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Argyrophilic nucleolar organizer region (AgNOR) counting in astrocytic gliomas: prognostic value

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Summary. In 87 astrocytic gliomas the number of AgNORs/nucleus was retrospectively studied and data correlated with the histological type of the tumors and survival. All patients were treated by the same surgical team and with uniform criteria. Statistically significant differences ($p<0.01$) were found in relation with the AgNOR averages among the histological types of tumors. A statistically significant linear correlation ($p<0.05$) between the AgNOR values and survival of the patients was also found. Patients with mean AgNOR values higher than 2.23 and lower than 2.9 survived an average of 11.5±9.1 months vs. a survival in average of 24.4±34.1 months with mean AgNOR values under 2.23 ($p<0.05$). Patients with AgNOR values higher than 2.9 survived, on average, 7.7±3.9 months.

AgNOR counting in astrocytic gliomas is a reproducible, easy, quick method with prognostic value. AgNORs may be successfully applied in routine material to assess the growth potential of astrocytic gliomas.

Key words: AgNOR, Astrocytoma, Glioblastoma, Prognosis

Introduction

Several grading schemes have been proposed for central nervous system (CNS) tumors that take into consideration the degree of tumor cellularity and nuclear anaplasia, the mitotic activity, the presence of tumor necrosis and, as far as glial neoplasms are concerned, the degree of vascular endothelial proliferation (Kernohan et al., 1949; Zülch, 1979; Daumas-Duport et al., 1988; Kleihues et al., 1993). However, the practice of grading in CNS tumors, especially of those belonging to the glioma group, presents considerable problems: the sampling error of the biopsy material; the possibility of evolution of an originally benign neoplasm to a more malignant one; and the inherent subjectivity in evaluating the microscopic criteria (Jellinger, 1978; Burger et al., 1985; Russell and Rubinstein, 1989). It thus becomes obvious that the prediction of the biological behaviour in an individual case is not always possible when conventional histological criteria are used.

For this reason new methods have recently been introduced to predict the biological behaviour of the tumors, based on the determination of tumor growing potential.

These methods included tritiated-thymidine uptake followed by autoradiography (Hoshino and Wilson, 1979; Franzini et al., 1989), bromodeoxyuridine (BrdU) uptake followed by anti-BrdU immunohistochemistry (Nagashima et al., 1985; Franzini et al., 1989; Hoshino et al., 1989, 1993) and flow cytometric studies of the deoxyribonucleic acid (DNA) contents of tumor cells (Hoshino et al., 1978; Nishizaki et al., 1989; Appley et al., 1990; Assietti et al., 1990). For different reasons these methods have been replaced by more simple and quicker ones. These include the immunohistochemical determination of proliferating cell nuclear antigen (PCNA) (Allegranza et al., 1991; Karamitopoulou et al., 1993), Ki-67 antigen in fresh tissue (Burger et al., 1986; Zuber et al., 1988) or the demonstration of nucleolar organizer region (NORs) by means of an argyrophilic technique.

Nucleolar organizer regions are loops of DNA which contain the genes encoding ribosomal RNA (Crocker and Nar, 1987; Rüschoff et al., 1989). Since NORs are closely related to the ribosomal protein synthesis, they are claimed to reflect cellular proliferative activity. NOR configuration has become of increasing interest after the finding that the original silver staining technique of NOR-associated proteins can be applied to paraffin sections (Ploton et al., 1986).

Materials and methods

Two hundred and fifty astrocytic glioma cases were collected in a circumscribed region of northern Spain

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served by a single clinical team over a 15-year period. All cases underwent surgery which consisted in either a lobectomy or subtotal removal. For 87 of these cases, the full complement of clinical, follow-up, histopathological analysis and silver staining to detect nucleolar organizers was available.

Sixty-two out of the 87 patients also underwent radiotherapy (45 Gy whole cranial irradiation and 15 Gy local irradiation) and sixteen patients also underwent chemotherapy (VM_{26}, 40 mg/m^2 the first and second day plus CCNU, 60 mg/m^2 on the third and fourth day followed by a rest period of 5 weeks. The cycle was than repeated for a total of 6 times).

All tissue was fixed in 10% formalin and embedded in paraffin wax. The diagnoses were made on the basis of histological criteria, with histochemical and immunohistochemical confirmation where necessary. The classification and grading were performed independently by two experienced neuropathologists (FFC-S and JF) according to the World Health Organization Criteria (Zülch, 1979; Kleihues et al., 1993).

There were 46 astrocytomas, 9 of which were pilocytic, 14 low grade and 23 anaplastic, and 41 gioblastomas.

For the demonstration of AgNORs the colloid silver nitrate staining technique as modified by Howat (Howat et al., 1988, 1990) was used in 3 µm-thick sections of representative areas of the tumors (Fig. 1).

All assessments of staining were made blindly (i.e. without knowledge of histological diagnosis) by the same observer (JCF). The number of nuclear dots (AgNORs) from 100 randomly selected neoplastic cells was counted using a x100 oil immersion lens. Clusters of dots were scored as multiple NORs as accurately as possible. The mean number of AgNORs per cell was then calculated for each case. The results were statistically analyzed using the Student's t test, variance analysis and the Peason's linear correlation.

Results

Nine patients with pilocytic astrocytomas and 3 patients with low-grade astrocytomas out of 87 patients remain alive at the end of the study, with a minimum follow-up of 7 years.

The average survival of the deceased patients is graphically shown in Table 1 (Fig. 2). Differences in survival in relation to the histological type were statistically significant ($p<0.01$, variance analysis).

Statistically significant negative linear correlation was observed between survival and age of patients ($p<0.01$, $r=0.334$).

Results referring to number of AgNORs/nucleus are expressed in Table 1 for each histological type. Differences between the histological types were statistically significant ($p<0.01$ variance analysis). The mean number of AgNORs per cell for glioblastoma multiforme was significantly higher than for anaplastic astrocytoma (unpaired t-test, $t=2.41$, $p<0.05$), low-grade astrocytoma ($t=4.36$, $p<0.001$) and pilocytic astrocytoma ($t=5.66$, $p< 0.001$). A significant difference was also seen between the number of AgNORs for anaplastic astrocytoma and for low-grade astrocytoma ($t=2.78$,

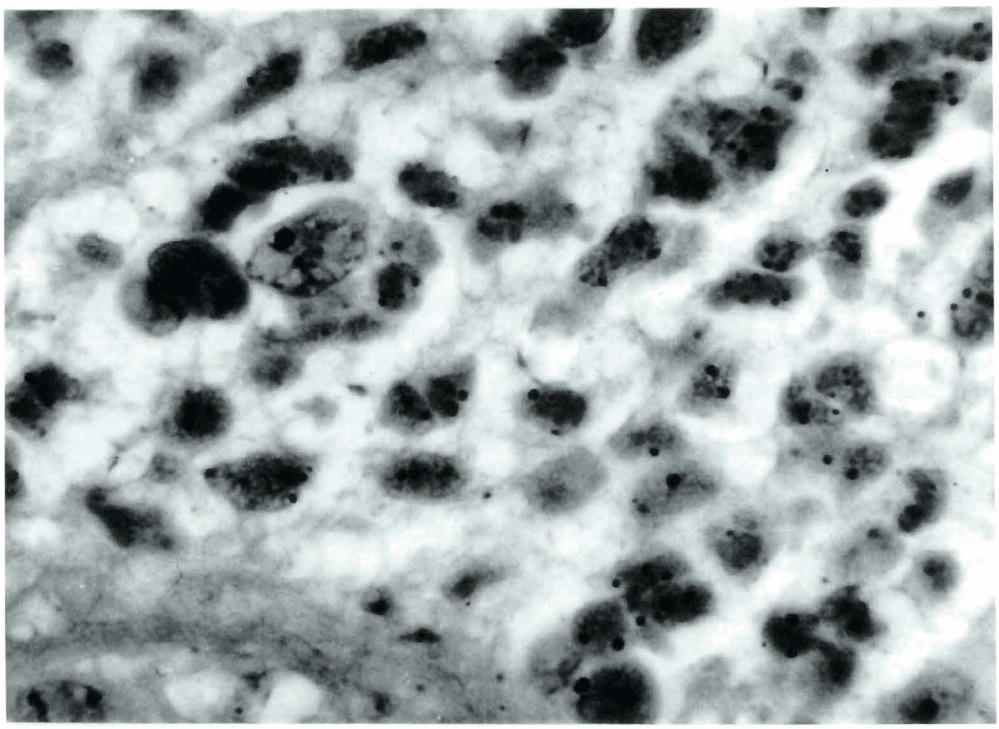

Fig. 1. Anaplastic astrocytoma. More than two AgNORs per nucleus in 80% of the tumour cells. Howat technique for AgNOR. x 1,000

p<0.01) and for pilocytic astrocytomas (t=2.77, p<0.01). Thus, the mean number of AgNORs paralleled the degree of histopathological malignancy, although some overlap was noted between the various types of tumor. No statistically significant differences between pilocytic and low-grade astrocytomas were found (t=0.20, p=0.0843). In the group of deceased patients, those which presented an AgNOR value higher than 2.23/nucleus and lower than 2.29 had an average survival of 11.5±9.1 months vs. those which had an AgNOR value lower than 2.23/nucleus, whose average survival was 24.4±34.1 months (p<0.05) (Fig. 3).

Patients with AgNOR values higher than 2.9 had an average survival of 7.74±3.87 (t=3.25, p<0.01) in relation to the survival of the patients with AgNOR values lower than 2.9.

A statistically significant negative linear correlation (p<0.05, r=-0.251) was observed between the AgNOR value and patients' survival in the group of patients which deceased in a period of 5 years after surgery.

Discussion

Astrocytic tumors are the most common type of the primary intracranial neoplasm in the mature nervous system (Vanderberg, 1992). Several aspects related to the cytogenesis and molecular biology of these tumors have benn recently incorporated by the new WHO classification of brain tumors (Kleihues et al., 1993). Based on the estimation of tumor growth potential, technical approaches have been developed in an attempt to define prognosis.

Our study demonstrates that the number of AgNOR increases with the degree of tumor malignancy. Our average values of AgNORs/nucleus were very akin to

Table 1. age, survival (months) and AgNOR/nucleus index according to histological diagnosis (average values and standard deviation).

HISTOLOGICAL TYPE (n)	AGE	SURVIVAL	AgNOR
Pylocitic (9)	11.56±8.82	-	1.70±0.29
Low-grade (14)	34.57±16.06	53.82±45.49*	1.73±0.40
Anaplastic (23)	52.52±15.37	17.87±22.38	2.11±0.40
Glioblastomas (41)	53.80±12.63	8.86±5.25	2.42±0.55
	p<0.01**	p<0.01**	p<0.01**

*: only 11 deceased patients; **: variance analysis, excluding pilocytic astrocytomas.

Table 2. AgNOR/nucleus value in astrocytic tumors (literature data).

REFERENCE	HISTOLOGYCAL TYPE	n	means±SD
Hara et al., 1990	Grade 2	5	1.6±0.18
	Grade 3	6	2.5±0.46
	Grade 4	3	2.8±0.31
Kajiwara et al., 1990	Low-grade	16	1.76±0.06
	Anaplastic	15	2.01±0.10
	Glioblastoma	20	2.51±0.12
Hara et al., 1991a	Grade 3 and 4	9	2.58±0.41
Hara et al., 1991b	Grade 2	4	1.80±0.13
	Grade 3	7	2.87±0.50
	Grade 4	11	3.13±1.13
Martin et al., 1991	Low-grade	10	1.3±0.20
	High-grade	9	1.6±0.17
Shiraishi et al., 1991	Low-grade	7	1.98 SE=0.23
	High-grade	27	2.41 SE=0.39
Korkolopoulou et al., 1993	Grade I	5	1.79±0.26
	Grade II	5	2.78±0.84
	Grade III	7	3.17±0.27
	Grade IV	16	3.88±0.40
Shibuya et al., 1993	Mod. anap. astroc.	2	1.71
	Highly anap. astroc.	4	2.20±0.14
	Glioblastoma	18	2.58±0.39
Present study	Pilocytic	9	1.70±0.29
	Low-grade	14	1.73±0.11
	Anaplastic	23	2.11±0.40
	Glioblastoma	41	2.43±0.55

SE: standard error; Mod.: moderately; anap.: anaplastic; astroc.: astrocytoma.

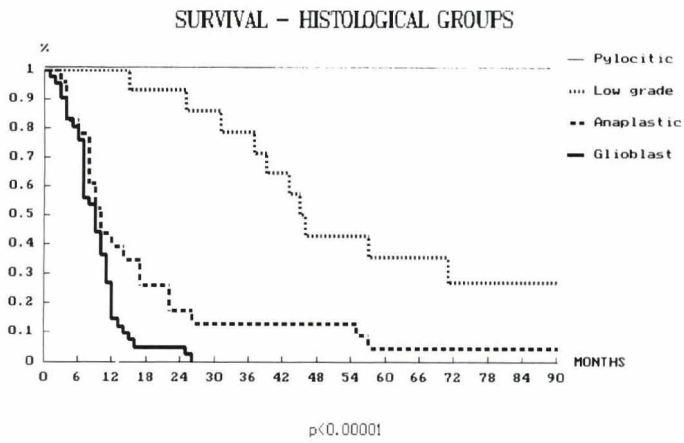

Fig. 2. Survival curves and histological types in 87 astrocytic gliomas.

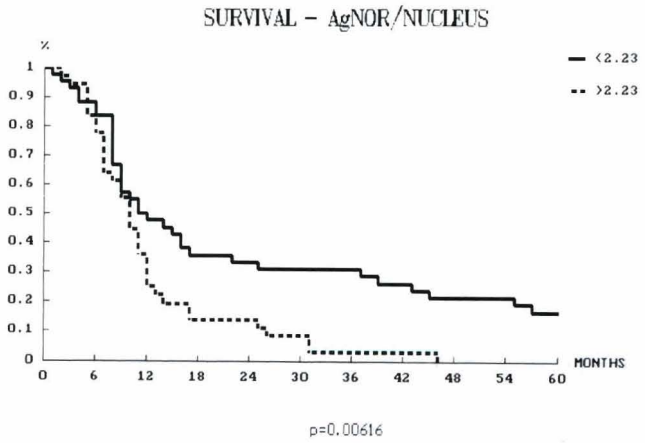

Fig. 3. Survival curves and average value AgNOR/nucleus (excluding pilocytic astrocytomas).

those obtained by other authors (Kajiwara et al., 1990; Shiraishi et al., 1991; Shibuya et al., 1993) (Table 2).

Differences in AgNOR values obtained by different investigators in relation to histological diagnosis are explained by the fact that there is no standardization of evaluation criteria of AgNORs (Louis et al., 1992). This is specially true in relation to intranuclear NOR groups, which were designated with different names: «AgNUS» (Maier et al., 1991); NOR-conglomerates (Martin et al., 1991); or compound AgNOR (Louis et al., 1992). Nevertheless, the reliability of the technique within a working team is very high, reaching 93% according to Louis et al. (1992). The real problem appears when comparing the results of different teams of investigators. As far as we know only Kawijara et al. (1990) have previously analyzed the prognostic value of AgNOR determination in 32 adult patients from a series of 51 astrocytic gliomas. These authors observe that the group of patients whose AgNOR value is <1.80 has a better prognosis than those patients with AgNOR values equal or higher than this number, comparing groups of patients dead or alive between 5 and 10 years after surgery.

We have not found an AgNOR value in our study which could be considered as a reliable «border» value between malignant and benign cases and which could be applied in all laboratories. Louis et al. (1992), in a paper about differential diagnosis of CNS tumors, indicate that a number of AgNORs higher than 1.5 or the presence of compound-AgNORs is diagnostic of neoplastic process vs. reactive gliosis.

NOR staining has been reported to be useful in determining the proliferative potential of other types of central nervous system tumors: for meningiomas (Chin and Hintorn, 1989; Orita et al., 1990; Kunishio et al., 1994); for myxopapillary ependymomas of cauda equina (Ross et al., 1993); to discriminate reactive gliosis vs. low grade astrocytoma, cerebellar granullar layer vs. meduloblastoma cells (Louis et al., 1992); and also in the evaluation of choroid plexus tumors (Centeno et al., 1993).

Other investigators have correlated AgNOR values in astrocytic tumors with other proliferation cell markers. Hara et al. (1990a, 1991a,b) found a positive correlation between AgNOR and percentage of Ki-67-positive cells in two different series of 9 and 14 tumors, respectively, using serial frozen cuttings, assuming that AgNOR value is representative of tumor growing potential.

Kajiwara et al. (1990) obtained a good correlation between AgNOR average value and positive BrdU cell index in 16 cases out of 51 astrocytic tumors. Shibuya et al. (1993) found a statistically significant linear correlation between AgNOR values and BrdU-positive cell index, and Ki-67-positive cells in 200 brain tumors, although they point to the possibility that AgNOR values may not measure cellular proliferation directly. However, AgNOR values may reflect other features of biological malignancy.

In contrast, Maier et al. (1991) did not find any relationship between AgNOR values and Ki-67 values, nor BrdU index in 34 gliomas, and the differences between low-grade and high-grade tumors are not significant either. Finally, Korkolopoulou et al. (1993) detected a positive linear correlation (r= 0.91) between AgNOR number and PCNA-positive cell index in 82 different CNS tumors, establishing that both techniques are representative of tumoral growing potential. All of these studies, including ours, infer that the prognostic value must be ratified with parallel clinical studies.

After all these considerations and the results of our investigation AgNOR value is undoubtedly another factor which contributes to assessing the prognosis of tumors and, in itself, can be useful to classify the grade of malignancy of an astrocytic glioma. This fact is particularly relevant in those cases with very scarce material (stereotactic biopsies). We think that this easy and simple method should be incorporated into routine neuropathological diagnosis of the tumors, especially astrocytic gliomas.

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