Gene Expression Microarray Analysis Reveals YKL-40 to Be a Potential Serum Marker for Malignant Character in Human Glioma

Meena K. Tanwar, Mark R. Gilbert, and Eric C. Holland

INTRODUCTION

GBM is the most common and most malignant primary brain tumor in adults (1). Primary GBMs arise de novo with no evidence of previous lower-grade glioma. Secondary GBMs arise from pre-existing anaplastic astrocytomas, grade III glioma, or diffuse astrocytoma, grade II. Of the gliomas, grade IV GBM has the worst prognosis with a median survival of ~12 months, even after surgical resection, radiation therapy, and chemotherapy. Patients with anaplastic astrocytomas have an average survival of 3 years, whereas patients with grade II gliomas have the best prognosis, with >5 years median survival (2). Histologically, GBMs are characterized by high mitotic activity, nuclear atypia, microvascular proliferation, and necrosis. However, in the future, molecular subgrouping of the gliomas could aid in the classification of these tumors and may eventually help to better predict survival (3).

Several distinct prognostic subgroups exist within each of these clinical grades, and consequently the survival of glioma patients can be quite variable (4, 5). Currently, the determination of treatment response and tumor progression is monitored using imaging techniques such as molecular resonance imaging or computed tomography. Often extensive tumor progression occurs in the interval between different imaging studies and/or goes undetected from lack of contrast enhancement. An alternative measure or marker of tumor burden may potentially permit early detection of treatment failure and allow for more rapid changes in therapeutic strategy.

Development of serum markers that are specific for a particular tumor type (6) or that correlate with biological properties of the tumor could be used for diagnosis and as a measure of response to treatment, and would greatly enhance patient care. If serum markers were identified that correlate with the activity of certain signaling pathways within the tumor, patients could be directed to clinical trials using specific agents aimed at that particular tumor biology. With such a set of markers that could predict and monitor response to therapy, patients would be better advised of the likely benefits of aggressive treatment. One method for identification of candidate markers is using gene expression microarray analysis (7, 8). Although many studies have been done on gene expression profiling of specific tumor types, and differentially expressed genes in these tumors have been reported, few of these studies have resulted in clinical application.

To identify potential serum markers as genes overexpressed in GBMs relative to lower-grade gliomas and normal brain, we performed cDNA microarray gene expression profiling of a series of GBMs and lower-grade gliomas. In this analysis, we found that the most differentially expressed gene in the series of GBMs versus normal brain was YKL-40. YKL-40 encodes a secreted protein with sequence similarity to glycosyl hydrolases (9) and has been identified previously as being expressed in other cancer types such as breast and colon (10, 11). Little to no differential expression of YKL-40 was seen in lower-grade tumors relative to normal brain. The expression of YKL-40 protein, confirmed by Western blot analysis of tumor extracts, was also specific to GBMs and undetectable in lower-grade gliomas and normal brain. To ascertain whether YKL-40 could be an accurate marker for GBM presence and tumor burden, we measured YKL-40 serum levels in patients diagnosed with various types and grades of glioma, using a YKL-40-specific ELISA assay. Our data indicate a significant difference in YKL-40 serum concentrations between normal subjects and GBM patients, as well as between lower-grade glioma and GBM patients. Furthermore, there was a trend toward high YKL-40 levels and high tumor burden, although this did not reach statistical significance. These preliminary data suggest that YKL-40 levels may be an efficient and accurate indicator of glioma presence and grade.

MATERIALS AND METHODS

Tissue Collection. Patient tumor and normal brain samples were collected from patients at M.D. Anderson Cancer Center at the time of surgical resection. Normal brain sample controls were obtained as overlying cortex during routine resection of deep intercerebral metastases. Specimens were snap frozen in liquid nitrogen and stored at −80°C until use. All of the patients signed consent for collection and analysis of samples.

Tissue Preparation and Microarray Analysis. For RNA and protein extraction, the tissue was ground to powder using a mortar and pestle in the presence of liquid nitrogen. Nine grade IV GBMs, 5 grade III gliomas, and 5 grade II gliomas were used in the array analysis. RNA was isolated from 100 to 400 mg of tissue per sample. Standard TRIzol preparation protocol (Life
Technologies, Inc., Rockville, MD) and reagents were used for total RNA isolation. To isolate polyadenylated RNA, the total RNA was twice passed over an Oligotex mRNA isolation column (Qiagen, Valencia, CA) following the manufacturer's protocol. The RNA samples were quantified using a spectrophotometer and visualized on a Tris-borate EDTA gel for quality assurance. The mRNA samples were converted to cDNA and subsequently hybridized to the Human V cDNA microarray by Incyte Genomics (St. Louis, MO). The data were analyzed using GEM Tools 2.5 software.

**Protein Extraction.** Sample collection and preparation was performed as described above. Protein extraction was performed using lysis buffer [100 mM NaCl, 30 mM Tris-HCl (pH 7.6), 1% NP40, 30 mM NaF, 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture tablets (Roche, Indianapolis, IN), and the tissue was homogenized on ice using a sterile, plastic disposable pestle. The samples were allowed to incubate on ice for 30 min; then centrifuged at 14,000 × g for 10 min at 4°C. The supernatant was transferred to a fresh microfuge tube, and protein quantitation was performed using the BCA Protein Assay kit (Pierce, Rockford, IL) and a standard spectrophotometer.

**Western Blot Analysis.** Fifty micrograms of each protein sample was separated on an SDS-PAGE gel. The protein samples were transferred overnight to nitrocellulose membrane (Osmonics Fischer-Scientific, Pittsburgh, PA). For protein detection, the membrane was incubated in 5% milk in TBS-T for 1 h at room temperature. The membrane was then incubated for 1 h with 4 mg of YKL-40 polyclonal antibody (Metry Biosystems, Mountain View, CA) in 5% dried milk-TBS-T. The membrane was then washed three times in TBS-T, 5 min each, and incubated with the secondary antirabbit antibody. 1:2000 dilution (Roche) for 1 h. Again the membrane was washed three times in TBS-T, and antibody-bound protein was detected by adding enhanced chemiluminescence reagent (Amersham Pharmacia Biotechnology, Piscataway, NJ) for 1 min and exposing the membrane to Kodak film.

**Blood Collection and Serum Separation.** All of the blood samples were collected from patients at M.D. Anderson Cancer Center diagnosed with glioma through collaboration with the Neuro-Oncology Clinic. Normal blood samples were collected from volunteer subjects with no known malignancies. The blood samples were allowed to clot at room temperature for no more than 72 h, and were then centrifuged at 4°C for 5 min at 1000 rpm. The serum (upper phase) was aliquoted and stored at −20°C until use.

**YKL-40 ELISA Assay.** YKL-40 levels were determined, in duplicate, for all of the serum samples, using the YKL-40 ELISA kit from Metry Biosystems according to the manufacturer's protocol. Protein concentrations were determined as absorbances using the Bio-Rad Benchmark Microplate Reader.

**Statistical Analysis.** The histological grade of each glioma according to the WHO criteria, as well as the patient disease status was determined for each sample collected. Disease status was stratified into a high tumor burden group and a low tumor burden group. Patients with newly diagnosed tumors awaiting initial surgery or who underwent only stereotactic biopsy were considered to have a high tumor burden. Patients with radiographic evidence of recurrent tumor growth were also included in this group. Patients who underwent a gross total or near total resection, had no active disease, or had evidence of response to treatment were placed in the low tumor burden group. Statistical analysis of data in each group was done using Student's t test.

**RESULTS**

**Experimental Design.** We were interested in studying gene expression changes that occur in malignant and nonmalignant gliomas compared with normal brain tissue to find potential clinical markers for this disease. Our approach was to start with a microarray analysis using the Incyte Genomics Human V cDNA microarray. Tumor mRNA samples were labeled with cy-5 and hybridized against the cy-3-labeled normal mRNA control. For this purpose, we chose to pool samples of normal brain mRNA, generating a constant control to be used on each chip analyzed (Fig. 1). The normal brain samples were obtained from patients with deep metastatic brain tumors, cases in which removal of overlying normal cortex was required for safe resection of the metastatic tumor. mRNA was isolated from normal frontal, temporal, parietal, and occipital cortex samples, and combined to make one common control. To preserve the quality of the mRNA during tissue collection, all of the specimens were snap frozen in liquid nitrogen immediately after removal from the patient during surgery and subsequently stored at −80°C.

Because each tumor mRNA sample was normalized against a pooled control, we were able to compare gene expression changes across different tumors. The differential expression patterns obtained were the comparative value of gene expression in a given tumor versus normal brain and represented an elevation or depression of gene expression in tumor tissue over normal tissue. For a given array hybridization, >97% of genes analyzed fell within a 2-fold variance between tumor and normal brain (Fig. 2A).

**YKL-40 Is Overexpressed in a Subset of GBMs.** There were a number of genes significantly up-regulated in the GBM samples but not in the lower-grade tumors, compared with the normal control. Of these genes, the most highly elevated gene expression was seen for chitinase-3-like-1, also known as human cartilage glycoprotein-39 and YKL-40 (Fig. 2B). YKL-40 mRNA has also been shown to be elevated in GBM samples by serial analysis of gene expression analysis (12) and by oligonucleotide array analysis (13). These independent methods of determining gene expression levels additionally validate our findings.

Review of the literature reveals that the gene for YKL-40 is located on chromosome 1q32 and is a mammalian member of family 18 glycosyl hydrolases, a gene family that includes bacterial and fungal chitinases (9, 14). YKL-40 has significant sequence similarity to the chitin-degrading enzyme, chitinase; it has been shown to bind chitin but retains no chitinase activity and has not been determined to have any other enzymatic activity or function (15). The full spectrum of mammalian polysaccharide structures that bind to YKL-40 and the function of this protein in human glioma biology are unknown. YKL-40 is secreted by chondrocytes, synovial cells, neutrophils, and the osteosarcoma cell line MG-63 (9, 16, 17).

Several lines of circumstantial evidence indicate that YKL-40 may promote degradation of the ECM or angiogenesis. YKL-40 is not present in normal adult cartilage but is detectable in human arthritic cartilage and during periods of cartilage generation, remodeling, and degradation. For example, patients with rheumatoid arthritis have high levels of serum-detectable YKL-40 (18). In addition, elevated YKL-40 levels have been detected in patients with hepatic fibrosis and cirrhosis (19), and in bovine breast tissue during mammary gland involution after cessation of lactation (20), processes that involve tissue destruction and ECM remodeling.

Because of the potential role of YKL-40 in ECM degradation, we compared its differential expression to that of other proteins involved
in this process such as endoproteases and endoglycosidases present on the microarray, many of which have been shown to be important factors in glioma biology (Fig. 2C). Although some of these genes demonstrated elevated mRNA levels in the tumor samples, when graphed together with YKL-40, their relative differential expression levels were barely detectable because of the substantial elevation of YKL-40 mRNA in the GBM samples. Differential expression values of YKL-40, endoglycosidases, endoproteases, and other ECM components on the array are shown in Table 1.

To determine whether the elevation in YKL-40 mRNA levels correlated with protein production, we analyzed protein extract from human GBMs, lower-grade gliomas, and normal brain tissue by Western blot analysis. We found that YKL-40 protein was dramatically elevated in a subset of the GBMs, and essentially below the detection limit for Western blot analysis, in both lower-grade tumors and normal brain extract (Fig. 2D).

**YKL-40 Levels Are Stable in Collected Serum.** In a recent publication, YKL-40 serum values were found to increase significantly over time when blood samples (before serum collection) were stored at room temperature for >24 h or at 4°C for >72 h before serum isolation (21). To determine whether our serum collection protocol introduced variable measurements because of collection techniques, we performed a time course experiment. We collected two vials of blood from each of two normal, healthy subjects. The blood samples were immediately centrifuged, and 150 µl of serum was collected from each vial and stored at 80°C. Next, one vial from each subject was kept at room temperature, and the other was kept at 4°C. Serum was collected from each vial at time points of 6 h, 24 h, 72 h, and 7 days, stored at 80°C, and subsequently analyzed by ELISA as described in “Materials and Methods.” We found that YKL-40 levels did not show any significant increase or decrease through continuous storage at either room temperature or at 4°C (Fig. 3A).

**YKL-40 Levels in the Serum of Patients Correlate with Glioma Grade.** On the basis of the above data, YKL-40 appears to be a promising candidate serum marker for a subset of GBMs. To test whether YKL-40 could be used as a clinical marker correlating with glioma grade and tumor burden, we collected serum samples from patients diagnosed with gliomas of various grades and 14 normal subjects.
healthy subjects. Serum levels of YKL-40 were measured in 45 patients with GBM (grade IV), 20 patients with lower-grade glioma (grades II and III), and 14 controls (Fig. 3B). The grade II and III gliomas were grouped together for this analysis based on the results of YKL-40 gene expression described above, which indicated that both histological grades had low expression. Comparisons of serum YKL-40 levels were made between glioblastoma and controls, glioblastoma and lower-grade gliomas, lower-grade gliomas and controls, and high versus low tumor burden in patients with glioblastoma. The results of these comparisons are reported in Table 2.

Table 1 Differential gene expression values of ECM-related genes

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Fig. 3. YKL-40 in the serum of glioma patients and normal controls, and as a function of tumor burden. A, time course of YKL-40 levels in serum from two normal volunteers. YKL-40 levels appear stable in serum stored at room temperature (———) and at 4°C (— — ——) over time. B, categorical illustration of YKL-40 serum levels in normal controls, lower-grade gliomas, and GBMs. * designates the mean YKL-40 value of each subgroup. C, YKL-40 levels displayed as a measure of tumor burden in patients with grade IV GBM and compared with normal controls. Tumor burden was defined, at the time of serum collection, as presence of tumor based on pre- or postoperative patient status.

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significant differences were detected between YKL-40 serum levels in patients with glioblastoma compared with controls ($P < 0.001$) and with patients with lower-grade gliomas ($P = 0.037$), as well as between lower-grade tumors compared with controls ($P < 0.001$). No significant differences were detected between high versus low tumor burden in serum YKL-40 from patients with GBM. The lack of statistical significance in the tumor burden analysis is because of a single outlier in the low tumor burden group, illustrated in Fig. 3C; however, there is no clinical explanation for the high serum YKL-40 levels in this patient.

**DISCUSSION**

Levels of YKL-40 protein can be reproducibly measured in the serum of patients with malignant primary brain tumors, reflecting the fact that YKL-40 is a secreted glycoprotein. Our data suggest low but detectable YKL-40 in serum of most normal controls and that significantly higher levels are detected in serum from patients with GBM compared with the lower-grade gliomas. The origin of YKL-40 production in normal individuals is not known. Consistent with the gene expression and Western blot data, not all of the high-grade gliomas manifested elevated serum levels of the YKL-40 protein. Furthermore, in some cases, the diagnosis attributed to a patient was made significantly earlier and may not have reflected the status of the tumor at the time of blood draw. This contributed to the large SD for YKL-40 levels in GBM samples. Because not all of the patients had molecular resonance imaging scans done at the time of blood drawal, it was not possible to correlate radiographic tumor volume to YKL-40 levels. Therefore, we categorized the patients into low and high tumor burden based on clinical grounds such as pre-versus postresection, postradiation, and recurrence. Comparison of the high versus low tumor burden samples from patients with GBM demonstrated a trend that was complicated by a single outlier. This outlier likely reflects the difficulty in truly determining a “low tumor burden” status based solely on clinical estimations.

The role of YKL-40 in the biology of GMBs is unclear. Given the relative specificity of increased expression in GMBs relative to anaplastic gliomas, it is possible that YKL-40 protein is an integral component of GMB biology and/or may contribute to GMB-specific histological characteristics, which distinguish them from grade II and III gliomas. Alternatively, or potentially complementarily, YKL-40 expression may be the cause or effect of the signaling abnormalities that characterize GMBs such as elevated Akt or Ras pathway activity.

The fact that not all of the GMBs express YKL-40 also indicates that it is involved in the biology of only a subset of these tumors, potentially a molecular subdivision of GMBs. It is possible that for those tumors that do express large amounts of YKL-40, its levels could be a useful indicator of GMB subtype or disease status. Although YKL-40 levels showed a wide range of variability, statistical analysis of this data strongly suggests that in YKL-40 producing high-grade gliomas, serum levels may be a valuable indicator of disease activity. The utility of the YKL-40 levels as a surrogate marker of tumor burden, response to treatment, or relapse remains to be determined. This resolution will require a prospective trial in which serum samples are analyzed in patients with tumors demonstrating elevated expression of the YKL-40 gene at the time of diagnosis with subsequent measures during the course of the illness. Such a prospective study is planned, evaluating both gene expression in the tumor tissue along with serial measurement of YKL-40 protein concentrations in serum.

**ACKNOWLEDGMENTS**

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