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MNI overexpression with varying tumor grade is a promising predictor of survival of glioma patients

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Abstract

Gliomas have substantial mortality to incidence rate ratio and a dismal clinical course. Newer molecular insights, therefore, are imperative to refine glioma diagnosis, prognosis and therapy. Meningioma 1 (MNI) gene is a transcriptional co-regulator implicated in other malignancies, albeit its significance in glioma pathology remains to be explored. IGFBP5 is regulated transcriptionally by MN1 and IGF1, and is associated with higher glioma grade and shorter survival time, prompting us to ascertain their correlation in these tumors. We quantified MN1, IGFBP5 and IGF1 expression in 40 glioma samples and examined their interrelatedness. MN1 mRNA-protein inter-correlation and gene’s copy number were evaluated in these tumors. Publicly available TCGA datasets were used to examine the association of MN1 expression levels with patient survival and for validating our findings. We observed MN1 overexpression correlated with low grade (LGGs) and not high grade gliomas (HGGs), and is not determined by copy number alteration of the gene. Notably, gliomas with upregulated MN1 have better overall and progression-free survival. IGFBP5 expression inversely associated with MN1 expression levels in gliomas but correlated positively with IGF1 expression in only LGGs. This suggests a potential grade-specific interplay between repressive and activating roles of MN1 and IGF1, respectively in the regulation of IGFBP5. Thus, MN1 overexpression, a promising predictor of overall and progression-free survival in gliomas, may serve as a prognostic biomarker in clinical practice to categorize patients with survival advantage.

Introduction

Gliomas are clinically recalcitrant and constitute one of the two most common types of primary brain and central nervous system (CNS) tumors (1, 2). Epidemiological data suggest gliomas comprise < 1.6% of all new cancer diagnoses, but have substantially higher mortality to incidence rate ratio (MIR=0.80) as compared to breast (0.28) and prostate (0.26) cancer that have significantly higher morbidity (Fig. 1A) (3, 4). Until recently World Health Organization (WHO) recommended
evaluation of tumor histology alone as the standard for glioma diagnosis (5). Gliomas based on their cytomorphologic similarity to non-neoplastic glial cells, namely the astrocytes, oligodendrocytes and ependymal cells, are classified as astrocytomas, oligodendrogliomas and ependymomas, respectively. Gliomas are further graded based on the WHO consensus-derived scale of I to IV (5). While the tumors of WHO grades I and II are categorized as low grade gliomas (LGGs), those ascribed to grades III and IV are considered high grade gliomas (HGGs) (6-9). Gliomas of all grades with the exception of WHO grade I are typically diffuse invasive tumors. They infiltrate the surrounding brain parenchyma extensively and very early in their course, making the complete surgical resection extremely difficult and unlikely (10).

Besides their diverse cellular origin, gliomas show substantial molecular and genetic heterogeneity. Different studies reported certain molecular signatures to be associated with specific glioma subtypes (7, 11). These molecular determinants were incorporated by WHO to revise the classification system for glioma diagnosis in 2016 (12). While the new WHO classification system is still to be widely accepted problems are becoming evident and further revisions are being proposed (13-17). Like other cancers, gliomas are characterized by aberrations in several molecular pathways that confer a growth advantage to these tumors (10). Insulin-like growth factor (IGF) system is one such pathway that has been implicated in Gliomas (18, 19). The IGF pathway involves concerted interactions of two ligands IGF1 and IGF2, three receptors comprising IGF1 receptor (IGF1R), IGF2 receptor (IGF2R) and insulin receptor (IR), and six IGF-binding proteins (IGFBP1 to IGFBP6) (20). IGF ligands mediate their growth-promoting effects by binding IGF1R. This transduces the phosphatidylinositol 3’-kinase (PI3K) and mitogen activated protein kinase (MAPK) cascades to inhibit apoptosis and elicit cellular proliferation, respectively (20, 21).

The IGF system plays pleiotropic roles in the development of the CNS with certain variations between the human and murine species (22). IGF1 and its receptor-IGF1R express in the normal human brain and are known to be upregulated in gliomas (23-30). In a study comprising 39
astrocytomas (WHO grade II-IV), IGF1 expression was observed in tumors of all grades. Markedly, only the proportion of immunopositive cells and not the staining intensity correlated with the histopathological grade of gliomas (30). It is also reported that exogenous IGF1 induces proliferative and anti-apoptotic signalling through its receptor IGF1R in cultured glioma cells (28, 31).

In addition to their autocrine growth promoting roles, IGFs also have characteristics of a paracrine growth factor and therefore remain in circulation. These circulating IGFs in serum are bound by IGFBPs, which prolong their half-lives and control their exit from the vascular compartment (19). In the process, IGFBPs modulate spatiotemporal distribution and bioavailability of IGFs in a cell/tissue-type and species-specific manner. Of the six IGFBPs, IGFBP5 is evolutionarily the most conserved across vertebrates and is more than 97% identical between human, mouse and rat (32). IGFBP5 and IGF1 show spatiotemporal co-expression during brain development suggesting that their interplay is crucial to the process (22, 33). Also, the brain of IGF1 transgenic mice shows upregulated IGFBP5 expression (34). Independent compelling observations about opposing roles of IGFBP5 (oncogenic and anti-oncogenic) in different cancers have been reported (19, 35).

Nevertheless, in gliomas elevated levels of tissue IGFBP5 were associated with higher tumor grades and poor prognosis (36-38). Silencing IGFBP5 expression impedes invasion but promotes proliferation of GBM cells in vitro, thus having opposing effects on two cellular hallmarks of neoplastic state (39).

Expression of IGFBP5 is regulated in part by IGF1 through both IGF1R dependent and independent mechanisms (40, 41). Besides IGF1, several other biomolecules such as retinoic acid (RA), MN1, the transcription factor-activating enhancer binding protein 2 alpha (AP-2), prostaglandin E2, cortisol and vitamin D are known to regulate transcription of IGFBP5 in a cell-type and context-dependent manner (41, 42). RA, for example, can in opposing ways either induce or inhibit IGFBP5 expression mediated by the retinoic acid receptor/retinoic x receptor (RAR/RXR) (41). MN1 (Meningioma 1 gene, 22q12.1) was initially identified due to a balanced translocation (4;22) in a
meningioma patient showing absence of its expression. Therefore, the authors proposed it as a candidate tumor suppressor gene (43). MN1 is a transcriptional co-regulator that can both induce and repress RAR/RXR target genes. As a co-activator, MN1 acts synergistically with RAR/RXR to activate RA mediated expression of IGFBP5 (44). MN1 is also known to transcriptionally co-repress RAR/RXR target genes (45-47). Interestingly, deletions in MN1 have been implicated in patients with neurodevelopmental abnormalities (48).

Independent studies have examined MN1 and IGFBP5 in varied contexts described above, and associated the latter with poor prognosis in gliomas. It interested us that since MN1 transcriptionally regulates IGFBP5, are the expression levels of the two genes related and can MN1 help predict patient survival in human gliomas. Therefore, we undertook the present study to - i) determine copy number alteration and expression of *MN1* in gliomas, and assess their correlation ii) discern association of *MN1* expression with tumor grades and patient survival, iii) quantify *IGFBP5* and *IGF1* expression in these patients, and iv) ascertain whether expression of *IGFBP5* changes as a function of *MN1* and *IGF1* expression levels.

**Results**

**Gliomas show copy number alterations of *MN1* gene**

The gliomas in the present study (experimental dataset) were analysed for *MN1* CNA using qPCR assay. Representative qPCR amplification plots (Fig. 1B) depict the curves for *MN1* (gene of interest) and *RNaseP* (reference gene) where the difference in cycle threshold (ΔCt) values of 1, 0 and -1 correspond to one, two and four copies of *MN1* gene, respectively. We found *MN1* gene copy number alteration (CNA) occurred in gliomas (33% i.e. n=13/39, Fig. 1C), and it was more common in HGGs than in LGGs (p=0.03). Specifically, in HGGs copy number gain (n=9/39) was observed thrice more often than loss (n=3/39, Fig. 1C). None of the LGGs showed *MN1* copy number gain.
Similar to the experimental dataset, the TCGA datasets also showed CNA of *MN1* gene more frequently in HGGs than in LGGs (Fig. 1D). However, unlike the experimental dataset where copy number gain was more frequent in HGGs, in the TCGA datasets copy number loss (n=93) is ~4 times more frequent than gain (n=24) in HGGs, whereas LGGs showed similar number of cases with copy number loss (n=17) and gain (n=13, Fig. 1D). Accordingly, *MN1* copy number loss contributed considerably (p<0.0001) to the higher overall CNA seen in HGGs as compared to LGGs. Notably, gliomas in either the experimental or TCGA datasets did not evince complete loss of the gene. To understand the role of *MN1* CNA in glioma pathology it is vital to understand the molecular significance of the gene in gliomas. As a step in this direction, we examined whether gliomas with *MN1* CNA have altered gene expression and if those with normal (two) copies show expression similar to non-neoplastic brain.

**Altered *MN1* expression fails to conform to CNA but correlates with LGGs**

Relative *MN1* (mRNA) expression in the experimental dataset is represented as bar graph (Fig. 2A). *MN1* expression in the non-neoplastic brain is considered as baseline/zero. Gliomas with relative *MN1* expression values beyond the range of ~0.3 and 0.2 are categorized as cases with downregulated and upregulated expression, respectively. We found that LGGs frequently evince *MN1* overexpression as compared to HGGs (p=0.04). Green and red numerals mentioned against the bar connote normal and altered copies, respectively in the corresponding tumor (Fig. 2A). Tabulated summary (inset Fig. 2A) shows ~45% gliomas (n=18/40) have *MN1* overexpression, and most gliomas, including those evaluated for protein expression, rarely show correlation between the gene’s expression and copy number status (Fig. 2A, B, C and D). To statistically assess if the observed CNA could reliably predict corresponding change in *MN1* expression we fitted the regression model to our data. High p-value and a low coefficient of determination indicate lack of association (p=0.17; R²=0.05, Fig. 2B) between gene’s copy number and mRNA expression in gliomas. Also, a negative slope (-0.2289) of the regression line suggests that with an increase in
MN1 copies, gene expression tends to decrease. This signifies that alteration in MN1 copy number does not imply a relatable change in its expression.

To ascertain whether gliomas with altered MN1 transcript levels have proportionate protein expression, we probed cases that had sufficient tumor tissue in excess of the diagnostic requirement, for MN1 and β-Tubulin expression. We noticed MN1 protein expression in glioma cases with discernible transcript levels (Fig. 2C and D). Nevertheless, in cases such as G7 and G23, despite observed transcript overexpression (Fig. 2A) MN1 protein levels were not upregulated (Fig. 2D). This discordance may be because commercially purchased total RNA and protein (non-neoplastic brain), used for normalizing tumor MN1 mRNA and protein expression, respectively originated from different individuals. In the remaining cases, protein expression trend corresponds with but is not altered proportionate to the change in mRNA levels, suggesting a role of post-transcriptional regulatory mechanisms (Fig. 2A, C and D).

Interestingly in the experimental dataset, median MN1 expression is reasonably higher in LGGs than HGGs (p=0.02, Fig. 2e). Analyses of the TCGA datasets also uncovered that LGGs have an appreciably higher MN1 expression than HGGs (p<0.001, Fig. 3A). Further, to validate our observations of MN1 expression not correlating with its copy number status, we analysed TCGA datasets for MN1 expression and CNA (Fig. 3B). Pairwise Wilcoxon test revealed LGGs and HGGs with single copy of the gene had markedly reduced average MN1 expression as compared to cases with normal copy number (p<0.0001; Fig. 3B). The reverse was not true, because a gain in MN1 copy number did not signify an increased average expression in LGGs and HGGs (Fig. 3B).

Regression analysis to test association between MN1 expression and the gene’s CNA yielded a significant test statistic (p<0.0001) but had low R-squared values in both LGGs (R²=0.07, Fig. 3C) and HGGs (R²=0.12, Fig. 3D). Similar to the experimental dataset, in the TCGA dataset examining the distribution of data points along the regression line suggests that gene’s copy number does not predict the variation in its expression levels, which is evident also in Fig. 3B.
Since the TCGA dataset validated our findings of altered *MNI* expression correlating with LGGs, we wanted to test whether *MNI* overexpression can be used to predict survival and assess it in relation to the widely accepted predictors such as Isocitrate dehydrogenase (IDH) mutation and 1p19q co-deletion.

**MNI overexpression predicts favorable survival in gliomas**

IDH mutations seen in gliomas (including grade II and III astrocytoma, grade II and III oligodendroglioma and GBM) are known to confer survival advantage in patients (11, 49). Also, the co-deletion of chromosome arms 1p and 19q observed in only oligodendroglialomas (grade II and III) is positively associated with survival.(11, 49) We tested whether *MNI* overexpression can sub-type LGGs by predicting better survival. Despite HGGs having lower median *MNI* expression than LGGs, we also examined the relationship between *MNI* expression and HGG patient survival.

Survival prediction analysis was not possible with the experimental dataset, so we used the TCGA datasets.

Kaplan-Meier estimates of survival (with log-rank test) indicate that *MNI* overexpression associated with longer survival in LGGs (p=0.0001; Fig. 4A) and HGGs (p<0.0001; Fig. 4D). Expectedly, LGGs with IDH mutation (p<0.0001; Fig. 4B) and 1p19q co-deletion (p<0.0001; Fig. 4C) have prolonged overall survival (OS). Likewise, HGGs with IDH mutation (p<0.0001; Fig. 4E) and 1p19q co-deletion (p<0.0001; Fig. 4F) have better overall and median survival (Fig. 4G). NA values for median survival in LGGs that overexpress *MNI*, and those with IDH wildtype (Fig. 4G), indicate that values could not be computed because the group did not drop to 50% survival probability at the end of available data (Fig. 4A and zoomed in region of survival curve therein, and Fig. 4B). In such a case, it implies that median overall survival is greater than the last point on the survival curve.

Therefore, in LGGs with *MNI* overexpression median overall survival maybe ~180 months or more, this seems better than the median survival predicted by IDH mutation or 1p19q co-deletion.
Further, we performed receiver operating characteristic (ROC) analysis to evaluate sensitivity and specificity of three-month survival prediction for *MNI1* overexpression, IDH mutation and 1p19q co-deletion in the TCGA retrieved datasets. Patients with missing values for any of the three molecular predictors were excluded. This is because a comparison can be performed for the three molecular predictors only when all three values for each patient in the dataset are available. Accordingly 100 LGGs and 131 HGGs were included in the ROC analysis. We observed that area under the ROC curve (AUC) in LGGs for MNI1 expression was the largest (0.853) as compared to IDH mutation (0.521) and 1p19q co-deletion (0.553) (Fig. 4H). AUC value of ≥ 0.7, means that the predictor being analysed is a good classifier for predicting survival. However, in HGGs AUC estimates of the 3-molecular alterations/parameters being evaluated were not available because the ROC curves were below the diagonal reference line and hence could not be computed. The AUC values suggest that *MNI1* overexpression has better predictive ability for making three-month survival prediction in LGGs. Therefore, our findings suggest that *MNI1* overexpression may be considered as a predictor for classifying LGGs with longer overall survival.

Notably, Kaplan-Meier analysis also showed that glioma patients with *MNI1* overexpression, irrespective of low or high tumor grade (Fig. 5A and B, p<0.0001), have a markedly longer progression-free survival (PFS).

**IGFBP5 and MNI1 expression inversely correlate in gliomas**

MNI1 is a transcriptional co-regulator and is known to synergize both induction and repression of RAR/RXR target genes. *IGFBP5* is also a RAR/RXR target gene; therefore we examined its expression in gliomas. We found *IGFBP5* mRNA levels were upregulated in most gliomas (90%; n=36/40). *IGFBP5* mRNA levels seemed slightly higher in HGGs than LGGs, but the difference was not statistically significant (p=0.58, Fig. 6A). The case-wise analysis shows overexpression of *IGFBP5* transcripts in ~84% (n=11/13) LGGs and ~92% (n=25/27) HGGs and expectedly this is not a considerable difference (p=1.0). Further, to test whether *MNI1* expression affects *IGFBP5* levels,
we used a linear regression analysis and found the association was not appreciable in LGGs (p=0.99; R²<0.0001, Fig. 6B) or HGGs (p=0.19; R²=0.07, Fig. 6C). In HGGs despite no statistical correlation, apparently IGF5 expression tended to decrease with an increase in the MNI levels.

To verify the above findings, we examined the TCGA datasets. The TCGA datasets statistically strengthened (p<0.0001, Fig. 6D) our observation of slightly higher IGFBP5 expression in HGGs than in LGGs, and was in line with the previous reports that HGGs have higher IGFBP5 expression than LGGs. The statistical insignificance in the experimental dataset may be because of the smaller sample size. Interestingly, it emerged that IGFBP5 expression tends to decline (p<0.0001) with an increase in MNI expression levels in both LGGs (slope: -0.51, Fig. 6E) and HGGs (slope: -0.41, Fig. 6F). This suggests a negative correlation between MN1 and IGFBP5 expression in gliomas.

**IGF1 and IGFBP5 expression correlates in LGGs**

IGF1 regulates IGFBP5 expression transcriptionally and post-translationally in a cell-type specific manner (40, 42, 50, 51). Therefore, we quantified IGF1 expression in gliomas. In LGGs average IGF1 expression is apparently more than HGGs, but the difference is not significant (p=0.54, Fig. 7A). Further, in LGGs a positive correlation between IGFBP5 and IGF1 expression levels was indicated by the regression line though p-value seemed barely significant (slope=0.2637; p= 0.05; R²=0.33, Fig. 7B). Contrarily, IGFBP5 and IGF1 expression showed no association in HGGs (p=0.81; R²=0.002, Fig. 7C).

Our findings differ from the previous report that showed IGF1 expression was higher in grade III and IV tumors than grade II gliomas (30). Therefore, we assessed the larger sample size of TCGA datasets to verify grade specificity of IGF1 expression. Similar to the experimental dataset, in the TCGA datasets we found LGGs (grade II gliomas) have higher average IGF1 expression value than HGGs (p=0.03, Fig. 7D). Also, in the TCGA datasets we noted IGFBP5 expression correlates with IGF1 levels in LGGs (p=0.03; Fig. 7E) but not HGGs (p=0.96; Fig. 7F). This suggests in LGGs...
higher expression of MN1 may repress IGFBP5 expression while IGF1 mediated induction may contribute in maintaining discernible IGFBP5 expression.

**MN1, IGF1 and IGFBP5 have different roles in meningiomas**

Briefly, to quantify the expression of these molecules and investigate their relationship in a different brain tumor histology, we examined 39 meningioma tumor samples. These samples were collected from meningioma patients undergoing surgical removal of their tumors at the same hospital as the glioma patients. Meningiomas in the experimental dataset showed varying expression of MN1, IGFBP5 and IGF1 but relation to grade was not possible due to limited numbers of grade II (n=3) and grade III (n=1) tumors (data not shown). Therefore, we analysed meningioma datasets available in GEO datasets and the findings differed from that in gliomas (Supplementary Fig. 1).

We found IGFBP5 transcript levels correlated positively with MN1 mRNA expression in grade I and II meningiomas (Supplementary Fig. 1D and E), unlike gliomas where IGFBP5 and MN1 expression were inversely associated in LGGs and HGGs (TCGA datasets). In grade III meningiomas the association seemed positive (Supplementary Fig. 1F), and this may have been statistically relevant if more number of grade III samples in these GEO datasets were available for analysis. Further, IGFBP5 mRNA levels associated positively with IGF1 transcript levels in all the grades of meningiomas (Supplementary Fig. 1G, H and I). This suggests that in meningiomas both MN1 and IGF1 may independently or co-operatively activate IGFBP5 expression. This is in contrast to gliomas where IGFBP5 expression is likely regulated by a grade-specific opposing (repressive and activating) interplay of MN1 and IGF1. In view of these observations, and that meningiomas are considered less aggressive with better prognosis than gliomas, it is likely that IGFBP5 expression and its regulation by MN1 and IGF1 may have distinct meningioma-specific oncogenic roles.

Functional analyses along these lines will be insightful for making clinically relevant inferences.
Discussion

MN1 co-regulates gene transcription and is encoded by its gene located on chromosome 22 which is reported to show aberrations in human brain tumors including gliomas (52, 53). To our knowledge, alterations in MN1 have not been assessed systematically in human gliomas earlier. We find that MN1 overexpression predicts better median overall and progression-free survival of the patients. Also, a collective molecular dynamic of MN1, IGF1 and IGFBP5 may be a determinant of prognosis in gliomas, proving eventually to be of clinical relevance in predicting patient outcomes.

We observed a grade-specific overexpression of MN1 transcripts, with upregulated expression in 69% LGGs (n=9/13 grades I and II) as opposed to only 33% HGGs (n=9/27 grades III and IV) when compared to non-neoplastic/normal brain. A higher median MN1 expression correlates with LGGs in both the experimental (gliomas in the current study) and validation (TCGA) datasets. Chen et al. have reported MN1 overexpression in low-risk group gliomas comprising grade II and III tumor data retrieved from the Chinese Glioma Genome Atlas (CGGA) array database (supplementary Fig. S1 by Chen et al.) (54).

Further, we observed that copy number alteration in MN1 is more frequent in HGGs compared to LGGs. It was intriguing that a change in MN1 copy number did not lead to a relatable change in the gene’s expression in either the experimental or TCGA datasets. Similar discordance between gene copy number and expression is reported earlier in breast cancer and gliomas (55, 56). It is likely that in such cases altered pre-transcriptional regulation or post-transcriptional change(s) modify mRNA half-life, without requiring CNA to predicate altered gene expression. Thus, change in gene dosage does not necessarily underlie altered MN1 expression in gliomas.

Survival analysis tests the ability of a molecular/biochemical parameter or treatment to predict survival probabilities in patients. It was interesting to note that within the LGG and HGG categories, patients with upregulated MN1 expression showed a survival advantage. ROC analysis showed that for a three-month survival prediction in LGGs, high MN1 expression is a good classifier as it had
greater AUC values than IDH mutation and 1p19q co-deletion. However in HGGs, ROC analysis was not informative because ROC curves were below the reference diagonal line and hence AUCs could not be computed. ROC analysis to compute time-dependent AUCs for longer time duration (more than three months) for each of the 3-molecular predictors returned AUC values < 0.6. This means performance of the 3-predictors/classification models to predict survival for longer time had poor discriminatory power. This may be because total number of evaluable LGGs (n=100) and HGGs (n=131) in the datasets was reduced owing to filtering out of data points/patients where values of any of the 3-predictors were not available for comparison. Remarkably, MN1 overexpression in gliomas irrespective of tumor grade predicts longer PFS indicating that such patients were less likely to undergo disease progression. Thus MN1 overexpression in gliomas clearly confers a survival benefit on patients and holds promise as a prognostic marker for clinical use.

MN1 possesses properties of a transcriptional regulator but lacks DNA binding domains and therefore functions as a co-regulator (46, 53). It was demonstrated that cooperation between MN1 and RAR/RXR is crucial to RA mediated transcriptional regulation of IGFBP5 (44). IGF1 is also known to regulate expression of IGFBP5 (40, 50, 51). In the present study, we found higher median IGFBP5 expression in HGGs, whereas similar to MN1 overexpression greater median IGF1 levels correlated with LGGs. Therefore, it is likely that IGFBP5 may simultaneously be regulated by MN1 and IGF1 in gliomas.

In LGGs despite overexpression of MN1 and IGF1, the two known activators of IGFBP5, it was counter-intuitive that IGFBP5 expression correlated with HGGs instead. Therefore, we examined further and found that IGFBP5 expression was inversely correlated with MN1 levels in LGGs and HGGs. This suggests MN1 tends to repress IGFBP5 expression in gliomas. However, another modulator (IGF1) of IGFBP5 expression may oppose MN1 mediated repression to enhance IGFBP5 expression. This molecular interplay between repressive and activating regulatory forces possibly
dictates grade-specific IGFBP5 expression and contributes to associated clinical attributes such as poor prognosis, disease progression and shorter survival rate (Fig. 8).

Accordingly, IGF1 levels that correlated only with LGGs seem crucial in maintaining detectable and oncogenically relevant grade-specific IGFBP5 expression (Fig. 8A). In HGGs lower MN1 levels may result in lesser repression, causing accumulation of IGFBP5 due to IGF1 mediated induction of expression (Fig. 8B). Similar to previous report (36), IGFBP5 overexpression in LGGs and HGGs (TCGA datasets) were found to portend dismal median survival (table insets in Fig. 8A and B). Thus, IGBP5 levels in gliomas seem to be fine-tuned by the opposing actions of MN1 and IGF1 in a tumor grade-specific manner and accordingly predict patient survival.

Our study shows for the first time that MN1 overexpression correlates with LGGs, is not determined by the changes in the gene’s copy number, instead associates inversely with IGFBP5 expression and thus, predicts better overall median and progression-free survival in gliomas. Further, based on our analyses we propose that grade specific expressions of MN1, IGF1 and IGFBP5 underlie their mutual regulatory dynamic to govern the clinical course and characteristics of different glioma grades. Future functional studies should help validate the proposed molecular interplay and its significance to glioma pathology. Notwithstanding the significance of studies needed on this line, we find that MN1 overexpression with varying tumor grade may be leveraged as a predictor of better overall and progression-free survival in glioma patients.

Materials and Methods

Patients and sample collection

This study was approved by the Institutional Human Ethics Committee of the National Institute of Immunology (IHEC/VB/24/2008) and the Max Healthcare Ethics Committee and was performed in accordance with the Declaration of Helsinki. Samples were collected with due informed written
consents from the concerned patients. A part of the resected tumor tissues along with matched peripheral blood samples were collected with due informed written consents of the concerned patients, who were consecutively operated for intracranial gliomas during May 2008–August 2009. All the tumors were examined histopathologically and graded following the WHO criteria 2007(5).

Clinical details of samples analysed in the experimental dataset are presented in Table 1. The glioma cases were grouped and numbered such that the progressing numbers correlate with increasing grades. 40 glioma (G) patients included in the study represent primary tumors, except G15 and G20 that represented recurrent cases. The glioma patient G20 had surgical resections for astrocytoma (II), oligodendroglioma (III) and oligodendroglioma (II) in 1999, 2005 and 2006, respectively. Tumor tissues of earlier resections for both G15 and G20 were not available for analyses. All GBMs analyzed in the study were de novo.

Sources of publically available datasets

We assessed publically available glioma datasets for validating our findings. Molecular data and associated clinical information pertaining to diffuse glioma (grade II and III) and GBM (grade IV) datasets of The Cancer Genome Atlas (TCGA) were retrieved in March 2020 from cBioPortal for cancer genomics (57-59). Like grade II and III gliomas, GBM is also a diffuse tumor entity, but TCGA classifies these into two different cohorts. Nevertheless, according to accepted method of grouping gliomas as LGGs (grade I-II) and HGGs (grade III-IV) (6-9), and similar to the experimental dataset, we extracted grade III glioma data from the TCGA diffuse glioma dataset and collated it with that of the GBM cohort. RSEM data was not available for TCGA grade I (Pilocytic astrocytomas) dataset and hence could not be analysed as part of LGGs. For analyses, relevant data were cleaned by eliminating missing values. We also analysed meningioma GEO datasets - GSE88720; GSE16581; GSE43290; GSE85133 and GSE77259.

Isolation of Genomic DNA and total RNA from samples
DNA was isolated from tumor tissues, which were collected in RNA later (Ambion, Austin, TX), following standard phenol chloroform extraction method. DNA extraction kit (Qiagen, Valencia, CA) was used for isolating DNA from peripheral blood leukocytes (PBLs), as per the manufacturer’s protocol. Total RNA from tumor tissues, was isolated using Tri Reagent RT (Molecular Research Centre Cincinnati, OH), according to manufacturer’s recommendations. Potential genomic DNA contamination of total RNA was checked using GAPDH primers in a 20µl reaction volume of PCR. Total RNA was reverse transcribed to cDNA using High Capacity archive kit (ABI, Carlsbad, CA).

**Real-Time quantitation of gene expression**

mRNA transcripts of genes of interest (GIs -MNI, IGFBP5 and IGF1) in the tumor tissues were quantified by quantitative real-time PCR (qPCR). Commercially purchased total RNA from normal human adult ♂ and ♀ brains (Stratagene, La Jolla, CA) were used as controls. Primers specific for GIs and gene of reference (GR) were designed using Primer Express 3.0 software (ABI) and are detailed in Table 2. GAPDH was used as GR, as it showed stable expression in both control and test samples. Specificity of the primers was confirmed by BLAST search and their efficiency was tested. Amplification was carried out with SYBR® green (ABI) using 7500 Real-Time PCR System (ABI). All the assays were performed with 100nM of forward and reverse primers each, of the respective gene, in a final reaction volume of 20µl, employing universal cycling conditions recommended by ABI. GIs and GR were amplified in separate wells. The results were ratified, when from the triplicate cycle threshold (Ct) values, two were concordant. Relative expression levels of GIs in patient samples were calculated as described previously (56, 60). Data was analysed on SDS 7500™ Software v2.0.3 and Data Assist™ Software v2.0 (ABI).

**Immunoblotting**

Glioma cases having adequate tumor tissue were subjected to protein expression analysis by western blotting. Total protein was extracted by homogenizing the tumor tissue in cold RIPA lysis buffer and
clarified by centrifugation. Commercially purchased total proteins of non-neoplastic brain served as control (Biochain, Hayward, CA). Equal amounts of proteins were resolved on 12% SDS polyacrylamide gels and transferred to nitrocellulose membranes (Millipore, Billerica, MA). The membranes were blocked in 3% non-fat dried milk and 2% BSA (Cell Signaling Technology, Boston, MA) in PBS. Blots were incubated with goat polyclonal anti-MN1 (sc-27349; Santa Cruz Biotechnology, Dallas, TX), rabbit polyclonal anti-β-Tubulin (RB-9249-P1; Neo Markers) primary antibodies and secondary horse-radish peroxidase conjugated mouse anti-goat (sc-2354; Santa Cruz Biotechnology) and anti-rabbit (111-036-045; Jackson Immuno Research Laboratories Inc., West Grove, PA) antibodies. Subsequently, the immunoreactive signals were detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore), as per manufacturer’s instructions. The relative abundance of the proteins was quantified from scanned images of immunoblots using Labworks™ image acquisition and analysis software (v.4.0.0.8 UVP). Levels of β-Tubulin were used to normalize the protein expression.

**Copy number analysis by qPCR**

Copy number of MN1 was assessed using TaqMan® assay (Assay Id. Hs02424444_cn; ABI), and TaqMan® Rnase P detection (P/N: 4316831; ABI) as the reference gene, on 7500 Real-Time PCR System (ABI) using tumor genomic DNA as the test samples. Genomic DNA from matched PBLs of the respective patient was also assayed to determine whether copy number variations detected were de novo somatic events or inherited ones. Commercially purchased human brain genomic DNA (BioChain) and blood genomic DNA from 2 healthy volunteers served as non-neoplastic tissue and healthy controls, respectively. All samples and controls were run in triplicates, in 20µl singleplex reactions, comprising 20ng of genomic DNA, 1x TaqMan® Universal PCR Master Mix (ABI) and 1x primer probe mix, using universal cycling conditions recommended by ABI. The gene copy number per diploid genome was calculated as described previously (60). All possible precautions were taken to avoid presence of non-tumor genomic DNA in the test samples.
Data plotting and statistical analyses

GraphPad Prism (version 5.03), R studio (version 1.2.5033) and YaRrr, survminer, timeROC were used for making various plots and performing statistical analyses (61). Missing values were filtered out by R-packages. Therefore, number of cases in a particular TCGA dataset may vary between different analyses and the number of cases in a specific analysis are indicated where related data is presented.

Survival data including patients’ survival time and vital status (death/progression/event=1, living/censored=0 to censor data) were used from the TCGA datasets. The censored observations (patients that are alive at last follow up) are shown as marks on the survival curve for different time intervals (x-axis). The risk table depicts the number of patients under observation for a particular time interval. The survival curve is unchanged for a time interval when censored observations are recorded, but at the next time interval number of patients at risk is reduced by the number censored between the two time intervals as reflected in the risk table.

To determine grade-specific over-expression of MN1, IGFBP5, and IGF1 between LGGs and HGGs, a two-tailed non-parametric Mann-Whitney test was performed and contingency tables were also analysed using two-tailed Fisher’s exact test. The p-value, slope and coefficient of determination ($R^2$) from linear regression analysis were used to discern association, nature of association (positive/negative) between parameters and whether the model explains the variability in the response data, respectively. In the pirate plots for the RSEM data (TCGA datasets), dots, bar/line and band represent data points/cases, mean and confidence interval, respectively. The level of significance is indicated as *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

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Legends to Figures

Figure 1. Epidemiological data and copy number analysis of \textit{MNI} gene in gliomas. (A) Bar graph showing global estimates of age standardized incidence and mortality rates (ASR) per 100,000 people, in both genders and all ages in the year 2018, and is arranged according to top 20 cancer sites (3). (B) qPCR amplification plots with \textit{MNI} as the gene of interest and \textit{RNaseP} as the reference gene where $\Delta Ct = 1$ (left panel) $\Delta Ct = 0$ (middle panel) and $\Delta Ct = -1$ (right panel) correspond to one, two and four copies of \textit{MNI} gene, respectively. (C) Bar graph summarizing \textit{MNI} copy number details of gliomas examined in the present study. Red and green bars indicate cases that have altered and normal copy numbers, respectively. ‘n’ denotes the number of cases (LGGs or HGGs as indicated). (D) Bubble plot showing copy number status of \textit{MNI} gene in LGGs and HGGs from the TCGA datasets. Red and green bubbles represent cases with copy number alterations (CNA) and normal (2) copy number, respectively. Number of cases (n) in each category is indicated along the bubble.
(A) Estimated age-standardised incidence and mortality rates (ASR) per 100,000 (worldwide in 2018 both genders and all ages).

(B) 

\[ \Delta Ct = 1 \]

1 copy

\[ \text{GAPDH} \]

\[ \text{MN1} \]

\[ \text{Cycle number} \]

\[ \Delta Ct = 0 \]

2 copies

\[ \text{GAPDH} \]

\[ \text{MN1} \]

\[ \text{Cycle number} \]

\[ \Delta Ct = -1 \]

4 copies

\[ \text{MN1} \]

\[ \text{GAPDH} \]

\[ \text{Cycle number} \]

(C) 

HGGs n=7

LGGs n=11

HGGs n=15

>2 copies

<2 copies

2 copies

3 copies

4 copies

Giomas (experimental dataset)

(D) 

LGGs

HGGs

Giomas (TCCA datasets)
Figure 2. *MNI* expression and its relation with the gene’s copy number alteration in gliomas.

(A) Bar graph depicting relative *MNI* mRNA expression (normalized to non-neoplastic brain tissues) in the experimental dataset. Numerals indicated above/below each of the bars denote copy number of *MNI* gene in the corresponding glioma cases. *#Case G14 showed upregulated *MNI* transcripts but was not evaluable for the gene’s copy number. No of cases showing overexpression vs total no of cases analyzed for the two categories are mentioned below the X-axis. Inset tabulated summary of *MNI* mRNA expression and copy number data in gliomas. ↑, = and ↓ signify upregulated expression, comparable and down regulated transcript levels relative to the non-neoplastic brain, respectively. (B) Scattergram shows ability of *MNI* copy number to predict *MNI* expression in gliomas (n=39). Linear regression line fitted to the data is shown and ‘R²’ represents coefficient of determination. (C) Immunoblots and (D) graphical representation of densitometric evaluation of immunoblots, showing MN1 protein expression in representative glioma cases. β–tubulin was used as an experimental control. NN Brain indicates non-neoplastic sample used as control. (E) Graph summarizing relative *MNI* mRNA expression in LGGs (n=13) and HGGs (n=27), where the central bar and whiskers indicate mean (±SD) values.
**Figure 3.** *MNI* expression (RSEM) is not related to CNA in TCGA glioma datasets. Pirate plots showing *MNI* expression observed in (A) LGGs (n=249) and HGGs (n=417), and (B) classified according to *MNI* copy number alteration (CNA) in a grade wise manner (x-axis). P-values for statistical comparison of gene expression between glioma grades and cases with different copy number status are indicated above the plots. Regression analysis depicting *MNI* expression (log transformed RNAseq median or RSEM values) as a function of CNA of the gene in (C) LGGs (n=246) and (D) HGGs (n=412). Linear regression line fitting the data is shown in black, and ‘R^2’ represents coefficient of determination.
Figure 4. *MNI* overexpression predicts better overall survival in gliomas. Kaplan-Meier survival curves stratify LGGs by (A) *MNI* expression (n=247), (B) IDH mutation status (n=226) and (C) 1p19q deletion status (n=100). HGGs are classified into sub-groups based on association of probability of survival with (D) *MNI* expression (n=393), (E) IDH mutation status (n=354) and (F) 1p19q deletion status (n=139). P-values are computed using log-rank test that is used to evaluate significance. (G) Tabulated summary shows median overall survival for the different survival curves. ‘NA’ denotes values not available. (H) Receiver Operating Characteristics (ROC) combined curve of *MNI* expression, IDH mutation status and 1p19q deletion status in LGGs (n=100). Values of area under ROC curve (AUC) are indicated along with p-value for group wise comparison with *MNI* expression.
Figure 5. High MNI expression correlates with progression-free survival of glioma patients. Kaplan-Meier survival curves showing progression-free survival in LGGs (A) and HGGs (B) based on MNI expression levels in these tumors.
Figure 6. IGFBP5 expression, determining its association with MNI levels in gliomas. (A) Graphical summary of relative IGFBP5 expression in LGGs (n=13) and HGGs (n=27) of the experimental dataset. The central bar and whiskers denote mean (±SD). Linear regression plots to test association of IGFBP5 expression with MNI expression levels in (B) LGGs (n=11), and (C) HGGs (n=27). (D) Pirate plots showing IGFBP5 expression (log transformed RNAseq median values) in LGGs (n=249) and HGGs (n=417) from the TCGA datasets. Scatter plots show relatedness of IGFBP5 expression with MNI expression levels in (E) LGGs (n=249) and (F) HGGs (n=417). The black regression line represents the regression model fitted to the data, and ‘R²’ refers to coefficient of determination. Statistical test values comparing gene expression between the glioma grades are indicated above the respective plots.
Figure 7. *IGF1* and *IGFBP5* expression does not correlate in high grade gliomas. (A) Dot-plots representing relative *IGF1* expression between LGGs (n=13) and HGGs (n=27). Scatter plots show linear regression model assessing relations between expression levels of *IGFBP5* and *IGF1* in (B) LGGs (n=12) and (C) HGGs (n=25). (D) In the TCGA datasets *IGF1* expression is noticeably lower in HGGs (n=417) compared to LGGs (n=249). Regression analysis plots assessing correlation between expression levels of *IGFBP5* and *IGF1* in (E) LGGs (n=249) and (F) HGGs (n=417). In all regression analysis plots, line fitting the data scatter is represented in black and ‘R$^2$’ represents coefficient of determination.
Figure 8. Model summarizing the association between MN1, IGFBP5 and IGF1 expression in gliomas and their possible molecular interplay. MN1, IGF1 and IGFBP5 are represented by specific symbols. In (A) LGGs and (B) HGGs, the numbers of a particular symbol denote relative abundance of the specific molecule, based on the findings of the present study. MN1 possibly synergizes with an unknown or known (RAR/RXR) repressor at regulatory elements in the IGFBP5 promoter to repress its expression in gliomas. Schematic suggests that (A) in LGGs high MN1 levels transcriptionally repress IGFBP5, which offsets IGF1-mediated induction of IGFBP5 expression such that it is predictive of better median survival (table within the schematic), (B) whereas in HGGs low levels of MN1 likely relieve transcriptional repression of IGFBP5, tilting the balance in favour of IGF1 mediated increase in IGFBP5 expression. This in turn estimates poor prognosis and lower median survival in HGGs than LGGs (table embedded in the schematic).
Table 1. Clinical details of Glioma cases analysed in this study

| Glioma Cases | Histological Sub-types | Total No. Cases per grade |
|--------------|------------------------|---------------------------|
|              | Sub-type | No. of Cases |              |              |
| LGGs (n=13)  | Grade I    | PA 2         | n=3          |
|             | (G1-G3)    | SEGA 1       |              |
| HGGs (n=27)  | Grade II   | DA 5         | n=10         |
|             | (G4-G12, G14) | O 2 |              |
|             |           | OA 3         |              |
|             | Grade III  | AA 3         | n=7          |
|             | (G15, G18-G23) | AO 2 |              |
|             |           | AOA 2        |              |
|             | Grade IV   | GBM 20       | n=20         |
|             | (G24-G43)  |              |              |

AA, Anaplastic astrocytoma; AO, Anaplastic oligodendroglioma; AOA, Anaplastic oligoastrocytoma; DA, Diffuse astrocytoma; GBM, Glioblastoma multiforme; HGGs, High grade gliomas; LGGs, Low grade gliomas; O, Oligodendroglioma; OA, Oligo astrocytoma; PA, Pilocytic astrocytoma; SEGA, sub-ependymal giant cell astrocytoma.
Table 2. Details of primers used for qPCR based mRNA expression analysis

| Gene  | Primer sequences a | Amplicon size(bp) |
|-------|--------------------|-------------------|
| GAPDH | FP 5’ GCCACATCGCTCAGACACCAT 3’
      | RP 5’ ACCAGGCGCCCAATACG 3’ | 72          |
| MNI   | FP 5’ CAGAACCCCAACAGCAAAGAA 3’
      | RP 5’ GACAGACAGGCACTGCAAGTG 3’ | 90          |
| IGFBP5| FP 5’ CTACAAGAGAAAGCGAATGCAAAACC 3’
      | RP 5’ TCCACGCACCGAGCAGATG 3’ | 62          |
| IGF1  | FP 5’ GTGCTGCTTTTGATTTCTTGA 3’
      | RP 5’ GCACAGCGCCAGGTATEGAG 3’ | 76          |

a Forward and reverse primers are located in different exons, thus eliminating chance amplification from potential contaminating genomic DNA. FP and RP represent forward and reverse primers, respectively.
Abbreviations

AUC Area under the (ROC) curve
CNA Copy number alteration
CNS Central nervous system
FKPM Fragments per kilobase of transcript per million mapped reads
GAPDH Glyceraldehyde 3-phosphate dehydrogenase
GBM Glioblastoma multiforme
HGG High grade glioma
IGF Insulin-like growth factor
IGFR Insulin-like growth factor receptor
IGFBP Insulin-like growth factor-binding protein
IDH Isocitrate dehydrogenase
LGG Low grade glioma
MN1 Meningioma 1 gene
OS Overall survival
PFS Progression-free survival
RA Retinoic acid
RAR/RXR Retinoic acid receptor/retinoic x receptor
RNase P Ribonuclease P
RSEM RNA-Seq by expectation maximization
TCGA The Cancer Genome Atlas
ROC Receiver operating characteristic