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Commentary

Nature versus Nurture in Glioblastoma

Microenvironment and Genetics Can Both Drive Mesenchymal Transcriptional Signature

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Glioblastomas (GBMs) are the most common malignant brain tumors, but they are currently incurable. It is hoped that targeted therapies may prove effective in improving survival. Indeed, efforts to integrate multimodal molecular and clinical data for brain tumors have begun to yield a clearer picture of these aggressive neoplasms. The Cancer Genome Atlas (TCGA)[1] and the Repository of Molecular Brain Neoplasia Data (REMBRANDT)[2] both support numerous data sets for mRNA and miRNA expression, DNA copy number alterations, sequencing data, and both magnetic resonance and histological images. Many of the molecular underpinnings of GBM have been identified, the most abundant mutations have been catalogued, and perturbed signaling pathways have been elucidated.[1] Mutations in isocitrate dehydrogenase 1 and 2 have been identified, and a relationship to secondary GBM has been established.[3,4] A group of tumors with a CpG island methylator phenotype has been described, portending an improved prognosis.[5] These efforts and others have also resulted in the identification of three or four transcriptional classes of GBM.[4,6] Common to all of the major molecular classification schemes for GBM is a group that characteristically shows increased expression of mesenchymal genes. This mesenchymal expression signature has been associated with reduced survival and aggressive behavior.[6]

Despite these new insights into the biology of GBM, and the promise of more tailored therapy on the horizon, the diagnosis of GBM is still made using a more fundamental technique: microscopic examination of H&E-stained slides. Over the years, several different histopathological grading systems for GBM and other astrocytomas have been used, including the Kernahan system, the St. Anne/Mayo system, and the now current World Health Organization (WHO) system.[7,8] A consistent feature of all these diagnostic schemata is the inclusion of necrosis and vascular proliferation, two elements of the tumor microenvironment, as indicators of poor prognosis. The reduction in patient survival associated with these features has withstood the test of time and multiple confirmatory studies (reviewed by Brat et al[9]).

In this issue of *The American Journal of Pathology*, Cooper et al[10] report their correlation of histopathological features of the tumor microenvironment with gene expression patterns developed by the large multimodality genomics efforts. They identify an intimate association between necrosis in GBM and the mesenchymal transcriptional class. The degree of necrosis demonstrated a close association with genes that define mesenchymal class, including master regulators such as STAT3 and C/EBP-β.[11] Perhaps their most interesting finding was that increasing mesenchymal expression signature among non-mesenchymal-class tumors correlated with the extent of necrosis (Figure 1). This highlights the important contribution of the tumor microenvironment in dictating mRNA transcription and represents an important first step in integrating microscopic features and gene expression patterns in a more quantitative and sophisticated way.

Correlating Morphological Analysis with Large-Scale Genomics Data

The microscopic analysis of tumor morphology is not new; in fact, it represents the basis of most studies in anatomical pathology. Because of relatively recent technological advancements, however, quantitative mor-

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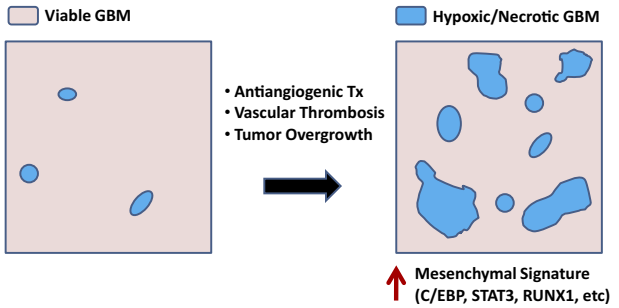

Figure 1. Increased necrosis in GBM promotes a mesenchymal expression signature.

phometry has become increasingly sophisticated. Improvements in imaging technology, including the ability to scan slides and store images with relative speed, increases in storage capacity, and production of powerful software platforms such as NIH Image and ImageJ have facilitated robust quantitative analysis of histological images on morphological grounds. Technology in molecular biology allowing for multimodal data acquisition of mRNA expression, genetics, and epigenetics has progressed even more rapidly. The field of glioma biology benefited from GBM being selected early as one of the tumors to be added to large public data sets such as the TCGA, which include not only expression profiles but also scanned slide and radiographic images for future correlation with molecular data.

Computer-based image analysis has been used previously to evaluate the TCGA data set. For instance, Cooper and colleagues[12,13] earlier used nuclear segmentation analysis to cluster TCGA tumors into three morphometric classes. Similar to clustering by transcriptional classes, which was reported by Verhaak et al,[4] morphometric clustering correlated with specific signaling pathways and survival profiles.[12,13] An analogous approach, using distinct criteria, generated five morphometric clusters from the TCGA data set and was predictive of therapeutic outcome.[14] These early results are encouraging, but thus far morphometric clustering has not shown the tight association with specific molecular aberrations that has been observed with transcriptional clustering.

The present contribution by Cooper et al[10] differs from previous efforts in that, rather than correlating intrinsic features of single cells (most often nuclear details) with TCGA data, they examined broader aspects of the tumor microenvironment, evaluating three components: necrosis, angiogenesis, and macrophage infiltration. Necrosis appeared to have the strongest relationship to the previously defined transcriptional classes, and in particular to the mesenchymal subgroup.

Necrosis, Hypoxia, and Aggressiveness in GBM

Necrosis is a strong predictor of behavior in malignant glioma.[9] Intratumoral necrosis likely results from multiple mechanisms. Focal necrosis occurs when the energy requirements of the highly proliferative tumor cells outpace their own energy supply. However, a more complex mechanism has been suggested for large areas of necrosis within GBM. These have been attributed to vascular thrombosis, which is often identified within tumors and seen in a high proportion of pseudopalisades in GBM.[15] Thrombosis of vessels may be related to a number of derangements in the coagulation cascade, but has specifically been attributed to the induction of tissue factor by tumor cells.[16] Tissue factor may promote coagulation in intratumoral vessels by directly inciting the coagulation cascade.[16] Finally, as antiangiogenic therapies such as bevacizumab are increasingly used in malignant gliomas, we will likely encounter more therapeutically induced hypoxia and necrosis.

How necrosis modulates gene expression is an area of active investigation. It may involve the induction of hypoxia-inducible transcription factors (HIF1-α and HIF2-α), the release of growth factors by dying cells, or eliciting necrosis-associated inflammation. HIF transcription factors are concentrated around necrotic regions in GBM, and they seem to promote a stem-like phenotype in tumor cells.[17,18] Li et al[17] demonstrated that HIF2-α was preferentially expressed in brain tumor stem cells in response to hypoxia, and knockdown of HIF2-α inhibited *in vitro* and *in vivo* tumorigenicity. Our research group found that hypoxia increased the expression of stem cell markers and increased the clonogenicity of GBM in a HIF1-α-dependent manner.[18] The findings reported by Cooper et al[10] thus suggest a broad link between necrosis, mesenchymal gene expression signature, stemness, and the hypoxic response.

It would be worthwhile to determine whether HIF proteins directly regulate the transcription of mesenchymal genes in glioma. Definitive answers to that question will require a chromatin immunoprecipitation approach, either microarray (ChIP-chip) or high-throughput DNA sequencing (ChIP-seq); however, the available data suggest that at least some mesenchymal genes are directly regulated by HIF. For instance, the hexokinase genes *HEXA* and *HEXB* are known targets of HIF1-α (reviewed by Keith et al[19]) and are among the genes that define the mesenchymal transcriptional class.[4] There is no known association of the so-called master regulators of the mesenchymal class and HIF proteins in glioma; however, C/EBP-β has been shown to be a target of HIF2-α outside the brain.[20] The present report provides some evidence supporting the notion that C/EBP-β could be regulated by HIF in glioma as well. For instance, the authors demonstrated that C/EBP-β is expressed highly in the pseudopalisading perinecrotic cells *in vivo*, and is induced in hypoxia *in vitro*.[10] The staining of pseudopalisading glioma cells around areas of necrosis has been reported previously for HIF1-α.[15] Other master regulators of the mesenchymal class, such as STAT3, may also modulate HIF activity. For instance, it was recently reported that NF1 loss, a genetic change that clusters in the mesenchymal transcriptional class, supports the activation of STAT3.[21] STAT3 was shown in some carcinomas to form a transcriptional complex with HIF1-α, and loss of STAT3 attenuated the ability of HIF1-α to induce targets such as VEGF.[22,23] It would be interesting to determine whether

STAT3 similarly supports transmission of HIF signals in glioma for such targets as C/EBP-β.

Limitations

The work described by Cooper et al[10] does have limitations. For instance, although the relationship between necrosis and the mesenchymal gene expression was clear, other components of the microenvironment (such as inflammatory infiltrates and angiogenesis) were examined less rigorously. This is largely due to limitations of the available data set. In the case of angiogenesis, histopathological analysis of the stained sections yielded only the descriptors 'present' and 'absent', precluding meaningful subsequent correlations. The analysis of inflammatory infiltrates was similarly constrained. It has been noted previously that the mesenchymal class contains higher proportions of inflammatory cells,[4] and inflammatory signatures were noted to be associated with increased necrosis in the authors' analysis of the data set. Although Cooper et al[10] did evaluate infiltration of macrophages and their relationship to gene expression, the authors relied on a categorical quantification for macrophages (0, 1+, and 2+), rather than the more continuous quantification (fractional area) used to quantify necrosis. Furthermore, a systematic evaluation of other nonmacrophage inflammatory cells was not performed, because no specific markers were used. Thus, a contribution of inflammatory cells or blood vessels to the mesenchymal transcriptional class has not really been excluded. Perhaps future additions to the TCGA will include immunohistochemical analysis of vessels and inflammatory cells, to allow for more robust evaluation of the tumor microenvironment.

The Future

The present work raises several important questions central to our understanding of glioma biology. For instance, it will be critical to determine more precisely how genetic alterations and the microenvironment interact within transcriptional classes. It is clear that certain genetic alterations cluster within specific transcriptional class, such as *IDH1* mutations in the proneural group and *NF1* deletion in the mesenchymal group.[4] *NF1* loss could directly regulate mesenchymal gene expression. Alternatively, *NF1* loss could indirectly promote the mesenchymal transcriptional class through a complex interaction with HIF signaling, or by generating a hypoxic/necrotic tumor environment through promotion of tumor overgrowth or vascular thrombosis. Valuable information may also be gleaned from the subsets of tumors that do not fit the typical profiles, such as mesenchymal tumors with minimal or no necrosis. It would be worthwhile to determine whether other activators of cellular stress, such as inflammation, can regulate the mesenchymal gene signature in tumors with low or absent levels of necrosis.

Other more practical questions will have to be answered as transcriptional classes become integrated into clinical decision making. Cooper et al[10] report that nonmesenchymal tumors become more mesenchymal with increasing necrosis. This finding suggests a certain degree of plasticity between transcriptional classes. It will be important to determine if regional differences in transcriptional class exist within individual tumors. If this occurs to a significant degree, sampling in the hypoxic core of a tumor may yield a mesenchymal signature, whereas more peripheral sampling could yield an alternative transcriptional class. Other clinical scenarios in which plasticity within transcriptional class may prove important is in the setting of tumor recurrence and after adjuvant therapy. Although transcriptional class switching has not been identified between the small subset of primary and recurrent tumors previously analyzed,[4] this finding will need confirmation in larger data sets. The use of chemotherapy such as bevacizumab, which specifically inhibits angiogenesis, may also drive a mesenchymal class by increasing intratumoral hypoxia. The translation of molecular advances into new therapeutic paradigms thus continues to grow more complicated.

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