YKL-40 and Matrix Metalloproteinase-9 as Potential Serum Biomarkers for Patients with High-Grade Gliomas

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Abstract

Purpose: Biomarkers can facilitate diagnosis, monitor treatment response, and assess prognosis in some patients with cancer. YKL-40 and matrix metalloproteinase-9 (MMP-9) are two proteins highly differentially expressed by malignant gliomas. We obtained prospective longitudinal serum samples from patients with gliomas to determine whether YKL-40 or MMP-9 could be used as serum markers.

Experimental Design: Serum samples were obtained concurrently with magnetic resonance imaging scans. YKL-40 and MMP-9 were determined by ELISA and the values correlated with the patient’s radiographic status and survival.

Results: High-grade glioma patients who underwent a surgical resection of their tumor had a transient increase of both YKL-40 and MMP-9 serum levels in the postoperative period. Glioblastoma multiforme (GBM) patients with no radiographic evidence of disease (n = 10 patients, 50 samples) had a significantly lower level of YKL-40 and MMP-9 than patients with active tumor (n = 66 patients, 209 samples; P = 0.0003 and 0.0002, respectively). Anaplastic glioma patients with no radiographic evidence of disease (n = 32 patients, 107 samples) also had a significantly lower level of YKL-40 compared with those patients with active tumor (n = 48 patients, 199 samples; P = 0.04). There was a significant inverse association between YKL-40 and survival in GBM, hazard ratio (hazard ratio, 1.4; P = 0.02), and anaplastic astrocytoma patients (hazard ratio, 2.2; P = 0.05).

Conclusions: YKL-40 and MMP-9 can be monitored in patients’ serum and help confirm the absence of active disease in GBM and YKL-40 in anaplastic glioma patients. YKL-40 can be used as predictor of survival in patients with high-grade glioma. Longitudinal studies with a larger patient population are needed to confirm these findings.

The current pathologic classification of high-grade gliomas [glioblastoma multiforme (GBM) and anaplastic glioma] encompasses a heterogeneous population of tumors with variable sensitivity to treatment. Tumor grade generally predicts prognosis, but for an individual patient, predictions are often inaccurate. For anaplastic oligodendroglioma, detection of 1p and 19q loss is an indicator of chemotherapy sensitivity and improved overall prognosis (1). There is no molecular indicator of treatment response or predictor of survival for anaplastic astrocytomas. The identification of promoter methylation of the O6-methylguanine-DNA methyltransferase (MGMT) DNA repair gene in some GBM patients was recently reported to confer a favorable treatment response to temozolomide chemotherapy and longer survival (2); however, this analysis must be done on tumor tissue that is frequently unavailable or noninformative.

Currently, tumor response is evaluated by noninvasive imaging, usually magnetic resonance imaging (MRI). However, MRI may be unreliable when assessing novel cytotoxic therapies, in discerning radionecrosis from tumor progression, and in predicting malignant transformation from a low-grade glioma. In other cancers, such as prostate cancer, serum tumor markers are used to diagnose malignancy, assess treatment response, and even predict survival. No such serum markers have been identified for brain tumors although ongoing research has identified potential candidate proteins (3,4).

YKL-40 is an extracellular matrix glycoprotein of unknown function, initially detected as a protein secreted by a human osteosarcoma cell line (5). Its name is based on its molecular weight of 40 kDa and three NH2-terminal amino acids, tyrosine, lysine, and leucine, and is also known as human cartilage glycoprotein 39 (5). It is a chitinase-like protein that has no catalytic activity against chitinase substrates due to amino acid substitutions in the region corresponding to the active site of chitinases (6). There is evidence that it may be involved in tissue remodeling (7). YKL-40 is secreted by...
articular chondrocytes and is detected in synovia and cartilage of patients with rheumatoid arthritis (7). YKL-40 expression promotes adhesion and migration of vascular smooth muscle cells in vitro, and it has been detected in vivo in atherosclerotic plaques and in the developing heart (8). It participates in differentiation of circulating monocytes into macrophages (9). In the central nervous system, it may be produced by activated macrophages in meningitis and encephalitis (10). A gene expression profile of gliomas showed that YKL-40 was highly differentially expressed compared with pooled normal brain using cDNA microarray analysis. YKL-40 was elevated in the serum of patients with GBM (11) and immunohistochemistry of high-grade gliomas showed increased YKL-40 expression in GBMs (12). Comparative genomic hybridization studies and gene expression array analysis of GBM tumor tissue revealed that YKL-40 was associated with chromosome-10 loss and poor clinical outcome (13).

Matrix metalloproteinase-9 (MMP-9) is a well-known matrix metalloproteinase associated with tumor infiltration and angiogenesis (14–17). MMP-9 belongs to the gelatinase subclass of MMPs and its expression has been detected both in tumor and endothelial cells in glioma (18). Increasing level of MMP-9 in glioma specimens correlates with higher tumor grade (14).

YKL-40 and MMP-9 are two secreted proteins that are central to glioma biology, making them candidates for serum markers of this disease. We studied serum samples from patients with high-grade gliomas to ascertain the usefulness of YKL-40 and MMP-9 as potential serum markers.

Materials and Methods

Patients. From August 2002 to March 2005, serum samples were collected prospectively from patients with histologically confirmed gliomas to assay YKL-40 and MMP-9 levels. Patients were allowed to enroll any time during the course of their illness. Patients with known glioma or suspected brain tumor identified by imaging, who had not yet undergone resection, were eligible for the study. Those patients enrolled before their initial surgical procedure and were retained in the study only after histologic confirmation of a glioma. Serum samples and imaging studies were obtained every 2 to 3 months when patients were evaluated in their follow-up visit. Serum samples would not be obtained or discarded if the patients had a concurrent inflammatory illness at the time of their appointment. All blood samples were obtained within 4 weeks of MRI scans with 118 samples (45%) obtained on the same day as the MRI and 93 samples (35%) within a week. In patients who underwent surgical resection, samples were obtained within 14 days postoperatively and serially 1 to 14 days postoperatively to determine the effect of surgery on the marker levels. Patients with rheumatic disease, acute infection, HIV, or other types of cancer were excluded from participating in the study. Pathology specimens from all patients enrolled in the study were centrally reviewed at our institution using the WHO classification scheme (19). This study was approved by the Memorial Hospital Institutional Review Board and all patients signed an informed consent.

Determination of level of serum marker. Blood samples were collected and allowed to clot for at least 1 hour at room temperature. They were centrifuged at 4°C for 10 minutes at 3,000 rpm. The serum was aliquoted and stored at −20°C. YKL-40 (Quidel Corp., San Diego, CA) and MMP-9 (Quantikine, R&D Systems, Minneapolis, MN) levels were determined by ELISA.

Immunohistochemistry. YKL-40 immunostains were done using formalin-fixed, paraffin-embedded tissue. After examination of all available H&E sections, the corresponding paraffin block with the most viable tumor was selected for immunostaining. Five-micron-thick sections were cut from each paraffin block. For antigen retrieval, slides were pretreated by steaming in citrate buffer for 30 minutes. Slides were then immunostained with a polyclonal anti-YKL-40 antibody (Quidel) at 1:500 dilution with standard avidin-biotin-peroxidase. Staining for YKL-40 was scored for the percentage of positive cells.

Determination of disease status. The patient’s disease status was assessed for each time point when a serum sample was collected. MRI was done with 1.5-T GE scanners (GE Medical Systems, Milwaukee, WI). All MRI studies included Fluid Attenuated Inversion Recovery, T2-weighted and T1-weighted before and after administration of Gadolinium contrast material (gadopentetate dimeglumine). Tumor size was determined by using the longest diameter (Response Evaluation Criteria in Solid Tumors criteria; refs. 20, 21) and the product of two longest perpendicular diameters of contrast-enhancing lesions on the gadolinium enhanced T1-weighted images. For non-enhancing residual anaplastic gliomas, the longest diameter and the largest cross-sectional area of expansile tumor were measured on Fluid Attenuated Inversion Recovery images. To ensure uniformity, all MRI scans were reviewed by two of the authors (A.H. and S.K.). Response for high-grade gliomas was assessed as complete response (CR), partial response (PR), stable disease (SD), or progression of disease (POD) according to the standard of Macdonald (22).

Statistical analysis. On examination of markers by response status, no consistent trend among PR, SD, and POD was found, which led to the collapse of these three response groups into one category. To test the association between marker and disease status (defined as CR versus combined PR, SD, and POD), we used all measurements in a logit model with generalized estimating equations that corrected for within-patient correlations (23). Because the distributions for both YKL-40 and MMP-9 were skewed, the data for both markers were log transformed before all statistical testing. Overall survival was defined as time from registration date to date of death or last follow-up. The effect of each marker on overall survival was analyzed using each marker separately as a time-dependent covariate in a Cox proportional hazards model (24). Each marker was used in two ways as a time-dependent covariate: (a) the value of the marker in the log-scale over time; (b) the change in each marker compared with the first value collected at registration to the protocol (difference in log values).

Results

Patient cohort. We enrolled a total of 143 patients (67 women and 76 men) with high-grade gliomas (Table 1). Seventy-seven patients had a GBM and 66 an anaplastic glioma. Among the anaplastic glioma patients, the most prevalent subgroup was anaplastic astrocytoma with 38 (57.5%) patients. The median age for patients with GBM was 55 years (range, 23-83 years) and for patients with anaplastic glioma, 46 years (range, 23-81 years). The median time from tumor diagnosis to enrollment was 1.4 months (range, 0-21 months) for GBM and 11.2 months (range, 0-137 months) for anaplastic tumors. We have serum levels linked to imaging in 137 patients for YKL-40 and in 134 patients for MMP-9. A median of 2 samples (range, 1-13) was obtained for patients with GBM and 4 samples (range, 1-12) for patients with anaplastic gliomas.

Normal subject YKL-40 and MMP-9 values. YKL-40 level was measured in 93 normal subjects (46 men and 47 women), healthy employees whose serum had been stored. The median age of the normal subjects was 37 years (range, 21-65 years). The median YKL-40 was 55.3 ng/mL (range, 13-101 ng/mL), with a mean of 59.3 ± 21.6 ng/mL. The mean value for YKL-40 established in our laboratory was higher than a previously published mean of 28 ng/mL (range, 15-166 ng/mL; ref. 25).
However, the prior data were obtained in women only\(^8\) of unknown age. The range of YKL-40 levels was similar in both groups. For MMP-9, serum was available for analysis from 52 subjects (13 men and 39 women). The median age of the normal subjects was 40 years (range, 23-64 years). The median MMP-9 was 315 ng/mL (range, 169-705 ng/mL), with a mean of 339 ± 163 ng/mL. The normal subjects were younger than the cancer patients and were intended to be used as a guide for YKL-40 and MMP-9 levels and not for formal comparison with the cancer population.

**Postoperative transient elevation of YKL-40 and MMP-9 serum levels.** In 30 patients with GBM and 14 patients with anaplastic glioma, levels were obtained preoperatively and serially over 2 weeks after resection. Tumor resection resulted in transiently increased levels of both YKL-40 and MMP-9. The levels of YKL-40 reached a peak 24 hours after resection (median, 264 ng/mL for GBM and 295 ng/mL for anaplastic gliomas; Fig. 1); the median levels of YKL-40 had a 4.4-fold increase relative to the baseline level for anaplastic glioma and a 3.2-fold increase for GBM at 24 hours. The maximum levels for MMP-9 occurred within 48 hours postoperatively (median, 805 ng/mL for GBM and 844 ng/mL for anaplastic gliomas). The median levels of MMP-9 had a smaller increase relative to the baseline level (1.2-fold for anaplastic glioma and 1.3-fold for GBM). Both YKL-40 and MMP-9 returned to baseline over the next 96 hours to 2 weeks.

**MMP-9 and YKL-40 serum levels correlate with no evidence of disease in GBM patients and YKL-40 with no evidence of disease in anaplastic glioma patients.** Over the course of the study, 10 patients with GBM had a radiographic CR, 13 had PR, 39 SD, and 29 POD. The median YKL-40 level for anaplastic glioma patients with a CR was 61 ng/mL; for PR, 76 ng/mL; for SD, 74 ng/mL; and for POD, 88 ng/mL. For anaplastic glioma patients, YKL-40 levels were also significantly associated with disease status (\(P = 0.04\)).

For GBM patients, the median value of MMP-9 was 116 ng/mL for patients with radiographic CR, 312 ng/mL for PR, 348 ng/mL for SD, and 360 ng/mL for POD. We found a significant difference in MMP-9 values for GBM patients with a CR compared with GBM patients with radiographic evidence of tumor (\(P = 0.0002\); Fig. 4). The majority of GBM patients with persistent radiographic CR had MMP-9 levels in the reference range (Fig. 5).

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### Table 1. Summary of patient cohort

<table>
<thead>
<tr>
<th></th>
<th>GBM</th>
<th>Anaplastic glioma</th>
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<tbody>
<tr>
<td>Total no. patients</td>
<td>(n = 77)</td>
<td>(n = 66)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
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</tr>
<tr>
<td>Median (range)</td>
<td>55 (23-83)</td>
<td>46 (23-81)</td>
</tr>
<tr>
<td>Sex (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>42 (55)</td>
<td>34 (52)</td>
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<tr>
<td>Female</td>
<td>35 (45)</td>
<td>32 (48)</td>
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<tr>
<td>Time from diagnosis to enrollment (mo)</td>
<td>1.4 (0-21)</td>
<td>11.2 (0-137)</td>
</tr>
<tr>
<td>YKL-40 Total no. samples</td>
<td>273</td>
<td>314</td>
</tr>
<tr>
<td>MMP-9 Total no. samples</td>
<td>267</td>
<td>315</td>
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<tr>
<td>YKL-40 Median no. samples (range)</td>
<td>2 (1-13)</td>
<td>3 (1-12)</td>
</tr>
<tr>
<td>MMP-9 Median no. samples (range)</td>
<td>2 (1-14)</td>
<td>4 (1-12)</td>
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<tr>
<td>YKL-40 Patients with samples correlated to MRI</td>
<td>(n = 71)</td>
<td>(n = 66)</td>
</tr>
<tr>
<td>MMP-9 Patients with samples correlated to MRI</td>
<td>(n = 69)</td>
<td>(n = 65)</td>
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\(8\) J. Dupont, personal communication.
For anaplastic glioma, the median value of MMP-9 was 285 ng/mL for CR, 212 ng/mL for PR, 253 ng/mL for SD, and 434 ng/mL for POD. For anaplastic glioma patients, MMP-9 values were not significantly associated with disease status \((P = 0.73)\).

**YKL-40 and MMP-9 levels and overall survival.** The median follow-up from entry for survivors with GBM was 4.6 months (range, 0-27 months) and for those with anaplastic glioma, 22 months (range, 0-29 months). The estimated 2-year survival from registration was 26% [95% confidence interval (95% CI), 12-39%] for GBM and 72% (95% CI, 59-84%) for anaplastic glioma. We analyzed YKL-40 as a time-dependent covariate in a Cox proportional hazard model for overall survival from entry for 75 patients with GBM (two patients with immediate postoperative measurements only were excluded) and 68 patients with anaplastic glioma (two patients enrolled with low-grade glioma progressed to anaplasia and were included using measurements after progression). The analysis was done in two ways: by using the actual value of YKL-40 and MMP-9 in the log scale and by looking at the change in the markers compared with the first level determined for an individual patient designated as baseline in the log scale. For GBM, there was a significant association between actual value of YKL-40 and survival, with a hazard ratio of 1.4 (95% CI, 1.1-1.9) per...
doubling of YKL-40 values ($P = 0.02$; Table 2). There was no significant association between the change in YKL-40 from baseline and survival ($P = 0.12$). For anaplastic glioma, there was a marginally significant association between the change from baseline and survival, with a hazard ratio of 1.7 (95% CI, 0.94-3.2) per doubling of YKL-40 compared with baseline ($P = 0.08$) but no significant association between the actual value of YKL-40 and survival ($P = 0.26$). In the anaplastic astrocytoma subset, we found a significant association between the actual value of YKL-40 and survival (hazard ratio, 2.2; 95% CI, 0.99-4.9; $P = 0.05$) and a marginally significant association with change from baseline (hazard ratio, 2.3; 95% CI, 0.98-5.2; $P = 0.06$).

The analysis of MMP-9 as a time-dependent covariate in a Cox proportional hazard model for overall survival showed that MMP-9 values were not significantly associated with survival for either anaplastic glioma ($P = 0.36$) or GBM patients ($P = 0.16$; Table 2). There was also no significant association in change from baseline in MMP-9 for GBM tumors ($P = 0.29$) or anaplastic gliomas ($P = 0.96$). In the anaplastic astrocytoma subset, there was no significant association between survival and actual value of MMP-9 ($P = 0.34$) or change from baseline ($P = 0.41$).

**Modest correlation of YKL-40 with MMP-9 in anaplastic gliomas but no correlation with tumor size.** The correlation of YKL-40 and MMP-9 values in the same serum sample of patients with anaplastic gliomas was moderate ($r = 0.52$) but the correlation was weaker for GBM ($r = 0.24$). To look at the correlation of the serum markers with tumor size, we measured tumor size on 225 MRI scans of GBM patients and 280 MRI scans of anaplastic glioma patients. Neither YKL-40 nor MMP-9 values correlated with tumor size determined by one or two dimensions for any glioma type.

**No significant correlation between YKL-40 serum levels and YKL-40 immunohistochemistry.** We were able to perform YKL-40 immunostaining in tumor specimens and compare them with the preoperative YKL-40 serum values in 18 patients. There was no correlation between immunostaining and preoperative serum levels of YKL-40 ($r = 0.08$). Twelve of these patients had a GBM. Comparative analysis of YKL-40 immunostaining to the preoperative YKL-40 serum values in these 12 patients also showed no correlation ($r = -0.18$).

**Discussion**

Elevated values of YKL-40 have been found in the serum of patients with various diseases including pulmonary sarcoidosis (26), liver fibrosis (27), systemic sclerosis (28), active inflammatory bowel disease (29), and bacterial endotoxia (30). Serum levels of YKL-40 are also elevated in patients with cancer although its role in cancer cells is unknown. In patients with recurrent breast cancer, elevated serum levels were related to metastatic disease and worse survival (31). Preoperative high serum levels of YKL-40 in patients with breast cancer were also related to shorter overall survival and it was an independent predictor of relapse-free survival (32). In colorectal cancer and small-cell lung cancer patients, YKL-40 is also considered an independent prognostic variable for poor survival (33, 34) and it is a potential marker for detection of ovarian cancer that may also predict recurrent disease and survival (25).

In a retrospective study, YKL-40 expression was increased in operative specimens of GBM that progressed after radiotherapy and was absent from tumor tissue of patients that responded to radiotherapy (35). MMP-9 can be detected in the cerebrospinal fluid of patients with brain tumors (36) and its expression in tumor tissue correlates with glioma grade (37).

At the cellular level, the YKL-40 protein has been associated with a survival advantage of GBM cells in response to hypoxia, ionizing radiation, and p53 inhibition (38). It may have a role in promoting cell proliferation as it was shown to promote proliferation of connective tissue cells in vitro (39). This mitogenic response to YKL-40 stimulation may involve both mitogen-activated protein kinase and phosphatidylinositol 3-kinase (40), two components of a signal transduction cascade known to be up-regulated in malignant glial cells. The presence of heparin and hyaluronan binding motifs in the sequence of YKL-40 suggests that it may interact with heparin-like molecules or hyaluronan present on the cell surface or extra-cellular matrix (8). YKL-40 may promote tumor angiogenesis independently of angiogenic factors as it was shown to promote migration of human umbilical vein endothelial cells (41).

Our data suggest that serum levels of YKL-40 and MMP-9 differentiate between GBM patients with and without evidence of disease; however, only YKL-40 was an indicator of survival in GBM patients. For GBM patients, a low absolute YKL-40 value identifies a subset that is more likely to do well. Those patients with persistently normal values have a longer survival and disease-free interval.

For anaplastic glioma, the YKL-40 level can also help identify patients with no evidence of disease, and the change from baseline is marginally predictive of survival. MMP-9 levels were not predictive of either evidence of disease or survival in anaplastic glioma patients. It seems that the anaplastic astrocytoma subtype has a significant correlation of survival with the actual value of YKL-40 and a marginally significant correlation with change in value from baseline. This may

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<th>Table 2. Summary of statistical analysis of survival</th>
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<tr>
<td>Total no. patients</td>
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<td>Total no. deaths</td>
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<tr>
<td>YKL-40 per doubling in actual value</td>
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<tr>
<td>YKL-40 per doubling in value compared with baseline</td>
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<tr>
<td>MMP-9 per doubling in actual value</td>
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<td>MMP-9 per doubling in value compared with baseline</td>
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correlate with increased biological aggression or even transformation to a higher tumor grade in some anaplastic astrocytomas, although we do not have histologic confirmation. Immunohistochemistry of tumor specimens suggested absent or reduced intensity YKL-40 staining in anaplastic oligodendrogliomas compared with the majority of GBMs (12). Currently, we only have serum sample data on 17 anaplastic oligodendrogliomas and 11 anaplastic oligoastrocytomas, an insufficient number to permit subset analysis in these anaplastic glioma subtypes. Different levels or expression of YKL-40 in these two subsets may affect our analysis for the entire anaplastic glioma group. It is possible that some of these relationships may be delineated with expansion of our patient number.

The heterogeneity and size of the population of patients enrolled in our study, together with a small number of events, are limitations that presently preclude other types of analysis, such as multivariate analysis controlling for known prognostic factors. Expanding the cohort of patients who enter the study uniformly at an early time point in their disease history, such as at diagnosis or before radiotherapy and chemotherapy treatment, will strengthen a future analysis.

YKL-40 expression in GBM tissue has been associated with radiation response determined by changes in tumor size between postoperative and postradiation MRI (35). We did not find any correlation between serum levels of either YKL-40 or MMP-9 and tumor size. The different methods used may account for the difference in the results in the two studies. In Pellosi et al.’s study, initial specimens were used to quantify protein expression and MRIs were obtained at early specific time points, but we obtained serial MRIs and used a different measurement criterion. Comparative analysis of preoperative serum levels of YKL-40 with tumor levels by immunohistochemistry showed no relationship between the two. Although sampling error may contribute to our immunohistochemical results and we had a limited number of patients to do this comparative analysis, our finding may suggest that sources of circulating YKL-40 other than the protein overexpressed by the tumor play an important role in the biology of high-grade malignant glioma. The detection of YKL-40 in the serum may be advantageous as the circulating protein can be found not only in those patients whose tumors overexpress the protein but also in patients whose tumors express small amounts of YKL-40 or in those in whom the protein cannot be detected with the current available methods.

Postoperative measurements of YKL-40 and MMP-9 have no clinical significance for at least 2 weeks after surgery because the values transiently increase in the immediate postoperative period. This may be related to tissue injury and subsequent remodeling. Peritumoral macrophages in small-cell lung cancer tumors (42) and even in glioblastomas (43) produce YKL-40. In inflammatory diseases, macrophages generate YKL-40 (44) whereas MMP-9 is increased with monocyte differentiation (45). Postoperative infiltration of these inflammatory cells may contribute to the postoperative elevation we observed.

In summary, YKL-40 has the potential to be used as a biomarker of prognosis for high-grade gliomas. A larger patient sample followed for an extended period will be required to confirm these initial data.

References


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