

## Urinary Biomarkers Predict Brain Tumor Presence and Response to Therapy

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**Abstract** **Purpose:** A major difficulty in treating brain tumors is the lack of effective methods of identifying novel or recurrent disease. In this study, we have evaluated the efficacy of urinary matrix metalloproteinases (MMP) as diagnostic biomarkers for brain tumors.

**Experimental Design:** Urine, cerebrospinal fluid, and tissue specimens were collected from patients with brain tumors. Zymography, ELISA, and immunohistochemistry were used to characterize the presence of MMP-2, MMP-9, MMP-9/neutrophil gelatinase-associated lipocalin (NGAL), and vascular endothelial growth factor (VEGF). Results were compared between age- and sex-matched controls and subjected to univariate and multivariate statistical analyses.

**Results:** Evaluation of a specific panel of urinary biomarkers by ELISA showed significant elevations of MMP-2, MMP-9, MMP-9/NGAL, and VEGF (all  $P < 0.001$ ) in samples from brain tumor patients compared with controls. Multiplexing MMP-2 and VEGF provided superior accuracy compared with any other combination or individual biomarker. Receiver-operating characteristics curves for MMP-2 and VEGF showed excellent discrimination. Immunohistochemistry identified these same proteins in the source tumor tissue. A subset of patients with longitudinal follow-up revealed subsequent clearing of biomarkers after tumor resection.

**Conclusion:** We report, for the first time, the identification of a panel of urinary biomarkers that predicts the presence of brain tumors. These biomarkers correlate with presence of disease, decrease with treatment, and can be tracked from source tissue to urine. These data support the hypothesis that urinary MMPs and associated proteins are useful predictors of the presence of brain tumors and may provide a basis for a novel, noninvasive method to identify new brain tumors and monitor known tumors after treatment.

Brain tumors are the most common solid cancer of childhood and are currently the leading cause of death of children, excluding trauma (1–3). In adults, the number of primary and metastatic brain tumors is steadily climbing, whereas mortality rates for most other tumor types have remained essentially unchanged (4). These grim statistics underscore the increasing need for the development of innovative tools to improve the outcomes of patients with brain tumors.

One of the most difficult issues in treating brain tumors is a lack of effective methods to detect novel or recurrent disease.

Currently, there are no generally accepted screening protocols for the discovery of asymptomatic brain tumors, particularly primary brain tumors. Early detection of tumors in other organ systems, both novel and recurrent, has frequently resulted in markedly improved patient outcomes. It would therefore be desirable to recapitulate the successes achieved in other organ systems through the development of noninvasive biomarkers capable of identifying brain tumors.

Despite recent advances in the imaging and treatment of brain tumors, the ability to prospectively diagnose new tumors or to detect tumor recurrence remains poor. Our laboratory has had a longstanding interest in developing novel, noninvasive biomarkers for the detection of tumors and their recurrence. Remodeling of the extracellular matrix and dysregulation of angiogenesis (the process of new blood vessel formation) are processes essential to the development and maintenance of many tumors. Matrix metalloproteinases (MMP), a multigene family of degradative enzymes, have been implicated in the establishment and maintenance of the vasculature required for tumor progression and metastasis, as well as in the initial angiogenic phase of tumor growth in experimental models and human tumors (5–7). In the central nervous system, MMPs have been associated with brain tumor development. Studies of primary brain tumors reveal that MMP-2, MMP-9, and several other MMPs are overexpressed in both experimental models and tissue samples from human patients (8–15).

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Received 5/22/07; revised 10/10/07; accepted 11/9/07.

**Grant support:** NIH grants PO1CA45548 (M.A. Moses) and DK065298 (M.A. Moses), K12CA90354 (E.R. Smith), the Warner Family Foundation (M.A. Moses and E.R. Smith), the Fellows Fund (R.M. Scott and E.R. Smith), and the Tara Bean Foundation (E.R. Smith).

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doi:10.1158/1078-0432.CCR-07-1253

Recent studies from our laboratory, now confirmed by others, support the premise that tumor stage and progression correlate with urinary levels of MMPs (16–20). Urinary levels of MMP-2 (gelatinase A) and MMP-9 (gelatinase B), and their complexes are elevated in patients with a variety of cancers, both organ confined and metastatic, both within and outside the urogenital tract. These studies were the first to suggest that the measurement of MMPs and related biomarkers in the urine of affected patients might represent a novel, noninvasive method of detecting disease status, progression, and therapeutic efficacy (16–20). Given that (1) MMPs are present in brain tumors and (2) that urinary MMPs have shown utility as noninvasive biomarkers for noncentral nervous system cancers, we initiated this study to determine whether urinary MMPs might have potential as noninvasive biomarkers to detect the presence of brain tumors.

## Materials and Methods

### Patient population

Specimens from pediatric and adult patients were collected in accordance with protocols submitted to and approved by the Institutional Review Boards of both Children's Hospital Boston and Brigham and Women's Hospital (Boston, Massachusetts). The number of patients is based on all patients presenting with nonmetastatic brain tumors over a 1-y period who were willing to participate in the study. All tumor patients had intrinsic brain tumors with pathologic confirmation of diagnosis. Tumor diagnoses included both primary glial tumors (glioblastoma, anaplastic astrocytoma, fibrillary astrocytoma, and pilocytic astrocytoma;  $n = 12$ ) and other primary central nervous system tumors (meningioma, choroid plexus carcinoma, ependymoma, medulloblastoma, atypical teratoid rhabdoid tumor, primitive neuroectodermal tumor, ganglioglioma, hemangioblastoma, and craniopharyngioma;  $n = 16$ ).

All of the pediatric patients (age 18 y and under;  $n = 11$ ) presented with previously undiagnosed and untreated tumors and had urine specimens collected before surgery. All patients had tumor evident on magnetic resonance imaging studies at time of specimen collection. Cerebrospinal fluid (CSF) and tissue were collected at time of surgery, on the same day as that of the urine collection. No pediatric patients had been treated with any chemotherapeutic agents or radiation before collection. No pediatric patients had known histories of vascular malformations or recent surgery (within 3 mo of specimen collection).

Adult patients (ages >18 y;  $n = 17$ ) included both patients with previously undiagnosed and untreated tumors and also other patients who had undergone previous surgical procedures (such as stereotactic biopsy or subtotal resection of tumor) with bulk residual disease on magnetic resonance imaging. All patients had tumor evident on imaging studies at time of specimen collection. No adult patients were currently receiving radiation therapy or chemotherapy at the time of collection. No adult patients had known histories of vascular malformations or recent surgery (within 3 mo of specimen collection).

The control patients were healthy age- and sex-matched volunteers. Evaluated as either a single group or divided into the clinically relevant classifications of pediatric (ages  $\leq 18$  y) and adult (ages >18 y) subgroups, no statistically significant differences in age or sex between tumor and control groups were present. No control subjects had any known histories of tumors, vascular malformations, or recent surgery (within 3 mo of specimen collection). CSF was collected from patients undergoing surgery with known congenital nonneoplastic lesions, including Chari I malformations and tethered spinal cords with fatty filia. Normal tissue was obtained from brain resected as part of epilepsy surgery, distant from the site of the epileptogenic focus. No patients

were critically ill at the time of analysis. No patients had systemic injuries. No patients were receiving any antiangiogenic therapies at time of analysis.

### Urine and CSF collection

Urine and CSF were collected as part of an Institutional Review Board–approved protocol. Once collected, urine and CSF were transported on ice to our laboratory, stored at  $-20^{\circ}\text{C}$ , then analyzed for MMP activity via substrate gel electrophoresis (zymography) and ELISA for MMPs and vascular endothelial growth factor (VEGF), as previously described by us (16).

### Tissue collection

Tissue specimens were obtained from the Department of Pathology, Division of Neuropathology at Children's Hospital Boston in accordance with the Institutional Review Board–approved protocol. Representative slides of brain tumor tissue were selected and 5- $\mu\text{m}$ -thick sections were prepared from paraffin-embedded tissue for immunohistochemistry staining with appropriate negative and positive controls.

### Urinary and CSF MMP analysis

**Zymography.** We first used the classic method for detecting the presence and activity of MMPs, substrate gel electrophoresis or zymography (16). This detection system not only measures the activity of the MMPs present in a particular sample but simultaneously identifies the entire panel of enzymes in the same sample that are capable of degrading a specific substrate, in this case, gelatin. Enzyme activity is detected by a zone of clearance in the gel where the substrate has been digested by the enzyme.

Samples were frozen immediately after collection and stored frozen ( $-20^{\circ}\text{C}$ ) until assay. Aliquots of each sample were centrifuged at 4,000 rpm for 5 min at  $4^{\circ}\text{C}$  and the supernatants were collected. Urine samples (30  $\mu\text{L}$ ) were mixed with buffer consisting of 4% SDS, 0.15 mol/L Tris (pH 6.8), 20% (v/v) glycerol, and 0.5% (w/v) bromophenol blue. Samples were applied, without boiling, into wells of a 4% acrylamide Laemmli stacking gel/10% SDS-acrylamide separating gel containing 0.1% (w/v) gelatin (Life Technologies, Inc.) on a mini gel apparatus. Gels were run at 15 mA/gel during stacking and at 20 mA/gel during the resolving phase at room temperature. After electrophoresis, the gels were soaked in 2.5% Triton X-100 with gentle shaking for 30 min at ambient temperature with one change of detergent solution. The gels were then rinsed and incubated overnight at  $37^{\circ}\text{C}$  in substrate buffer [50 mmol/L Tris-HCl buffer (pH 8), 5 mmol/L  $\text{CaCl}_2$ , and 0.02%  $\text{NaN}_3$ ]. After incubation, gels were stained for 15 to 30 min in 0.5% Coomassie Blue R-250 in acetic acid, isopropyl alcohol, and water (1:3:6); destained in acetic acid, ethanol, and water (1:3:6), and photographed.

**ELISA.** ELISA (Quantikine kits; R&D Systems, Inc.) were used to quantify levels of MMP-2, MMP-9, MMP-9/NGAL, and VEGF. Specimens, standards and reagents were prepared according to manufacturer's instructions. Protein concentration was determined via the Bradford method using bovine serum albumin as the standard. Levels were determined as nanogram per milliliter (ng/mL) for MMP-2, MMP-9, and MMP-9/neutrophil gelatinase-associated lipocalin (NGAL) or picogram per liter (pg/L) for VEGF.

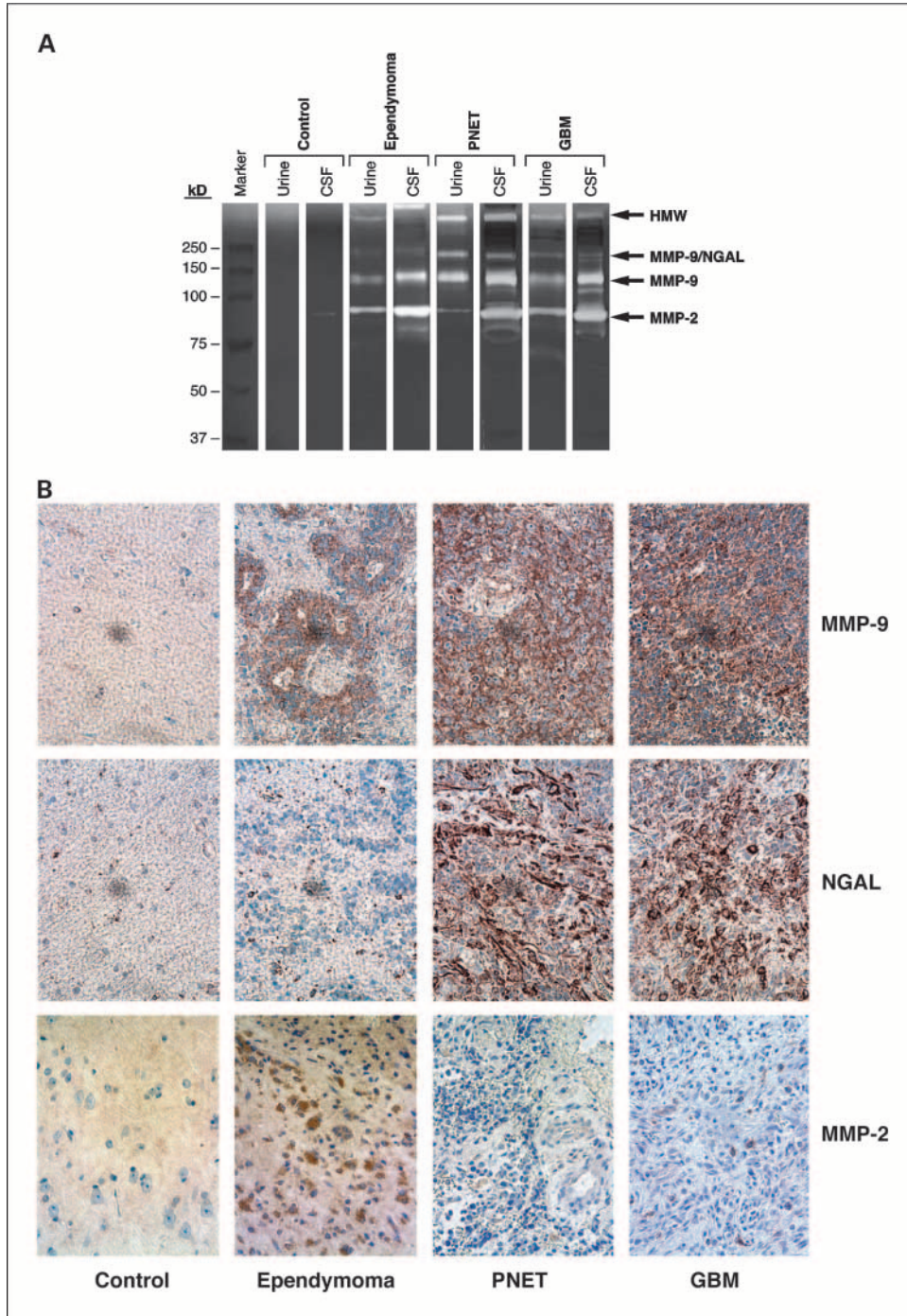
**Immunohistochemistry.** Immunohistochemical analysis of tumor tissue was conducted using monospecific antibodies against MMP-2, MMP-9, NGAL, or VEGF [MMP-2 (MAB 902; R&D Systems), MMP-9 (MAB 3309; Chemicon International), NGAL (MAB 1757; R&D Systems), VEGF (A-20 sc 152; Santa Cruz Biotechnology, Inc.), as previously reported by us (19)]. Five-micrometer-thick sections were cut from paraffin-embedded blocks of pathology specimens. Endogenous peroxidase activity was quenched with  $\text{H}_2\text{O}_2$  and the slides were then exposed to primary and secondary antibodies. All slides were blindly reviewed by a neuropathologist (AS) to confirm the adequacy of the material, the accuracy of the diagnosis, and immunohistochemistry results.

**Statistical analysis.** Patients with brain tumor and age-matched controls were compared with respect to urinary MMPs (ng/mL) and VEGF (pg/L) by the Mann-Whitney *U* test and presented using medians and interquartile ranges (21). Percentages of individuals positive for MMP-9, MMP-9/NGAL, and MMP-2 were compared using Fisher's exact test. Multiple logistic regression was applied to identify the combination of biomarkers that differentiate patients from controls (22). Receiver operating characteristic (ROC) curve analysis was done to calculate area under the curve for the independent predictors and to identify threshold values (i.e., cutoff points) that provide the optimal tradeoff between sensitivity of specificity. The likelihood ratio for a positive test was calculated as sensitivity/(1-specificity; ref. 23). AUC values and 95% confidence intervals (CI) were used to summarize

diagnostic performance of multivariate predictors (24). Statistical analysis was conducted using SPSS version 15.1 (SPSS, Inc.). Power analysis indicated that the sample sizes of brain tumor patients and controls provided 80% power ( $\alpha = 0.05$ ;  $\beta = 0.2$ ) to detect group differences in median levels of MMPs and VEGF (25, 26). Two-tailed *P* value of <0.05 were considered statistically significant.

**Results**

**Baseline characteristics.** Specimens from a total of 28 tumor patients and 23 control subjects were subjected to analysis. All patient specimens were initially analyzed as a single group. The



**Fig. 1.** Representative specimens from control subjects and patients with brain tumors were evaluated by (A) gelatin zymography (urine and CSF) and (B) immunohistochemistry (normal brain or tumor). Immunohistochemical analysis was done on tissue specimens from patients in this study. Brown staining of cells in prepared tissue specimens is indicative of immunoreactivity. Urine from the same patient was examined by zymography to identify the presence of active MMP species. There was a correlation between presence of a specific protein in the source tissue (as identified by immunohistochemistry) and the presence of enzyme in the urine, supporting the hypothesis that the tumor and surrounding tissue serves as the source of the proteins detected in the urine. Control specimens have no detectable MMP activity in the urine consistent with minimal immunoreactivity in the brain tissue. In contrast, the three brain tumor patient specimens show active MMP species in the urine on zymography, which correlates with increased amounts of MMP and NGAL staining in the tumor tissue. In addition, it seems that the relative intensity of staining for a given biomarker is consistent with the zymogram findings for the same individual protein. For example, the ependymoma sample exhibits stronger MMP-2 staining in the tissue and enzyme activity in the zymogram relative to the findings for MMP-2 in the PNET images. CTRL, control; EPND, ependymoma; PNET, primitive neuroectodermal tumor; GBM, glioblastoma multiforme; slide sections,  $\times 100$ .

**Table 1.** Demographics, MMPs, and VEGF for brain tumor patients and controls

Variable	Control group (n = 23)			Brain tumor group (n = 28)			P
	Median	IQR	Range	Median	IQR	Range	
Age (y)	22	4-55	0-71	32	8-58	1-73	0.37
MMP-9	0	0-0	0-6	0.6	0-5.7	0-294	<0.001*
MMP-9/NGAL †	0	0-0	0-12.8	0.8	0.2-1.0	0-1.1	<0.001*
MMP-2	0	0-0	0-3	4.3	0.8-8.8	0-27	<0.001*
VEGF ‡	25	0-250	0-391	753	451-957	25-4,462	<0.001*
Female sex, no. (%)		11 (48)			13 (46)		0.92

NOTE: Units are ng/mL for MMPs and pg/L for VEGF. MMPs, VEGF, and age were compared between groups with the Mann-Whitney *U* test and gender using the  $\chi^2$  test. This table provides the demographics (age and sex) of brain tumor patients and controls, in addition to levels of urinary levels of MMP-9, MMP-9/NGAL, MMP-2, and VEGF, as determined by ELISA. The median level, interquartile range, and overall range are presented, as are *P* values evaluating the significance of the difference between groups. This table reveals that the control and tumor groups are composed of individuals with statistically similar age and sex distributions, but that the control and tumor groups show statistically significant differences in urinary levels of MMP-9, MMP-9/NGAL, MMP-2, and VEGF.

Abbreviation: IQR, interquartile range.

\*Statistically significant.

† Based on 23 controls and nine brain tumor patients.

‡ Based on 19 controls and 21 brain tumor patients.

patient specimens were subsequently divided into the clinically relevant classifications of pediatric (ages  $\leq 18$  years;  $n = 11$ ) and adult (ages  $> 18$  years;  $n = 17$ ) and analyzed as adult and pediatric subgroups. There were no statistically significant differences in MMP or VEGF expression by age- or sex-based on ELISA data.

**Zymography.** Gelatin zymography was done on all urine and CSF specimens. MMP-2, MMP-9, MMP-9/NGAL, and other high molecular weight MMP species were detected in the urine and CSF of tumor patients. Control specimens had no detectable MMP activity except for trace MMP-2 bands, most commonly seen in the pediatric subjects, consistent with previous reports (27). MMP activity was detected in all tumor specimens, although not all species were present in all specimens. No control urine specimens had high molecular weight MMPs ( $> 92$  kDa), whereas high molecular weight MMPs were detected in the majority of tumor specimens.

Representative images of zymograms from several tumor patients and a control subject are displayed in Fig. 1. These data support the hypothesis that the presence of multiple MMP species and/or presence of high molecular weight MMPs are indicative of the presence of tumor (VEGF, one of the other biomarkers in this study, is not detectable by zymography.)

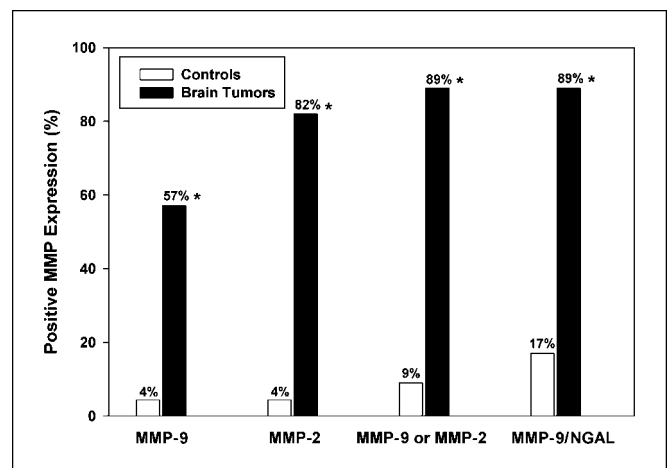
**ELISA.** Levels of MMP-2, MMP-9, MMP-9/NGAL, and VEGF in urine and CSF were quantitated using commercially available ELISA kits. Results were analyzed and tumor patients were compared with control subjects. As an initial analysis, the presence of any detectable level of urinary MMP-2, MMP-9, or MMP-9/NGAL by ELISA was detected with greater frequency in the urine of brain tumor patients than in the urine of the controls. Detailed analysis of the ELISA data revealed statistically significant increases in urinary levels of MMP-2, MMP-9, MMP-9/NGAL, and VEGF in tumor patients compared with control subjects.

Univariate comparisons between tumor patients and control subjects indicated no age or gender differences, whereas median levels of urinary MMPs and VEGF were significantly higher among tumor patients compared with control subjects ( $P < 0.001$ ). As shown in Table 1, median levels of urinary

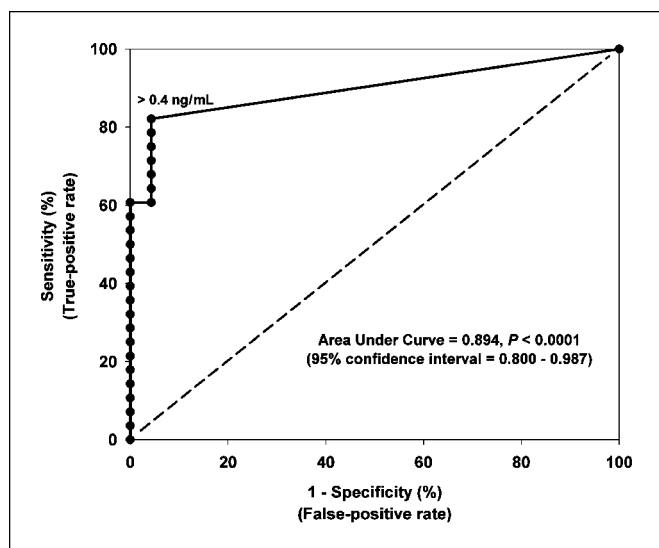
MMP-2 were 4.3 ng/mL (range, 0.8-8.8) for tumor patients and 0 ng/mL (range, 0-3) for controls. Median VEGF levels were 753 pg/L (range, 451-957) and 25 pg/L (range, 0-250) for tumor patients and control subjects, respectively.

Because the healthy pediatric population has been shown to exhibit low levels of urinary MMP-2 until puberty is completed, the data were also analyzed after subdividing the groups by age into pediatric (age 18 years and younger) and adult (age  $> 18$  years; ref. 27). With this subdivision, elevations in MMP-2, MMP-9, and VEGF levels remained significant in tumor patients relative to controls in both age groups. Given limited specimen availability, urinary MMP-9/NGAL data were not available for pediatric tumor patients, although urinary MMP-9/NGAL elevations in adult tumor patients were found to be significant relative to matched controls.

Despite clinical and surgical constraints, a limited number of CSF specimens were also available for analysis. Several patients did not have tumors located in regions near CSF cisterns, making CSF collection impractical. Other patients had surgical



**Fig. 2.** Urine of brain tumor patients contains significantly higher levels of MMP-2, MMP-9, and MMP-9/NGAL compared with control samples.



**Fig. 3.** Illustrates the ROC curve for MMP-2 along with the 45-degree line of nondiscrimination (*dashed line*). The curve identifies that the optimal threshold value for urinary MMP-2 is >0.4 ng/mL and shows excellent diagnostic performance in differentiating presence or absence of a brain tumor. The cutoff value of 0.4 ng/mL provides the best accuracy with an AUC of 0.894.

procedures associated with significant bleeding upon dural opening, thus contaminating the CSF with large volumes of blood. All available samples were tested for the presence of MMP-9, given the extensive literature documenting the association between increased MMP-9 expression and other pathologic conditions (see Discussion). CSF samples from patients with brain tumors were found to have significant elevations of MMP-9 relative to CSF samples from control subjects ( $P < 0.05$ ).

In urine, a significantly higher percentage of brain tumor patients were positive for MMP-9, MMP-2, and MMP-9/NGAL compared with controls (all  $P < 0.01$ ). Among the 28 tumor patients, 89% were positive for any MMP species, with 82% positive for MMP-2, 57% for MMP-9, and 89% for MMP-9/NGAL, whereas among the 23 controls, only 4% were positive for MMP-2 and MMP-9 and 17% were positive for MMP-9/NGAL (Fig. 2).

Multiple stepwise logistic regression analysis revealed that independent of age and sex, MMP-2 and VEGF were the two urinary biomarkers that significantly distinguished between brain tumor patients and control subjects. Results of the regression modeling clearly indicated that the multiplexed combination of both MMP-2 and VEGF provided significantly more diagnostic information in differentiating tumor from nontumor than either of the two biomarkers alone (both  $P < 0.001$ ). The multivariate model indicated that a urinary MMP-2 value of >0.4 ng/mL is 19 times more likely to indicate the presence of a tumor, and a urinary VEGF value of >350 pg/L is 9 times more likely to indicate tumor than nontumor, independent of age and sex.

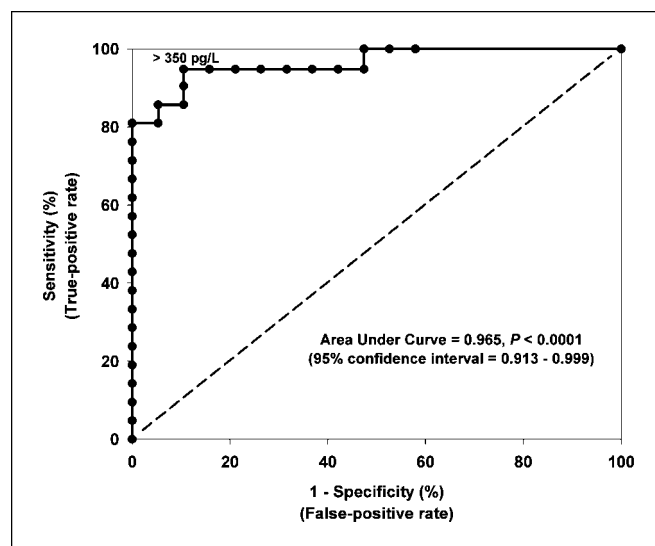
ROC analysis was applied to determine the optimal cutoff points for MMP-2 and VEGF for distinguishing tumor patients from controls. AUC for MMP-2 was 0.894, indicating excellent discrimination (95% CI, 0.800-0.987). The optimal threshold cutoff point was 0.4 ng/mL, which provided a sensitivity of 82.1% (23 of 28 tumor patients correctly classified) and a

specificity of 95.7% (22 of 23 controls correctly classified; Fig. 3). Overall accuracy using MMP-2 >0.4 ng/mL was 88.2%. The likelihood ratio of a positive test is defined as: sensitivity/(1-specificity) or  $82.1/4.3 = 19.1$ . This implies that a positive test result (i.e., >0.4 ng/mL) is >19 times more likely to be a true positive than a false positive. Urinary VEGF also provided excellent diagnostic accuracy (AUC, 0.965; 95% CI, 0.913-0.999). Based on the optimal cutoff value of 350 pg/L, sensitivity was 95.2% (20 of 21 tumor patients correctly classified) and specificity of 89.5% (17 of 19 controls correctly classified; Fig. 4). Overall accuracy using VEGF >350 pg/L was 92.5%. Likelihood ratio for a positive test for VEGF of >350 pg/L is  $95.2/10.5 = 9.1$ . Diagnostic performance for MMP-2 and VEGF is provided in Table 2.

Quantifiable measurement of VEGF levels and of the MMP species observed in ELISA corroborated the findings seen in the zymograms: that elevations of urinary levels of MMPs and VEGF are associated with the presence of brain tumors(16). These findings are statistically significant and are independent of patient age or sex.

**Immunohistochemistry.** A further objective of this study was to correlate expression of specific urinary biomarkers with expression of the same proteins in the tumor tissue—the putative source of the biomarker. To address this aim, individual tumor specimens were subjected to immunohistochemistry with monospecific antibodies to MMP-2, MMP-9, NGAL, or VEGF. Zymography data from the urine of the same patient was then compared with the tissue to determine whether high levels of enzyme activity in the zymogram were concordant with expression of the same protein in the tumor tissue by immunohistochemistry. All slides were blindly reviewed by a neuropathologist (AS) to confirm the accuracy of the diagnosis and immunohistochemistry results.

Expression of MMP-2, MMP-9, NGAL, and VEGF was detected in tumor tissue by immunohistochemistry. Presence or absence of tissue staining for a given protein correlated with



**Fig. 4.** Illustrates the ROC curve for VEGF. Overall accuracy using VEGF of >350 pg/L was 92.5%. This was complimented by logistic regression modeling in which a VEGF value of >350 pg/L is 9 times more likely to indicate the presence of a tumor than nontumor. The ROC curve showed an excellent diagnostic accuracy with AUC of 0.965.

**Table 2.** Results of ROC curve analysis and diagnostic performance indices of MMP-2 and VEGF

<b>Biomarker</b>	<b>AUC</b>	<b>95% CI</b>	<b>P</b>
MMP-2 (ng/mL)	0.894	0.800-0.987	<0.0001
VEGF (pg/L)	0.965	0.913-0.999	<0.0001
<b>Cutoff points</b>	<b>Sensitivity (95% CI)</b>	<b>Specificity (95% CI)</b>	<b>Accuracy (95% CI)</b>
MMP-2 >0.4 ng/mL	82.1 (65.2-94.0)	95.7 (78.1-99.9)	88.2 (76.1-95.6)
VEGF >350 pg/L	95.2 (76.2-99.9)	89.5 (66.9-98.8)	92.5 (80.0-98.5)

NOTE: This table presents the results of ROC curve analysis and diagnostic performance indices of urinary MMP-2 and VEGF as predictors of the presence of brain tumor in patients. The table reveals that clinically relevant cutoff points can be identified, with high levels of sensitivity and specificity for the detection of brain tumors.

presence or absence of the same protein in the zymogram. Variations in urinary expression of MMP species were reflective of the specificity of staining in tissue specimens; i.e., the expression of specific MMP species in a given tumor, as identified by immunohistochemistry, correlated with identification of activity of the same MMP species by zymography in the urine from the same patient (Fig. 1). These data revealed increased presence of MMP-2, MMP-9, NGAL, and/or VEGF in the tumor tissue compared with control tissue, supporting the hypothesis that differential expression of the biomarkers could be tracked from tumor tissue to urine sample.

**Longitudinal follow-up.** In a limited number of patients ( $n = 5$ ), urine specimens were collected at the time of tumor diagnosis and also at time points after surgical treatment of their disease. Each of these tumor patients had evidence of increased urinary MMP activity at time of diagnosis, as shown by zymography, compared with specimens from control subjects. Gross total resection of tumor was documented by imaging, both in the immediate postoperative period (within 72 hour) and at the 1 year postoperative time point. In all five patients, total resection of the tumor was associated with subsequent clearance of the urinary MMPs (Fig. 5). In all of these patients, 1 year follow-up imaging and urine specimens were available and showed continued correlation between radiographic cure and sustained absence of urinary MMPs. This effect was observed in a variety of tumors, including both benign (pilocytic astrocytoma) and malignant (choroid plexus carcinoma). These findings support the hypothesis that the tumor serves as the source of the urinary MMPs, i.e., when the tumor is present, increased MMP activity is detected in the urine by zymography; when the tumor is removed, the urinary MMP activity markedly decreases. The findings also support the hypothesis that urinary MMP monitoring may be a useful tool to predict therapeutic efficacy and tumor burden in known cancer patients.

## Discussion

Despite recent advances in the imaging and treatment of brain tumors, the ability to prospectively diagnose new tumors or to detect tumor recurrence remains poor. Unlike other organ-specific tumors that have pre-existing screening tests, such as prostate-specific antigen and digital rectal examination for prostate cancer; carcinoembryonic antigen, colonoscopy, and testing for fecal occult blood for colon cancer; and

mammography and breast exams for breast cancer, there is currently no method to prospectively detect brain tumors until they have progressed to the symptomatic stage. In addition, follow-up surveillance of residual or recurrent disease is a reactive process, responding to changes in the clinical exam or relying on detecting changes in periodic imaging studies.

MMPs are detectable in the urine of cancer patients (16) and correlate with the presence and stage of disease in a number of tumors (16–20). In primary brain tumors, MMP-2, MMP-9, and several other MMPs have been found to be overexpressed in both experimental models and tissue samples from human patients (8–12). The current study is the first to show that the detection of these proteases in the urine of cancer patients can predict disease status in brain cancer patients.

Increased MMP activity serves to facilitate both tumor cell migration and the development of new tumor-related blood vessels (28). The relationship between MMPs and new blood vessel development prompted us to search for the concomitant presence of vascular mitogens, including VEGF, in settings where MMPs are elevated. Examination of brain tissue specimens has shown that VEGF expression is present in a wide range of brain tumor types (29–34); however, evaluation of the utility of urinary VEGF levels as biomarkers for brain tumors has been virtually nonexistent, with the exception of limited preliminary data generated from our laboratory (17). The combination of a strong rationale linking MMP and VEGF expression coupled with encouraging preliminary data from our laboratory led to our inclusion of VEGF as one of the biomarkers in this study.

Use of urinary biomarkers as a means to screen for, and follow, known brain tumors has several appealing features. Collection of urine is risk free and easy for the patient, in contrast to many radiographic studies. In particular, urine sampling avoids the need for sedation and its attendant risks, which are often required to accomplish radiographic studies in the pediatric population. Urinary sample collection and analysis is considerably less expensive than magnetic resonance imaging, with a 10- to 100-fold difference in cost at our institution. Sampling of urine could easily be done at shorter intervals than are currently practical for imaging studies, enabling earlier detection of recurrent disease. Significantly, the detection of MMPs is an assay of biological activity and, as such, is intrinsically different than standard imaging studies, most of which evaluate anatomic findings. At a minimum, measurement of urinary biomarker levels could provide an alternative means

of evaluating the presence or recurrence of a brain tumor, complementing existing techniques. At best, it could provide a more sensitive and responsive tool for identifying and monitoring brain tumors than what currently exists.

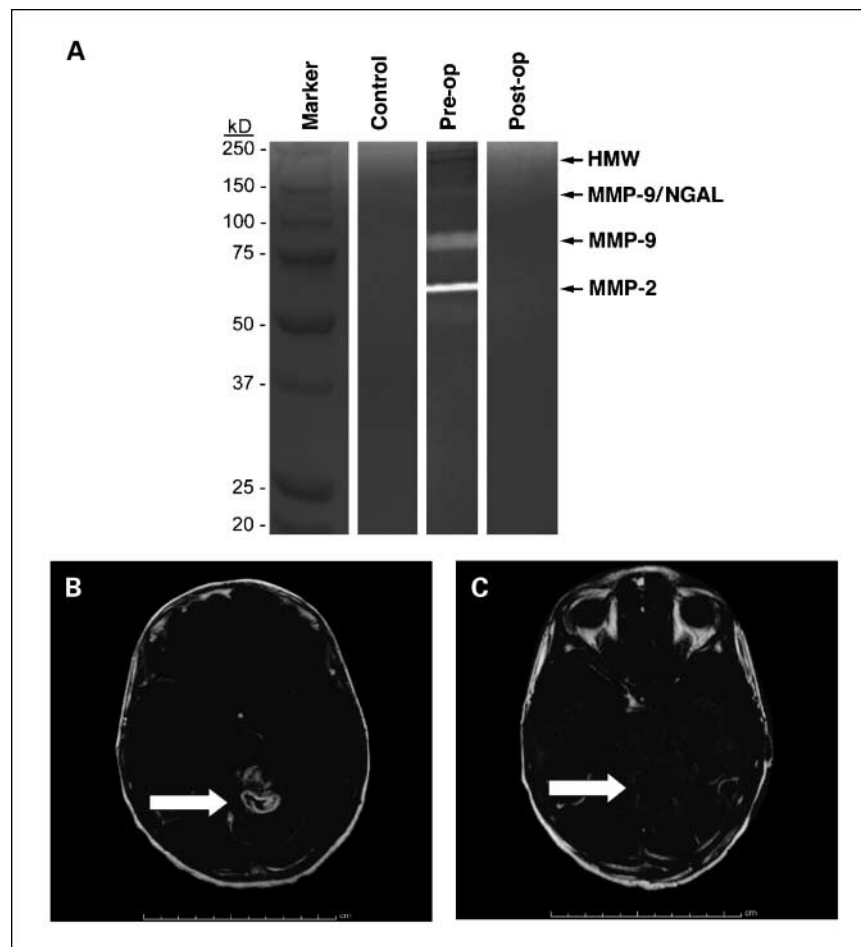
Implicit in the hypothesis that urinary biomarkers may be used to identify and follow brain tumors is the assumption that the elevation of the levels of these proteins is directly related to the presence of tumor. As such, two principles can be investigated to (35–37) test the validity of this assumption. First, the putative biomarkers should be present in the source tissue, presumably at increased levels compared with control tissue. Second, if the tumor is causative for the elevated levels of MMPs, then resection of the tumor should result in clearing of MMPs in the urine. We directly evaluated both assumptions in this study and find that the evidence supports our hypothesis. First, to determine whether the abnormal elevation of MMPs is related to the presence of the tumor, we obtained pathologic specimens from tumors in this series and matched specimens from normal brain. The tissues were analyzed for the presence of MMP-2, MMP-9, NGAL, and VEGF, proteins found to be elevated in the urine samples from the tumor patients. If the tumor is the source of the biomarkers, one would expect (1) to find these proteins present in the tumor tissue and (2) to detect them in lower amounts or absent in normal tissue, as was the case in our patients.

Our immunohistochemistry studies showed two important findings in support of our working hypothesis. First, as has

been reported by other groups, MMP-2, MMP-9, and VEGF are detectable in the tumor tissue, and expression was markedly lower or absent in control tissue (13, 38–42). Importantly, NGAL immunohistochemical evaluation of brain tumors has thus far only been described in this study and in a recent report from our group (43). Second, we report here the novel finding that the expression patterns of MMPs detected in the urine of tumor patients and control subjects mirror the staining patterns in the source tissue. Tumor patients with less MMP-2 activity in the urine had less MMP-2 staining in the tumor tissue, whereas tumor patients with greater MMP-9 activity in the urine had more MMP-9 staining in the pathology specimens.

Second, to show that these biomarkers should be elevated in the presence of tumor and then decline in the absence of tumor, we obtained long-term follow-up of urinary MMP levels from five patients in this study. In all cases, successful surgical treatments of the individual tumors were achieved with gross total resections, resulting in normalization of the MMP findings by zymography at 1 year postoperatively. Longitudinal follow-up samples show the absence of detectable MMP activity, that is, surgical and radiographic resection of a tumor results in subsequent clearing of the putative biomarker, a response durable at 1 year after surgery.

As mentioned above, the population of tumor types present in our population is heterogeneous. Although ongoing recruitment will ultimately allow more detailed subset analysis by tumor type, we were encouraged that our initial findings



**Fig. 5.** The loss of urinary MMPs after resection of a brain tumor, demonstrating that tumor presence is related to increased urinary MMP activity and removal of that tumor correlates with subsequent clearing of detectable urinary MMP activity. **A**, urine from a patient with a brain tumor (*preop*) contains MMP-2 and MMP-9 with strong zones of clearance indicating enzyme activity in the urine when the tumor is present (as seen in the preoperative magnetic resonance imaging; **B**). One year after a gross total resection of the tumor, with no residual/recurrent disease seen on the 1 y follow-up scan, the urinary zymogram reveals no observable MMP activity. The tumor of this patient is shown in the top axial T<sub>1</sub> postgadolinium magnetic resonance imaging (**B**; *arrow*) with the 1 y follow-up scan next to (**C**), showing no tumor in the resection cavity at the time that the postoperative urine was analyzed.

were validated in a wide range of brain tumor types. The heterogeneity of our population accurately reflects the disparate range of tumor types and patients seen in a tertiary care brain tumor clinic. The biomarkers that we have studied were selected because they are believed to be contributors to a general process common to the development of many tumors. As such, it would be expected to find that these putative biomarkers would be present across a wide range of tumors.

Although much of the data revealed by this work is promising, there are clearly a number of limitations inherent to this study. First, as a function of the challenge of obtaining tumor tissue, CSF, and urine from the same patient, it was difficult to obtain large sample numbers. Second, the tumors within the group are heterogeneous in type. Third, not all patients have long-term follow up or serial specimens available for analysis. Our investigations of these patients are ongoing with continued accrual of subjects, but we chose to present these data now as a proof of principle study. Even in our large tumor referral centers, not all patients met entry criteria or were willing to participate, and at times, we have been limited by the amount of specimen available for analysis. For example, some tumors were present in locations that did not provide adequate CSF. Finally, the preliminary nature of the current proof-of-principle study warrants further validation with larger patient samples followed longitudinally. Despite the limitations inherent to this population, we have successfully subjected the results from these patients to rigorous statistical analysis compared with appropriately matched controls to obtain significant and meaningful results.

This report documents our initial experience in assessing the utility of a panel of urinary biomarkers, MMP-2, MMP-9,

MMP-9/NGAL, and VEGF, as predictors of the presence or absence of brain tumors. Through the use of zymography, ELISA, and immunohistochemistry, we show that (a) MMP-2, MMP-9, MMP-9/NGAL, and VEGF can be detected in the urine of brain tumor patients; (b) levels of these proteins could predict the presence or absence of brain tumors with high degrees of sensitivity and specificity compared with control subjects; (c) these same proteins could be identified in the tumor tissue at levels reflective of those found in the urine, supporting the hypothesis that the proteins can be tracked from the tumor tissue to the urine; and (d) resection of the tumor results in subsequent clearance of the urinary biomarkers, a finding that is durable and consistent.

Early results from our ongoing protocol evaluating the role of urinary MMPs as noninvasive biomarkers for brain tumors are encouraging, particularly in light of this uniquely challenging cohort of cancer patients. These findings suggest that elevated urinary MMPs and VEGF may be indicative of the presence or recurrence of a brain tumor and that monitoring urinary levels of these proteins may have potential utility for improving the ability of clinicians to predict the presence of brain tumors. Moreover, the finding that the combination of MMP-2 and VEGF provided superior accuracy in predicting tumor presence compared with any biomarker alone substantiates the premise that biomarker accuracy can be improved through multiplexing. Ultimately, we envision that routine sampling of urinary MMPs and other biomarkers may enhance current methods of brain tumor detection and follow-up by facilitating earlier detection of both novel and recurrent disease through noninvasive surveillance for abnormal urinary biomarker profiles.

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