clustered in 10 loci of the human acrocentric chromosomes 13, 14, 15, 21, and 22. The transcriptional activity of these intranuclear segments plays a pivotal role in the formation of nucleoli, directing the syntesis of both ribosomes and associated proteins [5]. NORs associated protein [C23 (nucleolin) and B23 (nucleophosmin)] are selectively stained by silver impregnation technique in formalin-fixed paraffin-embedded tissues. After silver-staining, the NORs can be easily identified as black dots exclusively localized throughout the nucleolar area, and are called "AgNORs". The expression of AgNOR proteins was associated with several biological properties of neoplastic cells: metabolic activity, DNA content, histological grade of differentiation and, especially, the rapidity of cellular proliferation [31, 7, 26, 25].

The specific significance of AgNORs is not well understood. AgNOR quantification was used in tumor pathology as a parameter to distinguish malignant cells from benign or normal cells [11, 10, 9]. In the latest 20 years more than 400 studies was done in order to determine the correlation of AgNORs' quantity and quality with tumor aggressivness and rapidity of proliferation, with variable success rates that depended on tumor type [5]. A large number of papers have shown a linear correlation between AgNOR count and growth fraction in various human malignancies: breast cancers [32, 18], urinary bladder carcinoma [6], colorectal carcinoma [23], bone tumors [10], utererine cancer [12], endocrine cancers [22], skin cancers [2], lung cancers [28], brain cancers [8, 20]. Moreover, the method can be applied to small biopsies, can identify neoplastic clones with different proliferative activities and may stratify patients into different risk groups [25].

Because assessment of AgNORs offers an alternative approach to measure the tumor growth fraction and because there are a small number of articles dealing with AgNORs expression in astrocytic tumors, in this article we analyze possible correlation between AgNOR's morphology and AgNOR's number with histological tumor grade in order to establish a possible role for it as an objective indicator of tumor biological behavior, just after the resection of a cerebral astrocytic neoplasia.

Material and Methods

Selection of cases

16 primary cerebral astrocytic tumors stereotactic biopsied in the Department of Neurosurgery, Clinical Hospital „Prof. Dr. N. Oblu" Iaşi, and histopathologically diagnosed in Department of Neuropathology of the same hospital, were retrospectively identified. In all cases, tumor samples were fixed in 10% buffered formalin, included in paraffin, and stained with H&E according to standard procedure.

Table 1
Histological subtypes and grade of malignancy of the 16 investigated astrocytomas

Histological subtypes with grade of malignancy	No. of cases
Diffuse astrocytoma (grade II)	4
Anaplastic astrocytoma (grade III)	4
Glioblastoma multiforme (grade IV) With small cells With giant cells	8 4 4

All 16 cases were histopathological diagnosed and graded according to the WHO Classification of Brain Tumors [19] and divided into groups (Table 1). There were 4 cases of diffuse astrocytoma - grade II (GII), 4 cases of anaplastic astrocytoma - grade III (GIII), and 8 cases of glioblastoma multiforme - grade IV (GIV).

AgNOR Staining Technique

Five μm thick paraffin sections, obtained from paraffin blocks with the most representative tumoral areas, were selected and submitted to a combined staining technique: one – step silver nitrate method for AgNOR protein sites (modified after Ploton et al, 1986) [27] counterstained with periodic acid-Schiff staining for basement membrane of vascular components.

Briefly, slides were deparaffinized in xylene, and hydrated through descending concentrations of ethanol to double distilled, deionized water. The sections were stained with freshly prepared silver colloidal solution for 25 min in a thermostatically controlled environment (37oC). The working solution contained one volume of 2% gelatine in 1% aqueous formic acid and two volume of 50% aqueous silver nitrate solution. Sections were counterstained with periodic acid - Schiff solution, dehydrated through ascending grades of ethanol, cleared in xylene, and mounted in Canada balsam.

Quantification of the number of AgNORs

Histological sections, stained by the exposed method, were examined by eye, using in each case Olympus microscope, magnification 1000x, an oil-immersion lens, and cedarwood oil. In all cases we focused the image throughout the section thickness using at least 10 different microscopic fields in order to determine the homogeneous AgNOR quantitation throughout the tumor. Quantification of the AgNOR number has always been done in well preserved cells, excluding areas of tumoral necrosis, areas with staining artifacts or overlapping cells. We analysed the dense cellular areas and those with vessels that had maximal endothelial proliferation. Using the average formula we determined the mean AgNOR values for tumoral and vascular cells, taken individually and then together, for every histological subtype and then for every grade of malignancy. Mean number of AgNORs for each case was defined as the ratio between total number of black dots determinated in 100 individual tumor cells and the number of analysed cells. The same ratio was done for 100 individual vascular cells. At each determinated black dot were noted the size (subjective quantified in: small, medium and large), the appearance (granular or homogeneous), and any other morphological features encountered.

Results

The average values of mean AgNORs/nucleus (mAgNORs/nucleus) for each investigated tumor and its correlation with malignancy grades were represented in Table 2. Taking into account both tumoral and vascular cells, the mean values of mAgNORs/nucleus presented a linear increase with increasing grade of malignancy of cerebral astrocytomas from 1.96 for diffuse astrocytoma (GII), 2.34 for anaplastic astrocytoma (GIII), to 3.18 for glioblastoma multiforme (GIV). A distinct difference between the mean values of AgNORs/vascular nucleus was expressed: 1.52 for diffuse astrocytoma (GII), 1.90 for anaplastic astrocytoma (GIII), and 3.18 for glioblastoma multiforme (GIV).

The correlation between mAgNORs/tumoral nucleus and histological subtype of glioblastoma multiforme showed a distinct difference between small cells and giant cells variants, as giant cells glioblastoma expressed higher values (3.94) than small cells subtype (2.76).

The differential diagnosis between anaplastic astrocytomas and small cells glioblastomas became very difficult to determine based only on mAgNORs/tumoral nucleus because the mean values are almost identical (2.78 versus 2.76) as there were some overlaps of mAgNORs/tumoral nucleus between these two grades of malignancy: maximum value in anaplastic astrocytomas (GIII) was 2.91 and minimum value in small cells glioblastomas (GIV) was 2.47. Differentiation could be achieved by association of mAgNORs/vascular nucleus as there was no overlap between any investigated astrocytic tumor: maximum value in anaplastic astrocytomas (GIII) was 1.99 and minimum value in small cells glioblastomas (GIV) was 2.54 (Table 3).

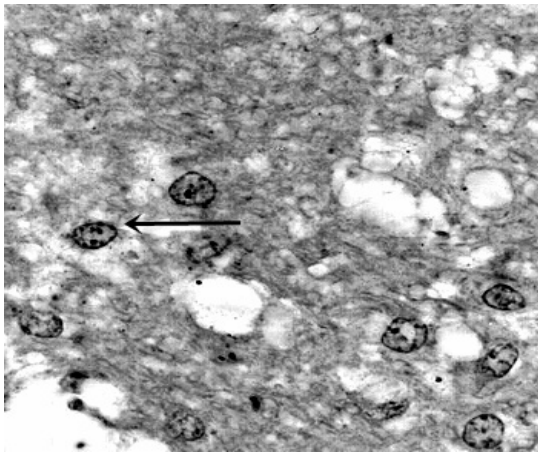

Figure 1 Diffuse astrocytoma (GII): 1 to 5 small black dots in tumoral nuclei (silver impregnation method, magnification x1000)

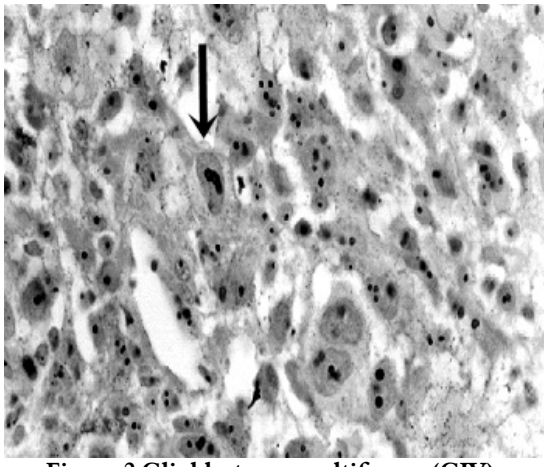

Figure 2 Glioblastoma multiforme (GIV): tumoral nuclei with 2 to 7 medium black dots scattered across nucleus area; one nucleus with bizarre great AgNOR dot (arrow) (silver impregnation method, magnification x1000)

Table 2

Mean values of AgNORs in tumoral and vascular nuclei of cerebral astrocytomas according to their histological subtypes and malignancy grades

MALIGNANCY GRADE	AVERAGE NUMBER OF AgNORs/NUCLEUS					
	tumoral cells		vascular cells		Whole tumor	
	Range	Group mean	Range	Group mean	Range	Group mean±SD
Diffuse astrocytoma (G II)	2.14 2.26 2.31 2.38	2.27	1.43 1.45 1.53 1.54	1.52	1.78 1.92 1.96 2.18	1.96
Anaplastic astrocytoma (G III)	2.63 2.74 2.85 2.91	2.78	1.82 1.82 1.98 1.99	1.90	2.28 2.30 2.36 2.42	2.34
Glioblastoma multiforme (G IV)	2.47 2.68 2.79 3.12 3.76 3.83 4.02 4.15	3.35	2.54 2.72 2.72 3.60 2.77 2.86 3.04 4.00	3.03	2.50 2.75 2.92 3.14 3.34 3.59 3.39 3.88	3.18

Table 3

Differential diagnosis between glioblastoma multiforme and anaplastic astrocytoma based on mAgNOR/tumoral nucleus and mAgNOR/vascular nucleus

NUMBER of AgNORs/ NUCLEUS	HISTOLOGICAL SUBTYPE					
	Anaplastic astrocytoma		Small cells glioblastoma		Giant cell glioblastoma	
	Range	Mean	Range	Mean	Range	Mean
mAgNOR/ tumoral nucleus	2.63 2.74 2.85 2.91	2.78	2.47 2.68 2.79 3.12	2.76	3.76 3.83 4.02 4.15	3.94
mAgNOR/ vascular nucleus	1.82 1.82 1.98 1.99	1.90	2.54 2.72 2.72 3.60	2.895	2.77 2.86 3.04 4.00	3.167

Table 4
Comparison between average values of AgNORs /nucleus in the literature and in the present study

Author	MEAN NUMBER OF AgNORs/nucleus								
	Diffuse astrocytoma (GII)			Anaplastic astrocytoma (GIII)			Glioblastoma multiforme (GIV)		
	tumoral nucleus	vascular nucleus	nucleus	tumoral nucleus	vascular nucleus	nucleus	tumoral nucleus	vascular nucleus	nucleus
Dumitrescu G. (2009)	***2,27***	***1,52***	***1,96***	***2,78***	***1,90***	***2,34***	***3,35***	***3,03***	***3,18***
Janczukowicz J. (2003) [17]			1.65			2.11			2.55
Choi YM et al. (1997) [4]			1.2 ±0.26			1.90 ±0.64			1.96± 0.57
Hara A. et al. (1991) [15]	1,52±0,07	1,80± 0,13		1,98± 0,23	2,87 ± 0,50		2,05 ± 0,29	3,13 ± 1,13	
Tokunaga Y. et al. (1997) [30]			2.04± 0.54			2.40±0.77			2.71 ± 1.13
Haberland C. et al. (1996) [14]			1,73			2,81			4,56
Tokiyoshi K. et al. (1992) [29]			1.68±0.87			1,85±1.03			2,76±1.26
Pedal W.P. et al. (1994) [24]			1,98			2,84			Small cells GM: 3.33 Giant cells GM: 4,24

Discussion

Analysis of the AgNORs' mean number is a widely accepted method for diagnostic assessment of a wide range of tumors, both on histological and cytological preparations. In 1986, Ploton et al. [27] have used and improved the silver impregnation method brought in histopathological practice by Goodpasture and Bloom in 1975 [13]. Ploton et al. suggested that the number of AgNORs/cell correlates with cellular activity and may be an indicator of

malignancy, because a great number of AgNORs represents a more active proliferation, and thus a more malignant cell [27].

AgNORs have found to be markers of proliferation in brain astrocytomas because from 1990 onwards some articles showed linear correlations between the number of AgNORs, Proliferating Cell Nuclear Antigen (PCNA), binding index of Ki-67, or mitotic rate and the histological grade [20, 3, 21, 17, 4]. Several studies have been conducted on the possibility of discrimination between astrocytomas with low malignancy and those with high malignancy, using only the mean number of AgNOR dots per nucleus (mAgNOR/nucleus) [16].

The present study was designed to verify the usefulness of AgNORs determinations in discriminating histological malignancy grades of cerebral astrocytic tumors. We found a wide range of AgNORs values within each histological grade, probably reflecting that well known morphological heterogeneity and biological behaviour variations of cerebral astrocytic tumors. Nevertheless there was a linear correlation between mean values of AgNOR/nucleus and histological grade.

Literature review on mean number of AgNORs in astrocytic tumors is summarized in Table 5 and data are compared with the results of the present study. It must be noted that the average values of AgNORs obtained in our study are in agreement with previous reports. All the authors obtained a gradual increase spectrum of the average number of AgNOR from diffuse astrocytomas, anaplastic astrocytoma, to glioblastomas multiforme and concluded that the number of AgNORs reflect the grade of malignancy in these neoplasias [17, 4, 15, 30, 14, 29, 24].

Janczukowicz (2003) found a statistically significant relationship between histological grade and expression of AgNOR in astrocytic tumors, but he stressed the presence of a small overlapping of extreme values between astrocytomas GII and GIII and between GIII and GIV [17].

We also observed a remarkable overlapping of the extreme values of the average number of AgNOR/tumoral nucleus between anaplastic astrocytomas (GIII) and small cells glioblastomas (GIV) probably because some of the anaplastic astrocytomas considered samples were in fact infiltration areas of a glioblastoma, the sample was too small and had no visible vessel or necrosis. Thus, AgNOR method may be particularly useful in small specimens or in biopsies from the infiltrating edge of an astrocytic tumor, where the usual histological features of malignancy may be absent. A further distinction between these two grades of malignancy could be made if mAgNOR/vascular nucleus is taken into account because none of the obtained values overlapped when comparing anaplastic astrocytomas with glioblastomas multiforme.

Hara et al. (1991) calculated mAgNOR/vascular nucleus of astrocytomas grade II, III and IV (1.80 ± 0.13, 2.87 ± 0.50, and 3.13 ± 1.13) and found it significantly higher than that of vascular nucleus of normal brain

without neoplastic transformation (1.26 ± 0.05) [15]. It must be noted that in the present study we did not found such great values for mAgNOR/vascular nucleus in anaplastic astrocytoma, probably because Hara et al. used 1979 WHO Histological Typing of Brain Tumors and we used 2000 WHO Brain Classification, where no vascular proliferation is needed to establish grade III of malignancy for these tumors. In the present study, the presence of increased average number of mAgNOR/vascular nucleus in anaplastic astrocytomas (GIII) and, especially, in glioblastomas multiforme (GIV) suggested that proliferative processes of tumor cells and vascular cells are interconnected. All these results showed that proliferative activity, both in tumoral cells and in vascular tumoral cells of the cerebral astrocytomas, increased with increasing grade of malignancy and therefore quantification of AgNORs is a useful marker in assessing the malignancy of these tumors.

Conclusion

A wide range of AgNOR values has been observed within each histological type and grade, probably reflecting variations in the biological behaviour. The number of AgNORs increased gradually from diffuse astrocytomas (GII) to anaplastic astrocytomas (GIII) and glioblastomas multiforme (GIV). Some overlaps of mAgNOR/tumoral nucleus have been detected between anaplastic astrocytomas (GIII) and glioblastomas multiforme (GIV) but the cause could be a wrong diagnostic of malignancy due to the small size of the stereotactic biopsies. The average number of mAgNORs/vascular nucleus appeared to be a more precious indicator as there was no overlap between any investigated astrocytic tumors.

AgNOR method proved to be a useful method for histological grading as it supplemented the information obtained with conventional histological assessment.

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