YKL-40 Is a Differential Diagnostic Marker for Histologic Subtypes of High-Grade Gliomas

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ABSTRACT

Purpose and Experimental Design: In modern neuro-oncology, no variable affects therapeutic decisions and prognostic estimation more than tumor classification. We showed recently that class prediction models, based on gene expression profiles, classify diagnostically challenging malignant gliomas in a manner that better correlates with clinical outcome than standard pathology. In the present study, we used immunohistochemistry to investigate YKL-40 protein expression in independent sets of glioblastomas and anaplastic oligodendrogliomas to determine whether this single marker can aid classification of these high-grade gliomas.

Results and Conclusions: Glioblastomas show strikingly more YKL-40 expression than anaplastic oligodendrogliomas. Only 2 of 37 glioblastomas showed completely negative YKL-40 staining in both tumor cells and extracellular matrix, whereas 18 of 29 anaplastic oligodendrogliomas were completely negative in non-microgemistocytic tumor cells and extracellular matrix. Tumor cell staining intensity was also markedly different: 84% of glioblastomas showed strong staining intensities of 2+ or 3+ whereas 76% of anaplastic oligodendrogliomas either did not stain or stained at only 1+. YKL-40 staining provided a better class distinction of glioblastoma versus anaplastic oligodendroglioma than glial fibrillary acidic protein, the current standard immunohistochemical marker used to distinguish diagnostically challenging gliomas. Moreover, a combination of YKL-40 and glial fibrillary acidic protein immunohistochemistry afforded even greater diagnostic accuracy in anaplastic oligodendrogliomas.

INTRODUCTION

In modern neuro-oncology, tumor classification is the variable that most affects therapeutic decisions and prognostic estimation. Among high-grade gliomas, anaplastic oligodendrogliomas and glioblastomas follow markedly different clinical courses. Anaplastic oligodendrogliomas remain the only subtype of high-grade glioma that commonly responds to chemotherapy (1, 2) and patients with these lesions have a much more favorable prognosis than patients with glioblastomas (3). The most widely used method of brain tumor classification is that of the WHO (3). The 2000 WHO system divides diffuse gliomas into astrocytomas, oligodendrogliomas, and oligoastrocytomas. Anaplastic oligodendroglioma and glioblastoma (an astrocytic tumor) are the most malignant forms of glial lesions in each of their two respective tumor lineages. These high-grade gliomas are characterized by dense cellularity and high proliferation indices as well as the presence of microvascular proliferation and/or necrosis (although, unlike glioblastoma, microvascular proliferation and/or necrosis are not required for diagnosis of anaplastic oligodendroglioma). Histologically, astrocytic tumors typically exhibit pleomorphic, spindle-shaped cells with copious eosinophilic cytoplasm and fibrillary processes. In contrast, oligodendrocytic tumors are characterized by monomorphic cells with rounded nuclei and perinuclear halos. Unfortunately, malignant gliomas often display intratumoral histologic variability or lack defining histologic features and, consequently, these tumors can be diagnostically challenging (4); as such, considerable interobserver variability can occur, resulting in limited diagnostic reproducibility (5, 6).

To develop more objective approaches to tumor classification in general, many recent investigations have focused on gene expression microarray analyses. In one such study of high-grade gliomas, we showed that class prediction models, based on gene expression profiles, provided a powerful tool to classify diagnostically challenging malignant gliomas (7). Although the prediction model used in the study used expression information from only 19 genes, many additional genes were differentially expressed between glioblastomas and anaplastic oligodendrogliomas. One gene, YKL-40, showed an average expression level in predicted glioblastomas 82-fold greater than the average expression found in predicted anaplastic oligodendrogliomas.

YKL-40, a secreted glycoprotein, is a member of the 18 glycosyl hydrolase family (8–10). Although the function of YKL-40 is not well characterized, it is expressed in numerous pathologic conditions of inflammation and tissue degradation and remodeling, including rheumatoid arthritis (11), hepatic fibrosis (12), and inflammatory bowel disease (13). In addition, high serum levels of YKL-40 have been detected in a number of cancers including breast (14), colorectal (15), and ovarian (16). Moreover, high levels of YKL-40 were associated with worse prognosis in all of these studies (14–16). Taken together, these findings suggest potential roles for YKL-40 in tumor invasion and angiogenesis. In fact, YKL-40 can facilitate migration of endothelial cells and promote the formation of branching tubules.
High YKL-40 serum levels have also been shown in glioblastomas; glioblastomas were found to have 3- to 62-fold elevation of YKL-40 levels over normal brain (21). Furthermore, there is evidence that YKL-40 levels correlate with grade in astrocytic gliomas (21). One recent study hypothesized that elevated levels of YKL-40 in glioblastomas might be due to the presence of increased numbers of macrophages (22) as the expression of YKL-40 in macrophages has been well documented (10, 23–26). Because there have been no in situ studies to date on YKL-40 expression in gliomas, the cellular localization of YKL-40 expression remains unknown. In the present study, we have used immunohistochemistry to investigate YKL-40 protein expression in high-grade gliomas and to determine whether this single marker can augment the histologic classification of high-grade gliomas. We have specifically chosen to examine YKL-40 expression in anaplastic oligodendrogliomas and glioblastomas as they comprise the highest-grade tumors of two distinct glial tumor lineages.

MATERIALS AND METHODS

Tissue Samples. Tissue samples were collected from Massachusetts General Hospital (Boston, MA) under the approval of the Massachusetts General Hospital Institutional Review Board. A total of 81 malignant gliomas were used in the study: 37 glioblastomas, 29 anaplastic oligodendrogliomas, and 15 anaplastic oligoastrocytomas. In initial experiments, a set of 19 glioblastomas and 18 anaplastic oligodendrogliomas were randomly selected for YKL-40 and glial fibrillary acidic protein (GFAP) immunohistochemistry. Subsequently, an additional 18 glioblastomas and 11 anaplastic oligodendrogliomas were randomly selected and stained with YKL-40 when the 15 anaplastic oligoastrocytomas were added to the study. In addition, specifically for the evaluation of reactive astrocytes, serial sections of regions with reactive gliosis in 16 of the above-mentioned gliomas and five pathologic brain samples that did not contain malignant primary brain tumor (e.g., metastases and vascular disease) were stained with YKL-40 and GFAP.

Immunohistochemistry. YKL-40 immunohistochemistry was done on formalin-fixed, paraffin-embedded sections with a polyclonal anti-YKL-40 antibody (Quidel Corporation, San Diego, CA). Antigen retrieval was achieved with a 5-minute incubation in 0.05% saponin/1% bovine serum albumin/PBS solution. Slides were incubated with a 1:400 dilution of primary antibody overnight at 4°C and visualized with an avidin-biotin complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) in the presence of 3,3’-diaminobenzidine tetrahydrochloride. GFAP immunohistochemistry was done in the absence of antigen retrieval with a monoclonal anti-GFAP antibody (1:1,000 dilution; DAKO, Carpinteria, CA).

For evaluation of tumor samples, staining for YKL-40 and GFAP was scored semiquantitatively for both cell number and staining intensity. The number of positive cells was given as 0%, <5%, 5% to 25%, 25% to 75%, and 75% to 100%. Signal intensity was graded on a scale consisting of 0 to 3+. When specifically investigating reactive astrocytes, the same signal intensity scale of 0 to 3+ was used to score only the reactive astrocytes and the cell number was also evaluated on a graded scale of 0 to 3+, indicating the number of positive cells relative to all reactive astrocytes in the microscopic field (0, none; 1+, few; 2+, some; 3+, many).

Statistical Analysis. Crude power calculations indicated that we had ~80% power to detect a difference in median percentage of positive cells, staining intensity, and extracellular staining intensity of 0.85 between glioblastomas and anaplastic oligodendrogliomas. This was based on two-sided 0.017 level Wilcoxon Rank Sum tests (corrected for the three comparisons) and assumed an SD of 1 unit (as seen in our data). The exact Wilcoxon Rank Sum test was used to compare medians of percentage of positive cells, staining intensity, and extracellular staining intensity among histologic subtypes; estimates and nonparametric 95% confidence intervals (95% CI) are reported for differences in these immunohistochemistry measures (27). McNemar’s test and the Wilcoxon Signed Rank test were used for comparisons of YKL-40 and GFAP within the same individuals; exact binomial 95% CIs were computed for probabilities of interest. A k-mean clustering algorithm (28) was used to cluster the subjects based on YKL-40 and GFAP staining (k = 2). We elected to use unsupervised k-mean clustering to avoid making assumptions about how to model the ordinal immunohistochemistry measures in a logistic regression and to avoid the problems of sparse data that would result. Histologic diagnoses were not used to train the clustering model and, therefore, the k-mean algorithm was not biased by these labels; there is, however, additional variability induced due to the estimation of the k-mean clusters. The histologic diagnosis was then assessed in the resultant two clusters. A Bonferroni correction for multiple comparisons was applied due to the three measurements of YKL-40 (i.e., intensity, percentage of positive cells, and extracellular staining intensity). Adjustments were not made for analyses within histologic subtypes as they were each of inherent scientific interest. All tests were two sided and the significance level was taken to be 0.05.

RESULTS AND DISCUSSION

YKL-40 Immunohistochemistry Distinguishes Anaplastic Oligodendrogliomas from Glioblastomas. In a recent gene expression-based microarray analysis, we identified a large number of genes differentially expressed between glioblastomas and anaplastic oligodendrogliomas (7). Class prediction models, based on gene expression profiles, were used to classify diagnostically challenging malignant gliomas and, utilizing this data, YKL-40 showed an average expression level in predicted glioblastomas 82-fold greater than the average expression found in predicted anaplastic oligodendrogliomas. Among these microarray samples, 22 of 35 predicted glioblastomas had YKL-40 RNA expression levels above the background of the assay, whereas only 1 of 15 predicted anaplastic oligodendrogliomas had YKL-40 RNA expression above background. To determine if differential YKL-40 RNA expression correlated with differential protein expression, we first did YKL-40 immunohistochemistry on six samples included in the original microarray study. Three glioblastomas that were positive for YKL-40 RNA showed YKL-40 staining by immunohistochemistry whereas no positive YKL-40 staining was seen in the three anaplastic oligodendrogliomas that did not express YKL-40 RNA (Fig. 1A and C). Thus, these preliminary
results suggested that YKL-40 immunohistochemical staining correlated with our YKL-40 RNA expression data.

Next, independent sets of glioblastomas (n = 37) and anaplastic oligodendrogliomas (n = 29) were immunostained for YKL-40. In addition to tumor cells, positive YKL-40 staining was also detected in blood vessels, extracellular matrix and, to a lesser degree, in a subset of reactive astrocytes. Fifty-four percent of glioblastomas showed positive YKL-40 staining in at least 25% of tumor cells (Fig. 1A and B; Table 1). In contrast, 69% of anaplastic oligodendrogliomas were positive for YKL-40 in less than 5% of tumor cells (Fig. 1C; Table 1). An even greater difference was seen in tumor cell staining intensity: 84% of glioblastomas showed strong staining intensities of 2+ or 3+ whereas 76% of anaplastic oligodendrogliomas either did not stain at all (62%) or stained at an intensity of 1+ (14%). Furthermore, when positive staining was found in oligodendrogliomas, it was most often in microgemistocytes and/or endothelial cells (Fig. 1E and 2C). Only 2 of 37 glioblastomas showed completely negative YKL-40 staining in both tumor cells and extracellular matrix. In contrast, 18 of 29 anaplastic oligodendrogliomas were completely negative in both non-microgemistocytic tumor cells and extracellular matrix. Normal brain was also negative for YKL-40 staining (Fig. 1G).

In order to determine if YKL-40 immunohistochemistry could be used to distinguish histologic subtypes of high-grade gliomas, we did statistical analyses on the semiquantitatively scored data. The percentages of positive cells were significantly higher in glioblastomas as compared with anaplastic oligodendrogliomas (P < 0.001; median location difference, 2.0; 95% CI, 1.0-2.0). Furthermore, staining intensities were also significantly higher in glioblastomas (P < 0.001; median location difference, 1.0; 95% CI, 1.0-2.0). The extracellular staining intensities in glioblastomas were not significantly different from those of anaplastic oligodendrogliomas (P = 0.186).

High-grade astrocytic gliomas are graded as either anaplastic astrocytoma (WHO grade III) or glioblastoma (WHO grade IV). In contrast, anaplastic oligodendroglioma (WHO grade III) is the highest grade designation given to oligodendrogial tumors. It is therefore possible that high-grade oligodendroglioma could encompass a wider range of malignancy when compared with either anaplastic astrocytoma or glioblastoma alone. Because YKL-40 expression has been shown to correlate with tumor grade in astrocytic tumors (21, 22), it was possible that the lower levels of YKL-40 expression found in our anaplastic oligodendroglioma sample set were associated exclusively with a lower grade as compared with the glioblastomas. We have two lines of evidence that suggest this is not the case. First, in a number of tumors that
displayed regional histologic heterogeneity, YKL-40 staining seemed to correlate with histology. For example, one diagnosed glioblastoma displayed 3+ intensity staining but only in 5% of the tumor cells. On histologic review, a large region of this particular tumor section exhibited histologic features more characteristic of an anaplastic oligodendroglioma in which YKL-40 staining was almost completely absent (Fig. 1D). Even within more diffuse histologic regions of oligoastrocytomas, astrocytic cells stained more intensely than the rounded cells more typical of an oligodendroglioma (Fig. 1F). Second, to examine this issue from another perspective, we did YKL-40 immunohistochemical staining on 15 samples of anaplastic oligoastrocytoma (Fig. 1F), a high-grade malignant lesion exhibiting histologic features of both astroglial and oligodendroglial differentiation; as for oligodendrogliomas, the grade III “anaplastic” designation is the highest given to oligoastrocytomas. The percent positive cells and staining intensity were significantly different between glioblastomas, anaplastic oligodendrogliomas, and anaplastic oligoastrocytomas (P < 0.001). For both percent positive cells and staining intensity, anaplastic oligoastrocytomas gave results intermediate to glioblastomas and anaplastic oligodendrogliomas (median location difference between glioblastomas and anaplastic oligoastrocytomas, 1.0; 95% CI, 0.0-2.0; between anaplastic oligoastrocytomas and anaplastic oligodendrogliomas, 0.51; 95% CI, 0.0-1.0). If decreased YKL-40 expression in anaplastic oligodendrogliomas was due exclusively to differences in tumor grade, anaplastic oligoastrocytomas would not be expected to display expression levels different from those seen in anaplastic oligodendrogliomas. Assuming a uniform distribution of malignancy within the “anaplastic” grade in both the oligodendroglioma and oligoastrocytoma sample sets, the finding that YKL-40 expression levels in anaplastic oligoastrocytomas are intermediate to those of glioblastomas and anaplastic oligodendrogliomas suggests that decreased levels of YKL-40 staining in anaplastic oligodendrogliomas can be attributed to biological differences among these tumors independent of grade.

At first glance, in light of the suggested roles for YKL-40 in cancer, the differential expression of YKL-40 between glioblastomas and anaplastic oligodendrogliomas seems counterintuitive as invasion and angiogenesis can be seen in both glioblastomas and anaplastic oligodendrogliomas. YKL-40 expression, however, has been correlated with specific signaling pathways. For example, YKL-40 is expressed selectively in murine mammary tumors initiated by neu/ras oncogenes (29) and

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Fig. 2 Comparison of YKL-40 and GFAP immunohistochemistry in high-grade gliomas. A and B, serial sections of a glioblastoma demonstrating similar staining patterns with YKL-40 (A) and GFAP (B). C and D, serial sections of an anaplastic oligodendroglioma exhibiting less YKL-40 immunopositivity (C) as compared with staining with GFAP (D). E and F, serial sections of an invasive tumor edge demonstrating the detection of fewer reactive astrocytes by YKL-40 (E) as compared with GFAP (F).
Table 1  Summary of YKL-40 immunohistochemistry results for glioblastoma, anaplastic oligodendroglioma, and anaplastic oligoastrocytoma

<table>
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<th>AO (n = 29)</th>
<th>AOA (n = 15)</th>
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<tr>
<td>75-100</td>
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(B) Staining intensity of cells positive for YKL-40

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(C) Staining intensity of extracellular matrix for YKL-40

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Abbreviations: GBM, glioblastoma; AO, anaplastic oligodendroglioma; AOA, anaplastic oligoastrocytoma.

Phosphorylation of both extracellular signal-regulated kinases 1/2 and AKT occurs in a dose- and time-dependent fashion on addition of YKL-40 in fibroblastic cell lines (20). The RAS and AKT signaling pathways cooperate to induce glioblastoma formation in mouse models (30) and activation of the RAS-mitogen-activated protein kinase and phosphatidylinositol-3-kinase-AKT signaling pathways is very common in human glioblastomas (31). Although an intriguing possibility, whether the differential expression of YKL-40 between glioblastomas and anaplastic oligodendrogliomas can be attributed to differences in these signaling pathways among high-grade glial tumors will require further investigation.

YKL-40 Is a Stronger Marker than Glial Fibrillary Acidic Protein for Distinguishing Histologic Subtypes of High-Grade Glioma. In current diagnostic pathology, immunohistochemistry detects GFAP, which is most often found in astrocytic gliomas. However, GFAP is not restricted exclusively to astrocytic lesions and anaplastic oligodendrogliomas may show positive GFAP staining (32). In order to determine if YKL-40 might provide a better marker for distinguishing histologic subtypes of high-grade glioma, we compared YKL-40 and GFAP staining in a subset of 19 glioblastomas and 18 anaplastic oligodendrogliomas (Fig. 2A-D; Table 2). As in our initial analysis above, YKL-40 staining was able to distinguish between glioblastomas and anaplastic oligodendrogliomas in this subset of tumors: the percent positive cells (P < 0.001) as well as the staining intensity (P < 0.001) were significantly different. In comparison, the percent GFAP-positive cells was also significantly different (P = 0.012) but GFAP staining intensity was not significantly different (P = 0.255).

Although both YKL-40 and GFAP were effective at distinguishing glioblastomas from anaplastic oligodendrogliomas, YKL-40 staining seemed to be a stronger marker. When a k-mean clustering algorithm was applied to the data set and asked to find two clusters, an algorithm using the GFAP data correctly identified 17 of 19 glioblastomas and 9 of 18 anaplastic oligodendrogliomas. In comparison, an algorithm utilizing the YKL-40 data correctly identified 19 of 19 glioblastomas and 10 of 18 anaplastic oligodendrogliomas. Interestingly, when both the YKL-40 and the GFAP data were used, the algorithm correctly identified 18 of 19 glioblastomas and 15 of 18 anaplastic oligodendrogliomas. Therefore, although YKL-40 provided a better class distinction than GFAP when distinguishing histologic subtypes of high-grade gliomas, using a combination of YKL-40 and GFAP immunostaining afforded even greater diagnostic accuracy with respect to identification of anaplastic oligodendrogliomas.

To determine which staining characteristics of YKL-40 were most responsible for improved diagnostic accuracy over GFAP staining, we compared YKL-40 and GFAP staining within single histologic subtypes. Within glioblastomas, YKL-40 staining was not significantly different from GFAP staining when comparing the percent positive cells (P = 1.0) nor cellular staining intensity (P = 1.0), suggesting that these two markers provide equivalent information for glioblastomas; both markers stained similar percentages of glioblastoma cells at comparable intensities. In contrast, the percent positive cells was significantly different between YKL-40 and GFAP staining within anaplastic oligodendrogliomas (P = 0.003); GFAP stained a greater percentage of anaplastic oligodendroglioma cells at much higher intensities (73% of samples had a YKL-40 staining intensity of 0 or 1+ versus 78% of samples with a GFAP staining intensity of 0 or 1+).

Table 2  Summary of YKL-40 and GFAP comparative immunohistochemistry results for glioblastoma and anaplastic oligodendroglioma

(A) Number of cells staining positive for YKL-40 and GFAP

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(B) Staining intensity of cells positive for YKL-40 and GFAP

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of samples displaying a GFAP staining intensity of 3+). Thus, the greater staining differential afforded by YKL-40 in comparison with GFAP could most likely be ascribed directly to significantly lower levels of YKL-40 staining in anaplastic oligodendrogliomas.

One of the complicating characteristics of GFAP staining is its ability to stain reactive astrocytes strongly; immunohistochemically positive reactive astrocytes can falsely exaggerate the impression of a positively staining glial tumor. In order to determine if decreased staining of reactive astrocytes might be one of the explanations for YKL-40 being a better marker compared with GFAP, we stained regions of reactive gliosis in 16 high-grade gliomas and 5 additional pathologic brain samples without glioma and scored these samples for positive staining of reactive astrocytes (Fig. 2E and F; Table 3). When scores were clustered into two groups (0 and 1+ versus 2+ and 3+), the YKL-40 and GFAP distributions of positive cells were significantly different (P = 0.003); GFAP immunohistochemistry detected greater numbers of reactive astrocytes in 11 of 21 samples (95% CI, 0.30-0.74) and stained the cells more intensely in 7 of 21 samples (95% CI, 0.15-0.57) than did YKL-40.

Anaplastic oligodendrogliomas are also associated with 1p/19q loss of heterozygosity; these genetic markers also predict chemotherapeutic response and better prognosis in these lesions (33, 34). Although 1p/19q loss of heterozygosity status was not available for the samples included in this study, we investigated 1p status in the anaplastic oligodendrogliomas used in our initial microarray analysis (7). Although the average YKL-40 mRNA expression was slightly lower in 1p loss of heterozygosity tumors as compared with 1p intact cases, these groups were not significantly different.4 Thus, it seemed that YKL-40 provided diagnostic information in addition to that afforded by 1p/19q status.

**SUMMARY**

In the present study, we used immunohistochemistry to investigate YKL-40 protein expression in high-grade gliomas and to determine whether this single marker can augment the histologic classification of high-grade gliomas. YKL-40 immunohistochemistry was able to distinguish anaplastic oligodendrogliomas from glioblastomas in a highly significant manner.

Moreover, the findings that regional histologic differences within tumors correlated with YKL-40 staining and that YKL-40 expression levels in anaplastic oligoastrocytomas were intermediate to those of glioblastomas and anaplastic oligodendrogliomas suggest that decreased levels of YKL-40 staining in anaplastic oligodendrogliomas can be attributed to biological differences among these tumors that are not simply reflective of malignancy grade. When compared with GFAP, the current standard clinical marker for distinguishing diagnostically challenging high-grade gliomas, YKL-40 staining provided a better class distinction. Moreover, using a combination of YKL-40 and GFAP staining afforded even greater diagnostic accuracy. In this study, we chose to focus on anaplastic oligodendrogliomas and glioblastomas, the highest-grade lesions of two distinct tumor lineages. In the future, it will be interesting to examine YKL-40 with respect to tumor grade within a single tumor lineage, such as comparisons specifically among glioblastomas and anaplastic astrocytomas, as well as in other types of central nervous system tumors.

**ACKNOWLEDGMENTS**

We thank Jennifer Roy for help with tissue acquisition; Molly Dorfman, Whitney Nugent, and Loc Pham for clinical data; and Kevin Park for photography.

**REFERENCES**


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**Table 3** Summary of YKL-40 and GFAP immunohistochemistry results for reactive astrocytes (n = 21)

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*Cell number: 0, none; 1+, few; 2+, some; 3+, many—as compared with the total number of reactive astrocytes.
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