NOVEL MOLECULAR PANEL FOR EVALUATING SYSTEMIC INFLAMMATION AND SURVIVAL IN THERAPY NAIVE GLIOMA PATIENTS

by richa

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Background

Inflammation is crucial to tumor progression. A traumatic event at a specific site in the brain activates the signaling molecules which trigger inflammation as the initial response within the tumor and its surroundings. The educated immune cells and secreted proteins then initiate the inflammatory cascade leading to persistent chronic inflammation. Estimation of the circulating inflammatory indicators Kynurenine (KYN), interleukin-6(IL-6), tissue-inhibitor of matrix-metalloproteinase-1(TIMP-1), and hTERT (human telomerase reverse transcriptase) along with neutrophil-lymphocyte-ratio (NLR)has prognostic value.

Aim

The present study aimed to assess the utility of chosen inflammatory marker panel in estimating systemic inflammation.

Methods

The markers were quantitively evaluated in 90 naive, molecularly sub-typed plasma samples. A correlation between the markers and confounders was assessed to establish their prognostication power. Follow-up on the levels of the indicators was done 3-mo post-surgery. To establish the validity of circulating KYN, it was also screened qualitatively by dot-immune-assay and by immunofluorescence-immunohistochemistry in tumor tissues.

Results

Median values of circulating KYN, IL-6, hTERT, Timp-1, NLR in isocitrate-dehydrogenase-*mutant/wildtype* (IDH-*m/w)* and within the astrocytic

subgroups were estimated, which differed from controls, reaching statistical significance (P<0.0001). All markers were negatively correlated with mortality (P<0.0001). Applying combination statistics, the panel of KYN, IL-6, hTERT& NLR achieved higher sensitivity and specificity (>90%) than stand-alone markers, to define survival. The inflammatory panel could discriminate between WHOgrades, IDH-*m/w* and define differential-survival between astrocytic IDH-*m/w*, therefore its assessment for precise disease prognosis is indicated. Association of KYN with NLR, IL-6, hTERT was significant. Cox regression described KYN, IL-6, NLR, hTERT as good prognostic markers, independent of confounders. Multivariate linear regression analysis confirmed the association of KYN and hTERT with inflammation marker IL-6, there was a concomitant significant decrease in their levels in a 3-month follow-up.

Conclusion

In the first evidence-based study of circulating-KYN in molecularly defined gliomas, the tissue expression is concomitant with plasma levels. A non-invasive model for assessing indicators of chronic systemic inflammation is proposed.

Key Words: Circulating; Glioma; Inflammatory marker; Kynurenine; Non-invasive; Prognostic.

CORE TIP: The current study is the first-ever analysis of the circulatory levels of Kynurenine (KYN), interleukin-6(IL-6), tissue-inhibitor of matrix-metalloproteinase-1(TIMP-1), and human telomerase reverse transcriptase (hTERT) along with neutrophil-lymphocyte-ratio (NLR) in a sizeable cohort of molecularly classified glioma samples. The ability of this panel to differentiate survival in glioma subgroups has been assessed. Evaluation of this inflammatory panel of potential biomarkers using the minimally invasive blood

sample, for prognostication and targeted personalized therapy in glioma, is suggested.

Introduction

The role of inflammation and its partakers in glioma, especially the molecular markers secreted at the tumor initiation stage, is still not completely worked out. Since the brain is unique in comparison to other organ systems, being confined within the blood-brain barrier (BBB), it presents a local innate immune-response to an inflammatory stimulus like tumor antigens[1] or injury, or oncogene over-expression, by the production of inflammatory molecules and/or metabolites[2]. Consequently, inflammation can be a cause or consequence of actively proliferating cells, an event that also initiates the recruitment of immune cells at the tumor site [3]. The stimuli, if persistent, can manifest as chronic inflammation, aberrant cell proliferation, and increased angiogenesis leading to the escape of specific molecules and cells into peripheral circulation [4]. Therefore, clinical management of inflammation associated with glioma initiation and progression is clinically recognized.

One of the significant inflammatory metabolites in the brain is Kynurenine (KYN) of the Kynurenine pathway; it is produced and secreted by endothelial cells and pericytes of the blood-brain barrier (BBB), which have been stimulated by inflammation. In the absence of effective immune regulation, unresolved inflammation is generated by aggressively proliferating cells. Excess KYN production is triggered by inflammation in the tumor environment (TE), which leads to local immune tolerance, and the inflammatory signals generated by the tumor results in KYN being transduced across the intact BB to be detected in systemic circulation[5,6]. Over-activation of KYN was first described in glioblastoma (GB) cell line and tumor tissue as early as 2012 by Opitz and colleagues [7]. Excessive production of this metabolite was linked to

increased tumor immunity and decreased survival as recorded by the Adams group [8] in cultured cells and 18 glioblastomas (GB) patient samples.

The tumor-infiltrating immune cells-neutrophils and lymphocytes are also markers of systemic inflammation [9]. Their involvement as local inflammation indicators in glial tumors has been documented by Zadora et al. [10].

Neutrophil recruitment initiates cytokines, which strengthens the initial *in situ* neuro-inflammatory response by activating more neutrophils and macrophages. Brain tumors, including glioma, express high levels of different cytokines, specifically interleukin-6 (IL-6), involved in multiple pathways of tumor pathology[11]. During gliomagenesis, IL-6 executes a critical role from other interleukins since it predominately triggers the pro-inflammatory cascade. In the last few years, a couple of blood-based investigations analyzing the expression of IL-6 in different grades of glioma suggest that IL-6 expression may indicate disease prognosis [12,13].

Involved in this scenario of brain inflammation is another set of proteins, the tissue inhibitor of matrix metalloproteinases (TIMPs), and known to perform multiple functions, including regulating inflammation in the TE. In this context, there is a lone study by Lin et al. reporting plasma levels of TIMP-1 to be associated with an inflammatory response in glioma, proposing a role of this molecule in prognosis [14].

In this landscape of inflammation partakers in the TE, the association between increased chronic inflammation and telomerase activity is often overlooked. The human TERT (hTERT) is an enzyme active in the immune system cells and regulates inflammation; however, its activity beyond a critical limit initiates proliferation. Several studies suggest that TERT activates the NF-kB target genes such as interleukin IL-6, which is crucial to inflammation and cancer progression [15-17]. Research groups, including ours, have demonstrated that

hTERT executes many vital functions independent of its telomere maintenance, including angiogenesis, inflammation, and stemness in glioma [18, 19]. These findings suggest that the feedback loop of the hTERT signaling pathway may reinforce the inflammatory signaling via cytokine secretion, leading to the development of chronic inflammation in the TE.

Mechanistically, a traumatic event at a specific site in the brain leads to the secretion of inflammatory molecules such IL-6 and KYN, which trigger the inflammatory cascade leading to overactivation of telomerase. The abnormal hTERT physiology is responsible for immune system dysfunction, followed by persistent chronic inflammation leading to a malignant phenotype. Further, this unresolved chronic inflammation also begins contributing to disease progression. The current exploratory study is the first-ever analysis of the circulatory levels of KYN, IL-6, NLR, hTERT, and TIMP-1 in a sizeable cohort of glioma samples categorized according to their histological grade and isocitrate-dehydrogenase (IDH) expression. The ability of this panel to differentiate survival in glioma subgroups has been assessed.

Materials and Methods

Subjects: Ninety treatment-naive samples, presenting with clinical symptoms like recurrent seizures, headache, increased intracranial pressure, and radiological diagnosis of glial tumor, were collected from the in-patient ward of the Neurosurgery department. Informed consent in writing was taken from all the participants of the study cohort (IEC/21/Res/11). Without any recent clinical history of inflammation or autoimmune disease, forty-five healthy subjects were taken as controls for blood samples. Whole blood was collected just before surgery; plasma and serum were separated and preserved at -80°C until further testing for target proteins was undertaken. A follow-up sample of all patients was done three months post-surgery. According to the established

lab protocol, the systemic levels of the marker KYN were correlated with its *in situ tumor* expression by IF-IHC for validation [18] (see supplementary file for detailed method-A).

Dot Immune-binding assay: Qualitative screening of circulating markers was done using 20ul of serum after removing high abundance proteins. Subsequently, the samples were loaded onto nitrocellulose membrane and incubated overnight at 4°c. Then the membrane was probed with KYN, IL-6 TIMP-1, and hTERT antibody (1:2000 dilution, Monoclonal, Santa Cruz Biotechnology, USA) for two hours followed by host-specific respective alkaline-phosphatase (AP) conjugated antibody tagging (Santa Cruz Biotechnology, United States; 1:2500 dilutions) for one hour. The label was detected using an AP substrate (BCIP, Sigma, Aldrich, United States) which produced a visible signal corresponding to the concentration of the target protein.

Quantification of circulatory markers: Quantification of the biomarkers in plasma samples was done by ELISA for KYN (Creative Diagnostics, United States), TIMP-1 (R&D systems, United States), and hTERT (Elabscience, United States) according to details provided in the instruction manual. The plasma concentrations were noted in ng/mL for KYN TIMP-1; and ng/L for hTERT.IL-6 test results were computed from the patients' clinical reports and compared with the baseline values earlier established for this marker in the lab [13]. The standard reference range of each marker was defined and set as per kit insert. Complete blood counts of all enrolled subjects were recorded from the pre-surgery blood profile; the neutrophil and lymphocyte counts were extrapolated into the mathematical formula and calculated [19] to establish the neutrophil-lymphocyte ratio in patients and controls.

Statistical analysis: Non-parametric Kruskal-Wallis test was used to assess the difference between samples of different histological grades for plasma values of all biomarkers. Mann Whitney test was applied on the groups stratified according to IDH status; for marker panel KYN, TIMP-1, NLR, IL-6, and hTERT, to differentiate their significance.

Analysis of area under the curve (AUC) for ROC was performed to define the cut-off values and sensitivity of the markers between the grades and within subgroups which attained ≥80% specificity. To establish the diagnostic accuracy of the markers, CombiROC software (https//: combiroc. eu) was used, and the predictive probability of the inflammatory panel for prognostication was subjected to ROC analysis.

To discern the influence of covariates on plasma maker levels, univariate analysis was carried out to identify the confounding factors, namely, age, site, EOR, therapy, KYN, TIMP-1, NLR, IL-6, hTERT. Parameters attaining a level of significance in this analysis were entered into the multivariate functionally to create the final model. Furthermore, the Cox proportional regression model was used to compute hazard ratios with 95% CI to establish the independent status of prognostic markers.

Overall survival (OS), defined as the time from randomization to death from any cause, was considered as a direct measure of clinical benefit to the patient. Patients alive or lost to follow-up were treated as censored. Curves defining total survival period with reference to the identified biomarkers were drawn on Kaplan-Meier estimates and differences compared between IDH-*m/w* subgroups for statistical significance using the log-rank test. Spearman's rho coefficient was applied to calculate the correlation of all five markers with OS within histological grades, between IDH-*m/w,* astrocytic *m/w*, and also in terms of KYN, TIMP-1, NLR, IL-6, and hTERT. Multivariate linear regression was

performed to describe the association of the four markers with inflammation. Paired t-test was performed for pre-op and 3-mo follow-up samples for the panel. The p-values of all statistical tests were two-sided ($p<0.05$).

Result:

Plasma samples from healthy controls (n=45) and glioma patients (n=90, IDH-*m* *n*=60 inclusive of astrocytic and oligo-component and IDH-*w*, astrocytic n=30) formed the study cohort. Details of patients, relevant demographics, and clinical data are presented in Table 1.

Qualitative screening: The presence/absence of KYN, IL-6, TIMP-1, and hTERT in circulation was screened on nitrocellulose- membrane by dot immune-binding assay, a colored signal (blue) indicated immune-complex formation and presence of the inflammatory molecule KYN (Figure 1), the signal intensity for each marker corresponding to the pathological grading of the tumor.

Quantification of biomarkers by sandwich ELISA: Median values of plasma KYN in grades II, III, and IV were 69.86ng/ml, 112.15ng/ml, respectively. Levels of circulating KYN, IL-6, TIMP-1, and hTERT emerged as substantially higher ($p<0.0001$) with increasing histological grade when the Kruskal-Wallis test was applied. The statistical difference between IDH-*m/w* groups (Table 2) using the Mann Whitney test was highly significant to KYN, $p<0.0001$, NLR; $p=0.0002$, TIMP1;$p=0.0405$, and hTERT; $p<0.0001$.

The association of plasma levels (pre-operative) of all markers yielded a positive correlation with the grade; the best value was for IL-6 ($r=0.5409$, $p<0.0001$), while the most significant inverse correlation of KYN ($r=0.6154$, $p<0.0001$) was attained with OS (Table 3). When the samples were molecularly stratified, there was an inverse correlation of all biomarkers with survival outcome, being worse for IDH-*was* compared to IDH-*m.* The Spearman coefficient for inflammatory markers KYN, TIMP-1, NLR, IL-6, and hTERT was

significant and positive for tumor grade, but there was an inverse association with OS, suggesting a poor prognosis with increasing systemic levels of these markers in therapy naïve glioma patients.

The tissue expression of KYN was concomitant with its plasma levels and increased with increasing histological grade (Supplementary Figure 1).

Determination of independence in prognostication: The relation of OS with KYN, TMIP-1, IL-6, NLR, hTERT, age, site, the extent of resection (EOR), and therapy was calculated using univariate and multivariate Cox-regression models to identify the plausible prognostic factors (Table 4). Univariate analysis delineated shorter patient survival to be associated with KYN, IL-6, NLR, TIMP-1, hTERT (p=0.0001), and age (p=0.0004). When multivariate Cox-regression model was applied, higher levels of KYN (p=0.0003), IL-6 (p=0.0004), NLR (p=0.0001) and hTERT (p=0.0026) were found to independently define prognosis. Based on these results, it was assumed that TIMP-1 could not be considered a sensitive marker for inflammation. So, the final maker panel of four markers, KYN, IL-6, NLR, and hTERT, was taken further for validation.

AUROC: The cut-off thresholds based on AUC for the four circulatory biomarkers were KYN: >22.89, IL-6:>62.5, NLR:>2.775, and hTERT:>1.309, at more than 70% sensitivity and 80% specificity, when comparing controls versus glioma patients. Levels of KYN, IL-6, NLR, and hTERT could significantly differentiate between histological grades, low & high grade, IDH-*m/w,* with more than 80% sensitivity (Supplementary Table 1).

Based on the AUROC analysis, optimal cut-off points were determined for the best balance of sensitivity and specificity for the highest value of likelihood ratio to predict survival through log-rank analysis, and thereafter Kaplan Meier curves were constructed for the four biomarkers (Figure 2a-2d). OS was even

better in patients with IDH-*m* astrocytic tumors than their IDH-*w* counterparts (Figure 2e).

Circulating concentrations of KYN, NLR, IL-6 and hTERT were set at 22.89ng/ml, 2.775, 62.5pg /ml and 1.309ng/L respectively. Patients with values exceeding these thresholds were observed to have a shorter survival period. The OS was defined for KYN (18 vs. undefined months, HR 3.176 with 95% CI 1.626 to 6.206, p = 0.0007), NLR (20 vs. 48 months, HR 0.4167 with 95% CI 0.204 to 0.8512, p = 0.0025), IL-6 (20 vs. 80 months, HR 0.25 with 95% CI 0.1318 to 0.4741, p = 0.0008), hTERT (18 vs. 48 months, HR 0.21 with 95% CI 0.1178 to 0.4741, p = 0.0006).

On follow-up, paired t-test between pre-and 3-mo post-surgery levels of KYN (p=0.0186), IL-6 (p=0.0107), NLR (p=0.0038), and hTERT (p=0.0016) showed a significant difference, and lower values post-intervention indicate that inflammation has plausibly reduced on de-bulking of the tumor.

CombiROC: The predicting accuracy of these chosen candidate markers to ascertain systemic inflammation status in glioma patients was increased substantially in combination as a panel rather than standalone markers. Applying CombiROC enhanced the sensitivity of the biomarker panel. The sensitivity of KYN, IL-6, NLR, and hTERT was 86.11%, 72.22%, 77.78%, and 80% individually, which increased to 94.4 % in combination with 96.7% specificity and an AUC of 0.983 as seen in combination VII (Figure 2f).

Based on the above results and multivariate linear regression, it can be inferred an association between the tumor secreted inflammatory molecules (Figure 3); therefore, these markers can be evaluated to assess systemic inflammation and prognosis.

Discussion

Inflammation is the first line of defense in response to tissue injury and/or infection. The link between a tumor and inflammation is a well-established fact and is one of the major attributes of malignancy [20]. Inflammation in the TE mediates all aspects of glial oncogenesis, including *in situ* progressive development of vasculature and tissue remodeling [21]. Thus, by and large, the inflammatory molecules orchestrate the extrinsic and intrinsic stimulus, thereby initiating and contributing to tumor progression [22].

Although KYN is an important inflammatory metabolite in the glial-onco-transformation process, there are limited studies on the role of this molecule in glioma, with three research groups recording the ratio of KYN and tryptophan in a very small number of patients. In reference is the study on the plasma of 18 GB patients by Adams et al. [8], who presented data to show that because of activation of the KYN pathway, the KYN/TRP ratio was significantly higher in GB as compared to healthy volunteers. The other group of investigators presented a different opinion on KYN as a marker, stating that there was no significant difference in the levels before and after surgical intervention, as seen in 10 GB samples[23]. However, the research by Lenzen et al. [24] documented significantly decreased serological values of KYN post a vaccine treatment with heat shock protein-peptide complex-96, but they did not provide any baseline data of KYN for comparison.

Therefore, the present therapy-naive dataset is unique as it establishes KYN levels in circulation for glioma, with values screened qualitatively at baseline and recorded quantitatively at two-time points, pre-and post-surgery. This marker was able to differentiate between IDH-*m/w* groups with high significance (Table 2), along with histological grades and OS (Figure 1& 2).

When a chronic inflammatory response is generated within the tumor, the KYN pathway proteins trigger the over-expression of pro-inflammatory cytokine IL-6

[8]. We noted a raised expression of the inflammatory marker IL6 in our enrolled glioma patients. Similarly, two recent studies on GB reveal that aberrant production of this circulating cytokine is directly associated with tumor growth and poor survival, demonstrating IL-6 as an independent prognostic factor for survival [13, 25]. These studies suggest that cytokine plays an important role in the *in-situ* inflammatory response within the tumor.

Despite the established association between telomerase activity and inflammation, the related molecular pathway in glioma has not been elucidated. Elevated telomerase activity for the sustained proliferation of tumor cells begins a smoldering response of acute followed by chronic inflammation [26, 27]. The work of Shervington and Patel [28] in glioma subtypes suggests a noteworthy variation of TERT protein expression levels in GB when compared with control. Two case reports presented from our lab also correlate hTERT expression to proliferation, stemness, and survival glioma subtypes [13, 29]. In a first-of-its-kind investigation, the correlation between tissue and blood concentrations of hTERT marker was also established by us in glioma [30]. Findings of the current analysis are also in line, and there was a significant difference among grades, IDH-*m* and *w*, and a positive association with KYN and IL-6.

In the present cohort analysis, the NLR score emerged as a good indicator of prognosis and survival in the molecularly typed sub-groups. Concurrence of this result can be found in studies conducted on smaller groups of GB patients, which show that there is a significant association of NLR with OS [31-34]. Our previous work on diffuse gliomas, along with the current study on IDH characterized gliomas, provides substantial laboratory-based evidence that NLR is a marker of inflammation and can be used as a prognosticator in the clinical setting [19].

There is an increasing acceptance of the proactive role of glial tumor cells in brain inflammation, leading to suppression of innate immune response via secretion of matrix metalloproteases [37, 38]. In a study conducted earlier in the decade, Sreekantreddy and his co-workers established GBM-specific up-regulation of serum TIMP1 by ELISA [39]. Similarly, while working with 36 patients, it was suggested by Cocker et al. that serum TIMP1 levels may serve as an independent predictor of survival in glioma subtypes [40]. In an extensive study on low-grade glioma patients, Zeng and his group established that plasma TIMP1 expression was significantly correlated with overall survival (OS) and relapse-free survival (RFS) in these patients [41]. However, our results are partially in contention with the above studies. The plasma levels of TIMP-1 were up-regulated in our patients compared to controls, but the marker did not reach significance as an independent prognosticator.

The systemic levels of target molecules discussed above can be considered to represent the cascade of events leading to chronic systemic inflammation in glioma, as depicted in Figure 3. The present work is a one of its kind experiment-based qualitative and quantitative estimation of circulating KYN in a substantial-sized cohort of molecularly defined gliomas. KYN can differentiate between histological grades, IDH-*m/w* in terms of patient survival, along with IL-6 and NLR, thus advocating assessment of this inflammatory panel of potential biomarkers using the minimally invasive blood sample for targeted therapy and prognostication in glioma.