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Two tumor markers, recoverin and vascular endothelial growth factor, have promise for application in patients with malignant glioma.

Cerebrospinal Fluid (Vascular Endothelial Growth Factor) and Serologic (Recoverin) Tumor Markers for Malignant Glioma

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Background: Clinically useful tumor markers have yet to be identified for malignant glioma. We report on two potential novel tumor markers, vascular endothelial growth factor (VEGF) and recoverin (protein A). VEGF is a highly specific endothelial cell activator that induces angiogenesis both *in vivo* and *in vitro*. Our study was designed to assess whether VEGF could be measured in the cerebrospinal fluid (CSF) of patients with cerebral neoplasms and used as a marker of particular tumors. We also studied serum recoverin levels in patients with various brain tumors and compared these to controls. Recoverin is a detectable serologic protein that is expressed in patients with cancer-associated retinopathy, a paraneoplastic syndrome.

Methods: In the VEGF arm, we used a solid-phase ELISA to determine the levels of VEGF. CSF samples from patients with anaplastic astrocytoma and glioblastoma multiforme (GBM) and with metastatic and nonastrocytic brain tumors were compared with nontumor control samples. In our recoverin study, an immunoenzymetric assay was used to measure the serum recoverin levels patients with glioma and compared with controls.

Results: In the VEGF arm, 89% of samples with malignant astrocytoma and 27% of nonastrocytoma samples had detectable levels of VEGF. VEGF was not detectable in normal CSF samples. The levels of VEGF were significantly higher in high-grade astrocytomas than in nonastrocytic tumors. Recoverin levels were 10-fold higher in patients with recurrent GBM relative to controls. In patients with low-grade glioma, anaplastic glioma, and GBM with no evidence of recurrence, a 3- to 5-fold increase was observed.

Conclusions: VEGF is detectable in CSF and may be a potential marker for differentiating astrocytic from nonastrocytic tumors. Recoverin is detectable in serum and may be a useful glioma tumor marker, especially for recurrent active disease. These markers may have application for tumor diagnosis, surveillance, and treatment response.

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Introduction

Astrocytomas are the most common primary brain tumors in humans. They are classified according to malignancy (astrocytoma, anaplastic astrocytoma, and glioblastoma multiforme [GBM]). The most recent statistics suggest that more than 18,000 new cases of brain cancer will be diagnosed in 2004 in the United States.¹ Despite advances in neuroimaging, microsurgical techniques, radiotherapy, and chemotherapy, the prognosis for these patients remains poor. With aggressive treatment, including surgical resection, focused radiotherapy, and systemic and/or local chemotherapy, the mean survival for these patients is still measured in months. Consequently, identifying areas of study to improve outcomes is an important focus of research.

One such area of research has been the identification of glioma tumor markers. In recent years, tumor markers have become an important area of cancer research. Advances in immunology, genetics, and immunohistochemistry have led to the discovery of more specific tumor markers, thus allowing for improved application of these markers in the treatment, surveillance, and diagnosis of patients with neoplasms. Despite these advances, no reliable marker has yet been identified for use in the diagnosis and surveillance of patients with malignant gliomas.² Moreover, tumor markers that can be readily obtained, quantified, and reproduced have proved elusive.

Allelic 1p chromosomal deletion has been shown to be a promising tumor marker in patients harboring anaplastic oligodendroglioma.^{3,4} However, despite high specificity, this test has limited sensitivity and is intensive and costly.⁵ Furthermore, it requires actual tumor tissue sample from a craniotomy or brain biopsy, making its usefulness in patient surveillance both cumbersome and difficult. A tumor marker from serum or cerebrospinal fluid (CSF) would be easier to collect, quantify, and reproduce and also more practical in clinical practice. In this paper, we report on our preliminary results of two tumor markers that may hold promise for application in patients with malignant glioma.

Materials and Methods

Patient Selection

Approval by the Institutional Review Board for both studies was obtained using the standard procedures and protocols of the Rhode Island Hospital. Patients were selected randomly from the Brain Tumor Service at our institute for both the vascular endothelial growth factor (VEGF) study and the recoverin study. Appropriate consent forms were signed.

VEGF

Samples of CSF were obtained either from the ventricular system in patients with external ventricular drains at

the time of surgery or by standard lumbar puncture at the time of hospital admission. Samples were obtained from 27 patients with high-grade astrocytomas, 39 patients with nonastrocytic central nervous system (CNS) neoplasms, and 14 patients with no known CNS neoplasm. Diagnosis was made via direct pathologic examination of biopsy tissue. Samples of CSF were collected and stored at -70° C until time of analysis. The samples were thawed on the day of assay.

We used the Quantikine Human VEGF Immunoassay (R&D Systems, Minneapolis, Minn) to determine VEGF

Table 1. — CSF VEGF Levels in Patients With High-Grade Astrocytomas

Patient No.	Diagnosis	Site	ng VEGF/mg TP
1	glioblastoma	unknown	0.00
2	anaplastic astrocytoma	lumbar	0.00
3	glioblastoma	lumbar	0.00
4	anaplastic astrocytoma	lumbar	0.08
5	glioblastoma	ventricular	0.41
6	glioblastoma	ventricular	0.87
7	glioblastoma	ventricular	0.90
8	glioblastoma	ventricular	0.98
9	glioblastoma	ventricular	1.22
10	glioblastoma	ventricular	1.48
11	glioblastoma	ventricular	1.58
12	glioblastoma	ventricular	2.05
13	glioblastoma	ventricular	2.38
14	glioblastoma	ventricular	2.67
15	glioblastoma	ventricular	2.85
16	glioblastoma	ventricular	4.49
17	glioblastoma	ventricular	6.47
18	glioblastoma	ventricular	6.53
19	glioblastoma	ventricular	7.14
20	glioblastoma	ventricular	8.24
21	glioblastoma	ventricular	8.60
22	glioblastoma	ventricular	8.79
23	glioblastoma	ventricular	9.06
24	glioblastoma	ventricular	10.11
25	glioblastoma	ventricular	11.51
26	glioblastoma	ventricular	16.57
27	glioblastoma	ventricular	17.08

CSF = cerebrospinal fluid
 VEGF = vascular endothelial growth factor
 TP = total protein

levels. Total protein (TP) content was determined using the BCA Protein Assay (Pierce Biotechnology, Inc, Rockville, Ill). Absorbances were read at 450 nm (VEGF) and 562 nm (TP), using a Multiskan Plus MK II spectrophotometer (Titertek Instruments Inc, Huntsville, Ala). The concentration of VEGF is expressed as ng VEGF/mg TP. Data are presented as mean \pm standard error. The student's *t*-test was used to compare means between groups.

Recoverin

We assayed the concentration of the calcium-binding protein p26 (recoverin) in the serum of 24 patients with histologically proven glioma. Included in the study were 5 patients with recurrent active GBM, 11 with anaplastic glioma, 6 with low-grade glioma, and 2 with GBM without clinical and radiographic signs of residual or recurrent disease at the time of testing. We also included a control group of 5 patients who had no known malignancy.

Blood samples were collected from all patients after an informed consent was obtained. The samples were refrigerated and brought to the laboratory for analysis the same day. Serum recoverin levels were determined using a competitive labeled antibody assay (Immunozyometric Assay for the Measurement of A-Protein in Plasma, Cytra Corp, Wrentham, Mass).

Results

VEGF

The level of VEGF in control subjects was exceptionally low (0.06 ± 0.06 ng/mg). Twelve of 14 control subjects had no detectable levels of VEGF. The two samples with detectable levels were noted to be grossly bloody. Twenty-four (89%) of 27 samples from patients with high-grade astrocytomas had detectable levels of VEGF ranging from 0.08 to 17.08 ng VEGF/mg TP with a mean value of 4.89 ± 0.96 ng VEGF/mg TP (Table 1).

A comparison of VEGF level and survival (days from diagnosis to death) yielded a correlation coefficient of 0.05. Ten (27%) of 37 samples from patients with nonastrocytic brain tumors had detectable levels of VEGF that ranged from 0.05 to 6.36 ng VEGF/mg TP with a mean value of 0.25 ± 0.17 ng/mg TP (Table 2). The positive samples included 4 lymphomas, 3 metastatic carcinomas, 1 melanoma, 1 myxopapillary ependymoma, and 1 radiation necrosis.

The level of VEGF detected in the CSF of patients with high-grade astrocytomas was significantly greater than those detected in the control group ($P=.00003$) and in patients with nonastrocytic tumors ($P=.00005$). VEGF levels in patients with nonastrocytic brain tumors were not significantly different from controls (Fig 1).

Table 2. — CSF VEGF Levels in Patients With Nonastrocytic Tumors

Patient No.	Diagnosis	Site	ng VEGF/mgTP
1	medulloblastoma	ventricular	0.00
2	medulloblastoma	ventricular	0.00
3	medulloblastoma	ventricular	0.00
4	metastatic carcinoma (prostate)	unknown	0.00
5	lymphoma	ventricular	0.00
6	lymphoma	ventricular	0.00
7	metastatic carcinoma (lung)	unknown	0.00
8	metastatic carcinoma	lumbar	0.00
9	lymphoma	lumbar	0.00
10	lymphoma	lumbar	0.33
11	lymphoma	unknown	0.00
12	metastatic carcinoma (breast)	ventricular	0.00
13	metastatic carcinoma (breast)	ventricular	0.00
14	metastatic carcinoma (colon)	unknown	0.00
15	pituitary adenoma	unknown	0.00
16	metastatic carcinoma (melanoma)	ventricular	0.00
17	metastatic carcinoma (lung)	unknown	0.00
18	metastatic carcinoma	unknown	0.00
19	metastatic carcinoma (lung)	lumbar	0.00
20	metastatic carcinoma (melanoma)	lumbar	0.00
21	metastatic carcinoma (melanoma)	lumbar	0.00
22	metastatic carcinoma	ventricular	0.00
23	metastatic carcinoma (melanoma)	unknown	0.00
24	metastatic carcinoma	unknown	0.00
25	metastatic carcinoma	unknown	0.00
26	metastatic carcinoma (lung)	unknown	0.00
27	metastatic carcinoma	lumbar	0.00
28	ependymoma	ventricular	0.00
29	metastatic carcinoma	ventricular	0.05
30	lymphoma	ventricular	0.08
31	lymphoma	ventricular	0.52
32	radiation necrosis	ventricular	0.11
33	metastatic carcinoma	ventricular	0.12
34	lymphoma	ventricular	0.16
35	metastatic carcinoma (lung)	ventricular	0.20
36	myxopapillary ependymoma	lumbar	1.31
37	metastatic carcinoma (melanoma)	ventricular	6.36

CSF = cerebrospinal fluid
VEGF = vascular endothelial growth factor
TP = total protein

Recoverin

We studied 24 patients with glioma (16 men and 8 women) with an average age of 47.5 years (Table 3). We found that the mean level of recoverin in the serum of patients with recurrent GBM was 33.3 pmol/L (range 27.4 to 41.2), which was approximately a 10-fold increase from the normal controls (mean 2.9 pmol/L, range 2.3 to 4.1). Serum levels of recoverin in patients with anaplastic gliomas showed a moderate increase (mean 9.8 pmol/L, range 7.0 to 14.9). Similar results were obtained for the patients with low-grade gliomas (mean 7.5 pmol/L, range 5.5 to 9.9). Patients with quiescent GBM had recoverin levels similar to that of the lower-grade tumors (mean 10.6 pmol/L, range 9.5 to 11.6). Fig 2 demonstrates the increase in the recoverin mean values, grouped according to the histologic grade of the tumors. Given the range of data and the relatively small size of the study, statistical significance was not demonstrable when comparing histologies except when comparing recurrent GBM with controls ($P=.005$ using student's *t*-test).

Discussion

Tumor marker is a term used for the biochemical measurement of a substance (eg, protein, genetic, chromosomal, amino acid, and metabolite) that is associated with a particular tumor. The marker can reside in any body fluid or cavity (eg, serum, urine, bile, CSF). Ideally, however, the fluid should be accessible, reproducible, and quantifiable.

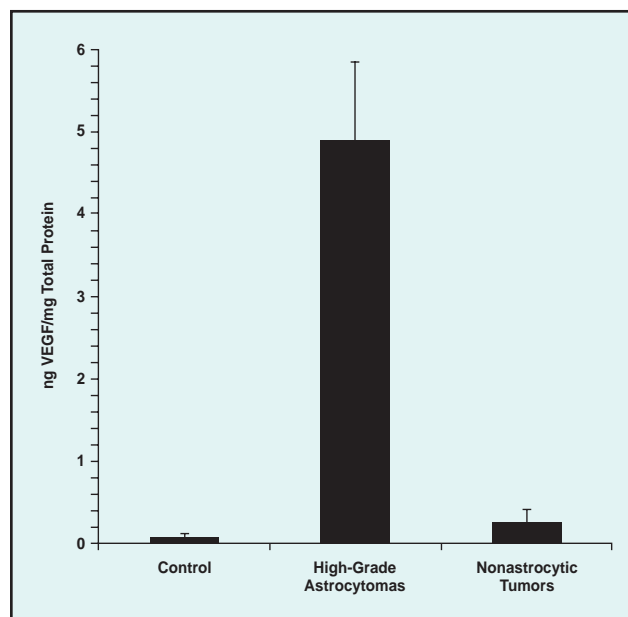


Fig 1. — Comparative vascular endothelial growth factor (VEGF) levels in cerebrospinal fluid (CSF). VEGF levels in the CSF of patients with high-grade gliomas ($n = 27$) are significantly higher than in control patients with no known central nervous system (CNS) neoplasm ($n = 14$, $P < .001$) or in patients with nonastrocytic tumors ($n = 37$, $P < .001$). VEGF levels in patients with nonastrocytic tumors did not differ significantly from control patients. Values are expressed as mean \pm SEM.

The most useful tumor marker should possess high specificity and sensitivity to the tumor, and it should be unique. For example, prostate-specific antigen (PSA) is a prototypical serum tumor marker that has a high specificity (96%) and moderate sensitivity (23%), and it is a serologic test that is easily obtained, is reproducible across different laboratories, and can be reliably measured. A PSA level of >10

Table 3. — Patient Demographics and Serum Recoverin Levels

Patient No.	Sex	Age	Diagnosis	Recoverin (pmol/L)
1	M	74	GBM	33.3
2	M	39	GBM	27.4
3	M	74	GBM	33
4	M	43	GBM	31.5
5	F	66	GBM	41.2
6	M	64	AO	14.1
7	F	36	AO	13.9
8	M	40	AO	7.1
9	M	54	AA	7
10	M	40	AO	4.7
11	M	56	AA	8.5
12	M	55	AO	14.9
13	M	39	AA	10.6
14	M	38	AA	9.6
15	F	45	AOA	8.1
16	F	38	AO	9.6
17	M	37	A	9.1
18	M	28	O	6.7
19	F	30	A	7
20	F	52	O	6.7
21	M	50	A	9.9
22	F	31	O	5.5
23	M	48	GBM-NERD	11.6
24	F	64	GBM-NERD	9.5
25	M	45	No tumor	2.3
26	F	36	No tumor	4.1
27	M	33	No tumor	2.8
28	M	42	No tumor	2.4
29	F	29	No tumor	2.8

GBM = glioblastoma multiforme (Kernohan's grade IV/IV)
 NERD = no evidence of recurrent disease
 Anaplastic glioma (Kernohan's grade II-III/IV): AO = anaplastic oligodendroglioma, AA = anaplastic astrocytoma, AOA = anaplastic oligoastrocytoma
 Low-grade glioma (Kernohan's grade I/IV): A = astrocytoma, O = oligodendroglioma

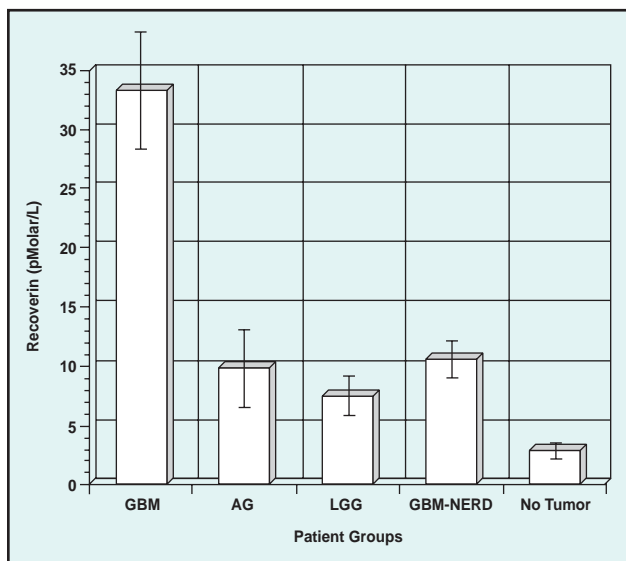


Fig 2. — Comparative serum recoverin levels in patients with active glioblastoma multiforme (GBM), anaplastic glioma (AG), low-grade glioma (LGG), GBM with no evidence of recurrent disease (GBM-NERD), and controls.

ng/mL has a high correlation with prostate cancer (67%).⁶ Consequently, this test can be used not only to confirm diagnosis, but also to monitor treatments and screen for cancer relapse. An ideal tumor marker may also be able to screen whole populations, especially high-risk groups, confirm histopathology, predict drug response, and provide a substrate target for further therapies.⁷⁻⁹

A biochemical substance that is specific to tumors is not a novel concept. In 1847, Sir Henry Bence Jones was the first to describe a urinary tumor marker as a method to confirm the diagnosis of a blood-born dyscrasia that resulted in kidney failure, wasting, and death. Many years later, the Bence Jones protein was found to be the IgG light chain associated with multiple myeloma. That same year, Sir Michael Foster described amylase as a marker for pancreatic cancer. The early part of the 20th century saw many advances in tumor marker biology. These discoveries revealed markers that were more specific and were associated with a vastly improved understanding of the pathophysiology of neoplasia. Among these markers were acid phosphatase (1930s) as a marker for prostate and bone cancers, urinary chorionic gonadotropins (1940s) for gestational trophoblastic neoplasms, and vanillylmandelic acid in the 1950s for neuroendocrine tumors.

The modern era of tumor markers has been influenced by the many technological advances in immunohistochemistry, molecular biology, genetics, and chromosomal and nuclear analysis.^{6,9} This explosion of knowledge has led investigators to develop more and more specific and sensitive markers for various cancers. Despite these advances, no reliable tumor markers have been described for malignant glioma. The reasons for this are numerous, including the relative low incidence of malignant glioma in the population, the lethality of most malignant gliomas (median survival for GBM is 13.2 months), the difficulty in

obtaining tissue samples to screen for potential tumor markers, and the limitations imposed by the blood-brain barrier to obtain serum samples of any potential tumor marker. However, the preliminary results of two important candidate tumor markers, VEGF and recoverin, show promise for clinical application in patients with malignant glioma. Given the great heterogeneity of malignant gliomas — hence, the nomenclature *glioblastoma multiforme* — these markers may prove to be more useful as tools for tumor surveillance and distinguishing tumor recurrence from radiation necrosis than as tools for diagnostic screening. Notwithstanding these limitations, both studies show a statistically significant increase in the levels of measured tumor marker (VEGF or recoverin) in malignant glioma than in nontumor controls. In addition, there appears to be a clear trend toward higher levels of tumor marker with increasing malignancy (Tables 2 and 3).

VEGF

VEGF is now well established as an important mediator of angiogenesis and the malignant progression of glial tumors.¹⁰⁻¹² Anaplastic astrocytoma and GBM are high-grade lesions and are characterized by an increase in cellular proliferation, mitotic activity, neovascularization, and necrosis. Several tumor cell lines, including glioma cell lines, have been shown both *in vivo* and *in vitro* to produce a variety of angiogenic growth factors such as the fibroblastic growth factors, vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), and platelet-derived growth factor (PDGF).¹³ Angiogenesis is now known to play an important role in tumor growth and metastasis. VEGF is a potent and highly specific endothelial cell mitogen with vascular permeability enhancing activities.^{14,15} *In situ* hybridization studies have shown a clear upregulation of VEGF mRNA activity in malignant gliomas, and VEGF levels are consistently highest in association with malignant gliomas.¹⁶⁻²⁰ Moreover, VEGF has been shown to have significant prognostic value when measured in resected tumor tissue of patients with astrocytomas.^{21,22} To date, attempts have failed to correlate the levels of VEGF found in tumor tissue and tumor cysts with the levels measured in serum.²² This is because VEGF levels in serum are highly variable, making them unreliable as tumor markers. Recently, however, VEGF has been shown to be not only measurable in the CSF of patients with metastatic brain tumors, but also a useful marker for carcinomatous meningitis.²³

Our study confirmed these earlier findings and demonstrated that VEGF is secreted into the CSF and can be detected in patients with primary or metastatic tumors of the brain. In contrast, VEGF appeared to be below the level of detectability in normal CSF (ie, patient controls). More important, we observed a statistically higher CSF VEGF level in patients with malignant glioma when compared to nonastrocytic tumors, making this a potentially

useful marker to distinguish brain metastases from primary brain tumors in patients who present with an unknown intracranial mass. This is somewhat surprising, given that metastatic tumors also express high levels of VEGF and may reflect a unique physiologic property of astrocytomas. However, a comparison of VEGF level and survival (days from diagnosis to death) yielded a correlation coefficient of 0.05, suggesting that VEGF levels in the CSF may not be of prognostic value. In addition, most samples were obtained from ventricular CSF. No definitive conclusion can be made regarding the correlation of ventricular vs lumbar VEGF levels in CSF. In light of the correlation between VEGF levels and carcinomatous meningitis, other factors, particularly the cytology of the samples, may be significant variables.²³ The paucity of patients with anaplastic astrocytomas (2 patients) also makes it difficult to assess the CSF VEGF level as a marker of increasing malignancy.

Recoverin

Recoverin is an intracellular signal transduction protein first described in the photoreceptor cells of the retina.²⁴⁻²⁶ The highest levels of expression are seen in the vitreous humor of the eye, but it can also be detected at low levels in the serum of normal adults. Recoverin was first identified by Thirkill et al²⁴ as a protein that binds antibodies expressed in patients with cancer-associated retinopathy (CAR), a paraneoplastic syndrome. As a result, recoverin gained interest as a potential serologic tumor marker. It was later suggested that recoverin, or cancer-associated retinopathy 3 (CAR-3) antigen, may be useful in distinguishing between neoplastic and chronic inflammatory disease of the pancreas, a tumor commonly known to cause paraneoplastic syndromes.²⁷ However, serum levels were elevated in only 51% of patients, thus limiting its potential use. Utilizing recoverin in other cancers has not been widely studied. Since the retina is technically an "outpouching" of the CNS, we postulated that serum levels of recoverin might also be elevated in patients with malignant glioma. The precise physiologic role of recoverin in gliomas is not known.

We found that serum recoverin levels were increased 10-fold in patients with active recurrent GBM compared with normal controls. A 2- to 5-fold increase was observed in the group of patients with low-grade or anaplastic glioma. These results suggest that recoverin levels correlate with the histologic grade of the tumor. Patients with GBM whose tumors were not growing (ie, quiescent tumors) had recoverin levels similar to patients with gliomas of lower histologic grade. Therefore, this assay may be particularly useful in following disease progression in patients with histologically confirmed glioma.

The advantages of a serum-derived tumor marker are evident. We found recoverin to possess similarities to PSA in its serum level profile: recoverin is expressed at low

levels (PSA <4 ng/mL in adult men; recoverin <4 pmol/L) in the blood of controls. Also, like PSA, recoverin in an intermediate level appears nonspecific for active tumor (PSA levels of 4-10 ng/mL are concerning but not specific for prostate cancer and may be secondary to trauma or infection; recoverin levels of 4-25 pmol/L may also be nonspecific). However, serum levels that are 5- to 10-fold higher than controls may have a higher association with active tumor (>10 ng/mL for PSA; >30 pmol/L for recoverin). Although our data are preliminary, this similar pattern of recoverin to PSA is intriguing. As with PSA, the 5- to 10-fold elevations may have the most predictive value for active tumor growth. We speculate that serum recoverin levels, as with other tumor marker levels, will be most useful as an adjunct to magnetic resonance imaging, rather than a screening tool, in following the course of individual patients with established malignant glioma.

Conclusions

Our results demonstrate that VEGF is secreted into the CSF and can be detected in patients with primary or metastatic tumors of the brain. It is below the level of detectability in normal CSF. The concentration of VEGF in CSF is higher in patients with high-grade astrocytomas than in those with nonastrocytic tumors. Therefore, VEGF levels in CSF may be a potential marker for the differentiation of astrocytic from nonastrocytic tumors of the CNS.

Serum recoverin level is a sensitive tumor marker for patients with glioma. Our data suggest that recoverin levels correlate with tumor histology. The specificity and sensitivity of this assay remains undetermined.

For both markers, we believe further investigation is warranted. In addition to larger patient analyses, future studies should focus on the extent to which these markers are elevated in other neoplastic and nonneoplastic diseases that involve the CNS and whether these markers can help differentiate between tumor regrowth and radiation necrosis. Correlating tumor tissue with CSF and serologic levels, as well as measuring sequential changes in tumor markers before and after surgery would also be important future areas of investigation.

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