Immunohistochemical Analysis of Progesterone Receptor and Ki-67 Labeling Index in Astrocytic Tumors

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BACKGROUND. Intracranial tumors such as meningiomas express steroid hormone receptors but little is known regarding progesterone receptor (PR) in astrocytic tumors. The authors evaluated expression of PR in 86 astrocytic tumors in relation to tumor proliferative potential.

METHODS. Paraffin embedded tumor sections were stained with polyclonal antiprogesterone antibody by the peroxidase-antiperoxidase method and with monoclonal MIB-1-Ki-67 antibody by avidin-biotin complex immunohistochemistry.

RESULTS. Sixty-three of the 86 astrocytic tumors (73%) showed positive PR immunoreactivity. PR expression was observed in 4 of 9 pilocytic astrocytomas, 13 of 24 Grade 2 astrocytomas, 15 of 20 anaplastic astrocytomas, and 31 of 33 glioblastomas. In addition to the tumor cells, cells of microvascular endothelial proliferation and the smooth muscle of tumor vessel walls were frequently PR positive. Glioblastomas had a significantly higher percentage of PR positive cells compared with anaplastic ($P < 0.0008$) and low grade ($P < 0.0001$) astrocytomas. Patients with PR positive astrocytomas were of an older age than patients with PR negative astrocytomas (48.71 ± 21.95 years vs. 37.09 ± 24.69 years; $P < 0.04$). The mean Ki-67 labeling index (LI) was significantly higher in the high grade (3–4) astrocytomas compared with low grade (1–2) astrocytomas ($P < 0.0001$). PR positive astrocytic tumors had higher Ki-67 LI than PR negative tumors. PR expression was not correlated with tumor recurrence and patient survival.

CONCLUSIONS. The current study suggests that PR in the astrocytic tumors correlates with histologic grade and PR may participate in the growth of these tumors and tumor angiogenesis. The measurement of PR in these tumors may indirectly represent tumor growth potential. ***Cancer*** **1997;80:2133–40.** © *1997 American Cancer Society.*

KEYWORDS: astrocytic tumor, histologic grade, progesterone receptor, immunohistochemistry, Ki-67, labeling index, angiogenesis, survival.

Steroid hormone receptors act as transcription factors that mediate the biologic effects of steroids by regulating gene expression at a predominantly transcriptional level. Certain intracranial tumors express sex steroid receptors; the presence of progesterone receptor (PR) has been widely reported in meningioma.[1–4] Steroid hormones could be involved in the development of meningiomas with a female gender predominance, as evidenced by their increase in size during pregnancy and subsequent decrease after parturition, and their association with breast carcinoma.[5] It has been reported that in poorly differentiated neuroepithelial tumors, the distributions of sex hormone receptors differ with the patient's gender.[6]

Human astrocytic gliomas are the most common intracranial tumors. Although not extensively studied, the presence of PR in human

astrocytomas has been demonstrated by other authors.[6–9] Two recent studies[10,11] have detected the expression of the PR gene in astrocytic tumors. Thus, studies regarding the biologic and clinical importance of PR in astrocytic tumors require further attention. Cell proliferation is a fundamental process controlled by highly coordinated mechanisms. Among the cell cycle-associated proteins, Ki-67 is detectable in all active parts of the cell cycle (G_1, S-, G_2, and M-phases), but absent in resting or quiescent G_0 phase.[12] The Ki-67 labeling index (Ki-67 LI) in astrocytic tumors reflect the histologic grade of malignancy.[13,14]

In the current study, we examined immunohistochemical expression of PR in a large number of astrocytic tumors. We also studied growth fractions of these tumors as assessed by Ki-67 LI and compared the expression of PR in astrocytic tumors with growth potential and with other clinicopathologic extents. In the current study, 73% of the tumors were PR positive; we found that the expression of PR correlated with the histologic grade of astrocytic tumors, and PR positive astrocytomas had higher growth potential.

MATERIALS AND METHODS

Tumor Samples

Eighty-six astrocytic tumors were obtained from 66 patients (34 males and 32 females) included in this study who underwent surgery in the department of Neurosurgery at the Nagasaki University Hospital, Nagasaki, Japan, from 1984 to 1995. Resected tumor samples were fixed in formalin and embedded in paraffin. Primary tumor tissue sections were not available from four patients with tumor recurrences. There were 62 primary and 24 recurrent tumors. The mean age of the patients was 54 years (range 1–80 years). The 5-μm tumor sections were used for histology (hematoxylin and eosin stain), and consecutive sections were stained immunohistochemically with antiprogesterone and Ki-67 antibodies.

Immunohistochemical Staining of PR

The deparaffinized tissue sections were placed in methanol containing 0.3% hydrogen peroxide for 30 minutes and washed in 0.05 M phosphate-buffered saline (PBS) (pH. 7.4) for 15 minutes. Tissue nonspecific activity was blocked by normal goat serum for 30 minutes, and sufficient primary antibody polyclonal rabbit antiprogesterone (3-CMO-BSA, Lot No. 071991w6; Chemicon Int. Co., Tamecula, CA) diluted 1:100 in PBS was applied and incubated for 1 hour at 37 °C. Subsequently, goat antirabbit immunoglobulin and rabbit peroxidase-antiperoxidase complex (Dako Co., Santa Barbara, CA), both diluted 1:100 in PBS, were applied and incubated for 1 hour at 37 °C. After each incubation, sections were washed in PBS for 15 minutes. The substrate chromogen 3,3′-diaminobenzidine-4 hydrochloride (Dojindo, Kumamoto, Japan) was applied for approximately 10 minutes. The sections were counterstained in Mayer's hematoxylin, dehydrated, and mounted. Two different breast carcinoma tissue sections served as positive controls. Appropriate negative controls were obtained by omitting the primary antibody, and comparison were made with the positive controls. Light microscopic visual assessment of PR positive cells was obtained as the percentage of total tumor cells in a given section. The entire tumor section was thoroughly searched for PR positive cells; the section was considered PR positive when at least 5% of the total tumor cells in that section were stained by the primary antibody.

Immunohistochemical Staining of Ki-67 Antigen

The staining method was essentially the same as described previously.[13] The deparaffinized tissue sections were rinsed in distilled water for rehydration. The sections were pretreated with 10 mM citrate buffer (pH. 6.0) in a domestic microoven (Hitachi MR-A330; Hitachi, Tokyo, Japan) for 15 minutes at 500 watts, after which the sections were cooled to room temperature. After pretreatment of the sections with 0.2% trypsin followed by a rinse in PBS, tissue endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 minutes. The subsequent steps were the same as for PR immunostaining, with the exception that the primary antibody was mouse monoclonal antibody MIB-1 ''paraffin Ki-67'' (Immunotech S.A., Marseille Cedex, France) diluted 1:100 in PBS and incubated overnight at 4 °C; the bridge antibody applied was biotinylated goat antimouse immunoglobulin (Vector Laboratories, Burlingame, CA) diluted 1:100 in PBS at room temperature for 1 hour. The avidin-biotin complex (Vector Laboratories) then was applied for 1 hour. The Ki-67 positive tumor cells were counted from the areas of a given tumor section in which the maximum numbers of tumor cells were immunostained. Both immunopositive and immunonegative cells from the ten (varied for available tumor cells) light microscopic high-power fields set with a video-monitoring screen were counted. The Ki-67 LI was obtained as the percentage of immunopositive cells from the total cells counted in the visualized fields of a given section.

Statistical Analysis

For statistical analysis, mean values and standard deviations (SD) were calculated; the *P* values were obtained by the chi-square test, Student's *t* test, Kruskal-

Wallis group test, and the log rank test (survival analysis).

RESULTS

Histology

The tumors were classified histologically according to the criteria of the World Health Organization classification of tumours of the central nervous system.[15] There were 9 pilocytic astrocytomas (Grade 1), 24 fibrillary/protoplasmic astrocytomas (Grade 2), 20 anaplastic astrocytomas (Grade 3), and 33 glioblastomas (Grade 4).

Immunoreactivity of PR in Astrocytic Tumors

Sixty-three (73%) of the 86 astrocytic tumors showed positive PR immunoreactivity. The PR positivity rates were 44% (4/9) in the pilocytic astrocytomas, 54% (13/24) in the Grade 2 astrocytomas, 75% (15/20) in the anaplastic astrocytomas, and 94% (31/33) in the glioblastomas (Table 1). There was a statistically significant higher positivity rate in high grade (3–4) compared with low grade (1–2) astrocytomas ($P < 0.005$, chi-square test). The PR staining patterns are shown in Figure 1. In most tumors, PR expression was observed either solely in the nucleus or solely in the cytoplasm. In the few tumor sections, PR was observed at the nuclear location in one area and at the cell cytoplasm in other areas of the same section, or both nuclear and cytoplasmic PR residing side by side (Figure 1D). In one of our positive controls, PR was identified solely at the nucleus and in the other, PR was cytoplasmic. In astrocytomas, small and large tumor cells; especially in the glioblastomas, the tumor cells in the pseudopalisading areas, mitotic cells, and multinucleated giant cells usually were PR positive. Cells of the microvascular endothelial proliferations (Figure 1E) and smooth muscle of the tumor vascular walls also were frequently PR positive.

In 44% (28/63) of the PR positive tumors, 60% of tumor cells strongly stained for PR, including 12 glioblastomas, 1 anaplastic astrocytoma, 3 Grade 2 astrocytomas, and 1 pilocytic astrocytoma in which more than 80% tumor cells were PR positive. The mean percentages (mean ± SD) of PR positive cells (Table 1) was 21.11 ± 29.34% in the pilocytic astrocytomas; 24.79 ± 29.32% in the Grade 2 astrocytomas; 32.0 ± 24.18% in the anaplastic astrocytomas; and 58.24 ± 22.13% in the glioblastomas. There was a statistically significant higher percentage of PR positive cells in the glioblastomas compared with the low grade (1–2) astrocytomas ($P < 0.0001$), and anaplastic astrocytomas ($P < 0.0008$, Student's t test). There were no statistically significant differences in the PR positivity rates and mean percentages of PR positive cells between the primary and recurrent tumors (Table 1). The PR positivity rate in the tumors from male patients was 72% and was 75% in tumors from female patients. The mean age of the patients with PR positive astrocytomas was 48.71 ± 21.95 years, and the mean age of the patients with PR negative astrocytomas was 37.09 ± 24.69 years; this age difference was statistically significant ($P < 0.04$). Again, the PR positivity rate in tumors from the patients age < 50 years was 48% (11/23), and that of tumors from the patients age > 50 years was 60% (38/63).

Ki-67 LI in Astrocytic Tumors

The staining pattern of the Ki-67 antigen is shown in Figure 1F, and the Ki-67 LI (mean ± SD) is shown in Table 2. The mean Ki-67 LI in pilocytic astrocytomas was 1.20 ± 1.56; in Grade 2 astrocytomas it was 1.78 ± 3.18; in anaplastic astrocytomas it was 13.47 ± 11.22; and in glioblastomas it was 15.69 ± 15.44. There was a statistically significantly higher Ki-67 LI in the anaplastic astrocytomas and in glioblastomas compared with the low grade astrocytomas (Grade 1–2; $P < 0.0001$). The relation between the Ki-67 LI and PR expression in the astrocytic tumors is shown in the Table 2. The mean Ki-67 LIs were 17.86 ± 16.46 and a mean of 2.0, respectively in the PR positive and PR negative glioblastomas; 14.89 ± 11.25 and 9.22 ± 11.18, respectively, in the PR positive and PR negative anaplastic astrocytomas; 2.68 ± 4.09 and 0.72 ± 0.89, respectively, in PR positive and PR negative Grade 2 astrocytomas; and 1.90 ± 1.60 and 0.64 ± 1.43 respectively, in PR positive and PR negative pilocytic astrocytomas. These differences showed no statistical significance. Again, tumors were graded as Grade 0: no or <5% positive cells; Grade 1: up to 25% positive cells; Grade 2: 25–50% positive cells; and Grade 3: > 50% positive cells (Table 2). Within the histologic grades of astrocytic tumors, mean tumor Ki-67 LI increased as the PR staining grade increased in astrocytomas with no statistical significance except in the glioblastomas ($P < 0.03$, Kruskal–Wallis four-groups test).

Tumor Recurrence, Patient Survival, and PR Expression in Astrocytic Tumors

Overall, PR expression in astrocytic tumors was not related to tumor recurrence and patient survival. Four patients with glioblastomas had at least one tumor recurrence, and all primary tumors were PR positive; the median time to first recurrence was 315 days. One of these four glioblastomas recurred three times as PR positive glioblastomas. Recurrent tumor samples from two glioblastoma patients were not available for analysis. The fourth patient had a PR positive glioblastoma at first recurrence and a PR negative glioblastoma at

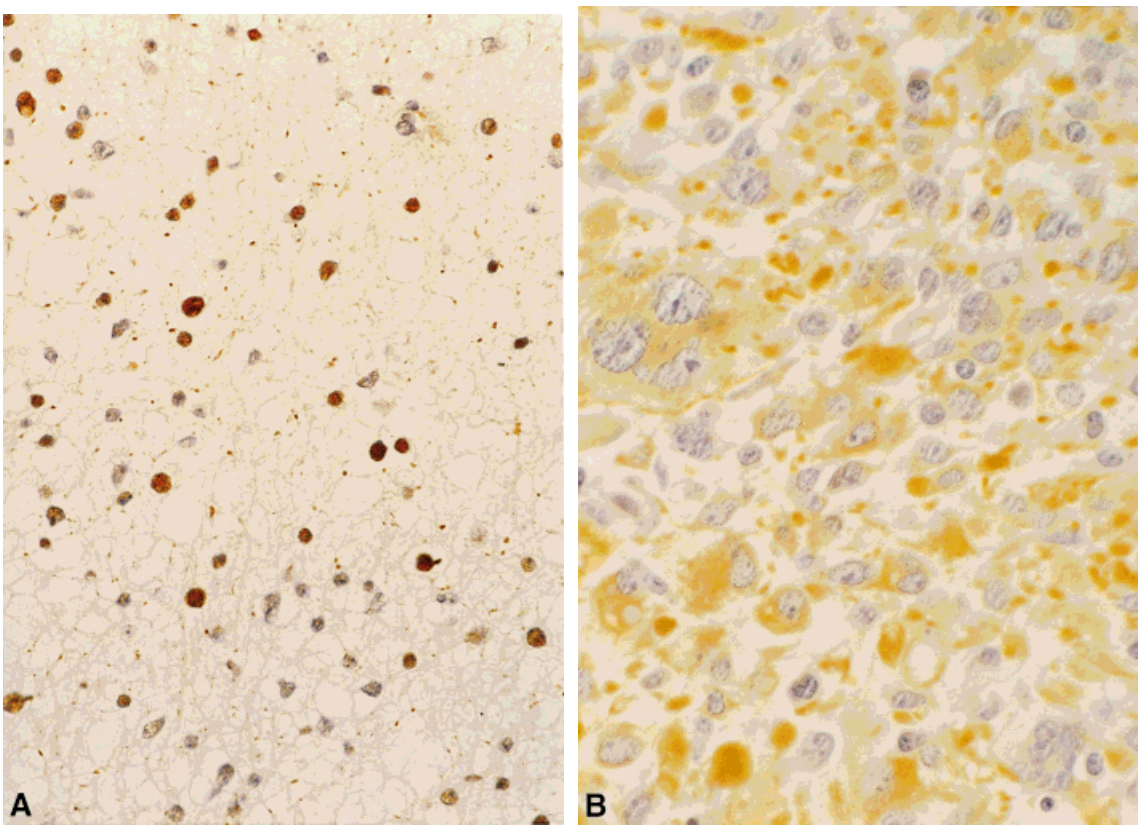

FIGURE 1. Representative sections of the astrocytic tumors immunostained with polyclonal rabbit antiprogesterone antibody. (A) nuclear progesterone receptor (PR) is shown in the Grade 2 astrocytoma; (B) cytoplasmic PR in the anaplastic astrocytoma; (C) nuclear PR in a glioblastoma; (D) cytoplasmic PR and a few nuclear PR in the glioblastoma pseudopalisading area; and (E) PR in the proliferative endovascular component of the glioblastoma. (F) Section of glioblastoma stained with MIB-1-Ki-67 antibody showed nuclear Ki-67 antigen. (PR, peroxidase antiperoxidase and Ki-67, avidin-biotin complex, original magnification, ×400).

second recurrence. Three patients with anaplastic astrocytoma had tumor recurrence, primary tumor tissues from two patients were not available for analysis, and the other primary tumor was PR negative. All recurrent anaplastic astrocytomas of these four patients were PR positive. Eight patients with primary Grade 2 astrocytomas had tumor recurrence at least once. Two PR positive Grade 2 astrocytomas recurred as anaplastic astrocytomas (one PR positive and one PR negative), and the other two Grade 2 astrocytomas (primary tumor tissues were not available for immunostain) recurred as PR negative anaplastic astrocytomas. Two PR positive and two PR negative tumors recurred as Grade 2 astrocytomas. One PR positive and one PR negative pilocytic astrocytoma recurred.

The patients were followed for at least 1 year; maximum follow-up time was 12 years. Our analysis of patient survival was restricted only to those patients without tumor recurrence. Survival data was available for 76% of patients with low grade (1–2) astrocytomas; of the patients, 82% were still alive at last follow-up. Two PR positive and one PR negative low grade astrocytoma patients died with survival times of 1541 mean days and 1527 days, respectively. Survival data were available for 31 patients with high grade (3–4) astrocytomas. In the group of PR staining Grade 0, there was only one patient (with a glioblastoma), who was still alive with a survival time of 953 days. The survival times of high grade astrocytoma patients was 364, 312, and 260 days with tumor PR staining of Grade 1, 2, and 3, respectively ($P = 0.441$).

DISCUSSION

To our knowledge, the current study appears to represent the largest series of astrocytic tumors immunostained for PR. We identified PR in 73% of the astrocytic tumors, with a significantly higher PR positivity rate in high grade (3–4) compared with the low grade (1–2) astrocytomas. The mean percentage of PR positive cells in the glioblastomas was significantly

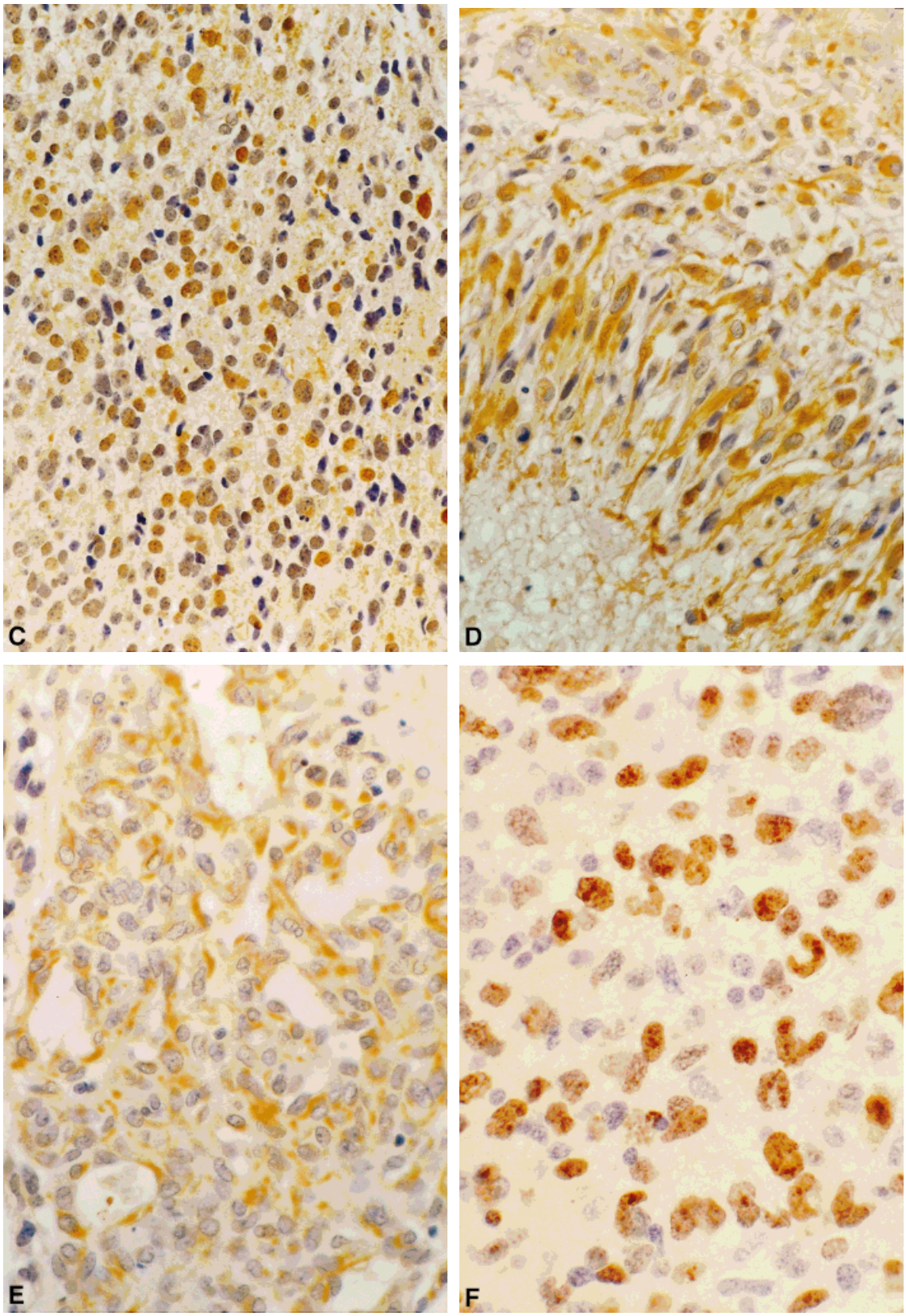

FIGURE 1. *(continued)*

TABLE 1
Expression of PR in 86 Astrocytic Tumors

Histology	Total no. of tumors	PR	
		Positive (%)	Positive cells (%)[a]
Pilocytic astrocytoma (Grade 1)			
Primary	6	3 (50)	15.0 ± 23.45
Recurrent	3	1 (33)	33.33 ± 41.63
Total	9	4 (44)	21.11 ± 29.34
Astrocytoma (Grade 2)			
Primary	17	9 (53)	28.24 ± 32.30
Recurrent	7	4 (57)	16.43 ± 19.94
Total	24	13 (54)	24.79 ± 29.32
Anaplastic astrocytoma (Grade 3)			
Primary	12	10 (83)	33.75 ± 21.55
Recurrent	8	5 (63)	29.38 ± 30.41
Total	20	15 (75)	32.00 ± 24.18
Glioblastoma (Grade 4)			
Primary	27	26 (96)	60.00 ± 24.55
Recurrent	6	5 (83)	50.00 ± 25.78
Total	33	31 (94)	58.24 ± 22.13[b]

PR: progesterone receptor.
[a] Mean ± standard deviation.
[b] Glioblastomas versus low grade (1 and 2) astrocytomas: $P < 0.0001$; and glioblastomas versus anaplastic astrocytomas: $P < 0.0008$ (Student's t test).

TABLE 2
Ki-67 Labeling Index and its Relationship to PR Expression in Astrocytic Tumors

Tumors	Ki-67 LI (Mean ± SD)
Pilocytic astrocytomas	1.20 ± 1.56
Astrocytoma (Grade 2)	1.78 ± 3.18
Anaplastic astrocytomas	13.47 ± 11.22[a]
Glioblastomas	15.69 ± 15.44[a]
PR staining grades[b]	
Pilocytic astrocytomas	
Grade 0 (5)	0.64 ± 1.43
1 (1)	0
2 (2)	2.8
3 (1)	2.0
Grade 2 astrocytomas	
Grade 0 (11)	0.72 ± 0.89
1 (6)	2.03 ± 4.04
2 (4)	3.57 ± 5.40
3 (3)	2.96 ± 3.05
Anaplastic astrocytomas	
Grade 0 (5)	9.22 ± 11.18
1 (8)	12.82 ± 11.01
2 (3)	18.47 ± 17.85
3 (4)	16.33 ± 8.26
Gliblastomas	
Grade 0 (2)	2.0
1 (3)	9.51 ± 3.66
2 (7)	14.19 ± 14.56
3 (21)	21.20 ± 18.50

PR: progesterone receptor; LI: labeling index; SD: standard deviation.
[a] Compared with low grade (1–2) astrocytomas: $P < 0.0001$ (Student's t test).
[b] Grade 0: no positive cells; Grade 1: up to 25% positive cells; Grade 2, 25–50% positive cells; and Grade 3, >50% positive cells.

higher than that in the other astrocytic tumors studied. The expression of PR in the astrocytic tumors also has been demonstrated by previous authors.[6–9,11] In their study, Carroll et al. did not find detectable PR mRNA in any of the 28 astrocytic tumors they analyzed by Northern blot analysis, but by using polymerase chain reaction-Southern blot analysis they detected strong bands of PR-mRNA in 62% of the glioblastomas and weak bands in 37% of anaplastic astrocytomas and 25% of low grade astrocytomas.[11] In the current study, we used the PR immunohistochemical method and the PR positivity rate in different grades of astrocytomas definitely was higher than the reported studies. Moreover, we noted not only the positivity rates but also the percentage of PR positive tumor cells and their areas of distribution in sections of astrocytic tumors. It has been suggested that the presence of steroid receptors in the malignant tissue may be the result of undifferentiation of cells and depression of the normally inactive gene.[16] Our results provide evidence that astrocytic tumors express PR and that in astrocytomas, the accumulation of PR increases as the tumor grade increases. This finding may indicate that PR has a significant role in the development of astrocytic tumors and toward the development of a malignant phenotype.

PR belongs to a superfamily of ligand-induced transactivators that exert their regulatory activity on discrete genes through DNA binding at multiple locations on specific sequences possessing a high level of dyad symmetry (hormone responsive elements).[17,18] They typically are located within several hundred base pairs upstream of the transcription initiation sites or alternatively within the coding region of the genes. After binding to specific sites in the promoter regions of the target genes, steroid receptors are able to elicit an increased rate of transcription initiation. In the current study, PR was observed in both the nucleus and the cytoplasm. By using the same antibody previously, we demonstrated the localization of PR in the cytoplasm and/or nucleus of human meningiomas.[3] Moreover, using the same antibody in immunoelectron microscopy we have detected PRs in the nucleolus, in the nucleus, and in the cell cytoplasm of meningiomas (unpublished data). The presence of PR in the cell cytoplasm of certain tumors has been demonstrated by other authors.[1,19] After entering the cell, the steroid hormones bind to the cytoplasmic receptor protein

and this complex then translocates to the nucleus. The interaction of the transformed complex with the nuclear chromatin determines the series of events leading to protein synthesis and/or cell proliferation.[20] The PR we detected in the astrocytomas meets the criteria of the classic two-step mechanism for steroid receptors that requires a cytoplasmic unoccupied receptor that after binding with progestin should translocate into the nucleus in occupied form. Hence PR in astrocytomas should be considered as functionally active to initiate cell proliferation.

In breast carcinoma, expression of PR correlates with better overall patient survival.[21] Another study[22] demonstrated that there was a trend toward a shorter disease free survival for PR positive primary/PR negative recurrent patients compared with PR negative primary/PR positive recurrent patients that may reflect a loss of hormonal regulation or an increase in disease aggressiveness. In the current study, we did not find any correlation between PR expression in astrocytic tumors, tumor recurrence, and patient survival. Further study is needed to clarify the role of PR in astrocytic tumor recurrence and patient survival.

The Ki-67 LI in astrocytic tumors noted in current study agreed with reported studies.[13,14] In their study Applanat et al.[23] did not find any correlation between the PR status and Ki-67 LI in human meningiomas. We found that PR positive astrocytic tumors had a relatively higher Ki-67 LI compared with the PR negative tumors and that the tumor Ki-67 LI increased as PR staining grade increased in astrocytomas; this tendency was observed in all grades of astrocytomas. Although all PR positive cells were not in the proliferative state as assessed by Ki-67 immunostain, our relative comparison suggests that expression of PR increases in parallel to the increased proliferative activity of astrocytic tumors and that PR has a profound effect in the growth of human astrocytomas. The increased PR expression in highly proliferative astrocytomas may stimulate the growth of malignancy and tumor aggressiveness, the degree of which may be determined by the percentage of PR positive cells in tumors.

Angiogenesis, the proliferation of capillary endothelial cells, is a vital component in the development, progression, and metastases of many human neoplasms.[24,25] Tumor secretes angiogenic peptides that contribute to tumor neovascularization[26] that provides the blood supply necessary for tumor growth. Among the astrocytic tumors, glioblastomas are highly malignant and the most neovascularized tumors. The overexpression of certain angiogenic factors such as acidic fibroblast growth factor, transforming growth factor (TGF-α and TGF-β), and epidermal growth factor (EGF) receptors in primary astrocytomas underlie the intense neovascularization,[27,28] of which TGF-β and EGF are female sex hormone-regulated growth factors.[29–32] In the current study, we found that most of the microvascular endothelial proliferations and tumor blood vessels expressed PR. These results suggest that the vascular endothelial cells secrete the PR that is involved in tumor angiogenesis most likely by interacting with the angiogenetic factors mentioned earlier. There are interactions between the sex steroid hormones and EGF receptors, and EGF expression in breast carcinoma is regulated by progestin and progestin action could be antagonized by EGF.[31,32] Progesterone can potentiate the action of EGF and other growth factors in the growth of cultured meningioma cells by increasing the number of receptors for the growth factors; EGF stimulation could possibly be completely prevented by the progesterone receptor antagonist RU38486 in cultured meningioma cells without progesterone.[30] RU38486 can act as an inhibitor of growth in PR positive cells via PR.[33] Human astrocytomas also express EGF receptors.[14,34] The possibility that there may be a good relationship between the EGF receptor and PR in the astrocytic tumors warrants future study.

The antiprogesterone mifepristone (RU486) has been used clinically with some success to treat patients with unresectable meningiomas.[35,36] It has been reported that in mice bearing a xenograft of a human glioblastoma cell line, treatment with RU486 for 4 weeks resulted in a significant reduction in tumor volume and weight.[37] The finding that antiprogesterone agents may be a complementary tool in adjuvant therapy in the treatment of unresectable, partially resected, or recurrent astrocytic tumors warrants additional study.

In conclusion, the current study demonstrated that the expression of PR in astrocytic tumors correlates with histologic grades. This study suggests that PR participates in the growth of astrocytic tumors and their angiogenesis. The high proliferative potential of PR positive tumors might have some impact on the treatment of these aggressive tumors.

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