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CASE REPORT

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ABOUT COVER

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AIMS AND SCOPE

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WJCO mainly publishes articles reporting research results and findings obtained in the field of oncology and covering a wide range of topics including art of oncology, biology of neoplasia, breast cancer, cancer prevention and control, cancer-related complications, diagnosis in oncology, gastrointestinal cancer, genetic testing for cancer, gynecologic cancer, head and neck cancer, hematologic malignancy, lung cancer, melanoma, molecular oncology, neurooncology, palliative and supportive care, pediatric oncology, surgical oncology, translational oncology, and urologic oncology.

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ORIGINAL ARTICLE

Observational Study

Novel molecular panel for evaluating systemic inflammation and survival in therapy naïve glioma patients

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Abstract

BACKGROUND

Inflammation is crucial to tumor progression. A traumatic event at a specific site in the brain activates the signaling molecules, which triggers 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. Therefore, estimation of the circulating inflammatory indicators kynurenine (KYN), interleukin-6 (IL-6), tissue-inhibitor of matrix-metalloproteinase-1 and human telomerase reverse transcriptase (hTERT) along with neutrophil-lymphocyte ratio (NLR) has prognostic value.

AIM

To assess the utility of chosen inflammatory marker panel in estimating systemic inflammation.

METHODS

The chosen markers were quantitatively evaluated in 90 naive, molecularly subtyped plasma samples of glioma. 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, tissue-inhibitor of matrix-metalloproteinase-1 and NLR in isocitrate-dehydrogenase-mutant/wildtype and within the astrocytic sub-groups were estimated, which differed from controls, reaching statistical significance ($P < 0.0001$). All markers negatively correlated

with mortality ($P < 0.0001$). Applying combination-statistics, the panel of KYN, IL-6, hTERT and NLR achieved higher sensitivity and specificity (> 90%) than stand-alone markers, to define survival. The inflammatory panel could discriminate between WHO grades, and isocitrate-dehydrogenase-mutant/wildtype and define differential survival between astrocytic isocitrate-dehydrogenase-mutant/wildtype. Therefore, its assessment for precise disease prognosis is indicated. Association of KYN with NLR, IL-6 and hTERT was significant. Cox-regression described KYN, IL-6, NLR, and 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-mo follow-up.

CONCLUSION

The first evidence-based study of circulating-KYN in molecularly defined gliomas, wherein the tissue expression was found to be 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, interleukin-6, tissue-inhibitor of matrix-metalloproteinase-1 and human telomerase reverse transcriptase along with neutrophillymphocyteratio 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.

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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, it presents a local innate immune response to an inflammatory stimulus, like tumor antigens[1], 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 KYN pathway; it is produced and secreted by endothelial cells and pericytes of the blood-brain barrier, which have been stimulated by inflammation. In the absence of effective immune regulation, chronic 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 blood-brain barrier to be detected in systemic circulation[5,6]. Over-activation of KYN

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was first described in glioblastoma (GB) cell lines and tumor tissues 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 Adams *et al*[8] in cultured cells and 18 GB patient samples[8].

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 cytokine secretion, 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 more critical role than 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 suggested 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), known to perform multiple functions, including regulating inflammation in the TE. In this context, there is a lone study by Lin *et al*[14] 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 telomerase reverse transcriptase (hTERT) is an enzyme active in the immune system cells and regulates inflammation; however, its activity beyond a critical limit initiates uncontrolled proliferation. Several studies suggest that TERT activates the nuclear factor kappa-B target genes such as 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, namely angiogenesis, inflammation, and stemness in glioma[18,19]. These findings suggested 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 as IL-6 and KYN, which trigger the inflammatory cascade leading to over-activation 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, neutrophil-lymphocyte ratio (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

Inclusion criteria

Patients with symptoms of recurrent seizures and headache radiologically diagnosed and histopathologically confirmed as glioma grade II, III, or IV.

Exclusion criteria

Patients less than 18 years of age or patients histopathologically confirmed as glioma grade I.

Subjects enrolled for observational study

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 (IRB/21/Res/11). Forty-five healthy subjects without any recent clinical history of inflammation or autoimmune disease, 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 3 mo post-surgery. According to the established lab

protocol, the systemic levels of the marker KYN were correlated with its *in situ* tumor expression by immunofluorescence-immunohistochemistry for validation (Supplementary File Method A).

Dot immune-binding assay

Qualitative screening of circulating markers was done using 20μL 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, United States) for 2 h followed by host-specific respective alkaline-phosphatase-conjugated antibody tagging (Santa Cruz Biotechnology, United States; 1:2500 dilutions) for 1 h. The label was detected using an alkaline-phosphatase 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 enzyme-linked immunosorbent assay (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 manuals. The plasma concentrations were noted in ng/mL for KYN and 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[18] to establish the NLR 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 and for marker panel KYN, TIMP-1, NLR, IL-6, and hTERT, to differentiate their significance. Analysis of area under the curve for receiver operating characteristic was performed to define the cut-off values and sensitivity of the markers between the grades and within subgroups that 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 receiver operating characteristic analysis. To discern the influence of covariates on plasma maker levels, univariate analysis was carried out to identify the confounding factors, namely, age, site, extent of resection, therapy, KYN, TIMP-1, NLR, IL-6 and, 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% confidence interval (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-mutant/wildtype (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 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-operative and 3-mo follow-up samples for the panel. The *P* values of all statistical tests were two-sided ($P < 0.05$).

RESULTS

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.

Table 1 Demographic features and concentrations of molecular markers of recruited subjects

No.	Factors	Controls	IDH-mutant	IDH-wildtype
1	Sample size, (*n*)	(*n* = 45)	60	30
2	Sex, M/F	M = 30; F = 15	M = 51; F = 9	M = 25; F = 5
3	Age (yr)	25 (21-50)	35.0 (13-75)	51.5 (25-76)
4	Site	-	Frontal = 37/Non-frontal = 23	Frontal = 12/Non-frontal = 18
5	EOR	-	STR = 38; GTR = 14	STR = 30; GTR = 0
6	OS	-	28.5 (1-158)	6.5 (1-38)

Values presented as median with range. M: Male; F: Female; EOR: Extent of resection; STR: Sub-total resection; GTR: Gross total resection; OS: Overall survival; IDH: Isocitrate dehydrogenase.

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 of each marker corresponded to the pathological grading of the tumor.

Quantification of biomarkers by sandwich ELISA

Median values of plasma KYN in grade-II, III, and IV were 69.86ng/mL, 112.15ng/mL, 237.318 ng/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 Mann Whitney test was highly significant for KYN ($P < 0.0001$), NLR ($P = 0.0002$), TIMP-1 ($P = 0.0405$) and hTERT ($P < 0.0001$).

The association of plasma levels (preoperative) of all markers yielded a positive correlation with the grade; however, the best value was observed for IL-6 ($r = 0.64$, $P < 0.0001$, Table 3), 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-*w* ascompared 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 and therapy was calculated using univariate and multivariate Coxregression 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 area under the curve 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 *vs* glioma patients. Levels of KYN, IL-6, NLR, and hTERT could significantly differentiate between histological grades, low and high grade and 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 and the highest value of likelihood ratio to predict survival through log-rank analysis, and thereafter Kaplan Meier curves were con-

Table 2 Concentrations of molecular markers of recruited subjects of glioma patients in terms of isocitrate dehydrogenase mutant and wildtype glioma

No.	Factors	Controls (*n* = 45)	IDH-mutant (*n* = 60)	IDH-wildtype (*n* = 30)	*P* value
1	KYN (ng/mL)	5.009 (0.052-34.43)	77.155 (1.56-387.44)	237.318 (53.43-894.95)	< 0.0001[c]
2	IL-6 (pg/mL)	37.009 (6.715-62.49)	68.62 (21.85-209.73)	197.672 (59.217-803.711)	< 0.0001[c]
3	NLR	1.59 (0.87-2.8)	3.67 (1.23-11.5)	5.585 (3.1-18)	0.0002[c]
4	TIMP-1 (ng/mL)	38.03 (10.26-73.99)	82.54 (25.09-131.30)	107.01 (37.175-185.09)	0.0405[a]
5	hTERT (ng/mL)	1.029 (0.075-1.58)	1.364 (0.1-6.886)	2.185 (0.565-8.84)	< 0.0001[c]

[a]*P* < 0.05.
[c]*P* < 0.001.
Median with ranges of all circulatory markers in controls, isocitrate dehydrogenase (IDH)-*m* and IDH-*w*. KYN: Kynurenine; IL-6: Interleukin-6; NLR: Neutrophil-lymphocytes ratio; TIMP-1: Tissue inhibitor metalloproteases-1; hTERT: Human telomerase reverse transcriptase; IDH: Isocitrate dehydrogenase.

Table 3 Spearman coefficient correlation of circulatory markers with overall survival, histological grade and Kynurenine

No.	Circulatory markers	Spearman r	95%CI	*P* value
	Correlation with OS			
1	KYN (ng/mL)	-0.6154	-0.7324 to -0.463	< 0.0001[c]
2	IL-6 (pg/mL)	-0.5531	-0.6854 to -0.3855	< 0.0001[c]
3	NLR	-0.5696	-0.698 to -0.4058	< 0.0001[c]
4	TIMP-1 (ng/mL)	-0.4831	-0.6312 to -0.3011	< 0.0001[c]
5	hTERT (ng/mL)	-0.4386	-0.5960 to -0.2489	< 0.0001[c]
	Correlation with grade			
6	KYN (ng/mL)	0.5409	0.3705 to 0.6761	< 0.0001[c]
7	IL-6 (pg/mL)	0.6400	0.4944 to 0.7507	< 0.0001[c]
8	NLR	0.4982	0.3190 to 0.6430	< 0.0001[c]
9	TIMP-1 (ng/mL)	0.2614	0.05118 to 0.4495	0.0128[a]
10	hTERT (ng/mL)	0.3000	0.06441 to 0.4834	0.0102[a]
	Correlation with KYN			
11	IL-6 (pg/mL)	0.4919	0.3115 to 0.638	< 0.0001[c]
12	NLR	0.4084	0.214 to 0.5717	< 0.0001[c]
13	TIMP-1 (ng/mL)	0.4718	0.2877 to 0.6223	< 0.0001[c]
14	hTERT (ng/mL)	0.3504	0.1485 to 0.5243	0.0007[c]

[a]*P* < 0.05.
[c]*P* < 0.001.
An extremely significant inverse correlation of kynurenine, interleukin-6, neutrophil-lymphocytes ratio, tissue inhibitor metalloproteases-1 and human telomerase reverse transcriptase was obtained with respect to overall survival. A significant positive correlation of interleukin-6, neutrophil-lymphocytes ratio, tissue inhibitor metalloproteases-1 and human telomerase reverse transcriptase was obtained with respect to grades and kynurenine circulatory levels. OS: Overall survival; KYN: Kynurenine; IL-6: Interleukin-6; NLR:Neutrophil lymphocytes ratio; TIMP-1: Tissue inhibitor metalloproteases-1; hTERT: Human telomerase reverse transcriptase; CI: Confidence interval.

structed for the four biomarkers (Figure 2A-D). 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 22.89 ng/mL, 2.775, 62.5 pg/mL and 1.309 ng/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: 1.626-6.206, *P* = 0.0007), NLR (20 mo *vs* 48 mo, HR 0.4167 with 95%CI: 0.204-0.8512, *P* = 0.0025), IL-6 (20 mo *vs* 80 mo,

Table 4 Univariate and multivariate Cox regression analysis of variables

No.	Variable	Univariate		Multivariate	
		HR (95%CI)	*P* value	HR (95%CI)	*P* value
1	KYN (ng/mL)	1.0037 (1.0026-1.0048)	0.0001[c]	1.0024 (1.0011-1.0037)	0.0003[c]
2	IL-6 (pg/mL)	1.0032 (1.0021-1.0042)	0.0001[c]	1.0022 (1.0010-1.0035)	0.0004[c]
3	NLR	1.2437 (1.1621-1.3312)	0.0001[c]	1.2085 (1.1138-1.3114)	0.0001[c]
4	TIMP-1 (ng/mL)	1.0150 (1.0075-1.0225)	0.0001[c]	1.0046 (0.9975-1.0118)	0.2042
5	hTERT (ng/mL)	1.2681 (1.1498-1.3987)	0.0001[c]	1.2303 (1.0749-1.4080)	0.0026[b]
5	Age(yr)	1.0245 (1.0108-1.0383)	0.0004[c]	1.0090 (0.9938-1.0233)	0.2165
6	EOR	1.3379 (1.8805-3.0328)	0.1726	NA	NA
7	Site	0.9527 (0.6221-1.4589)	0.8236	NA	NA
8	Therapy	0.8507 (0.2201-1.3578)	0.906	NA	NA

[b]$P < 0.01$.

[c]$P < 0.001$; indicated a significant association.

Results are presented as estimated hazards ratio with 95% confidence interval for specific variables. Variables with a significant P value (< 0.05) in univariate Cox-regression model were selected and analyzed using multivariate Cox-regression. CI: Confidence interval; EOR: Extent of resection; HR: Hazard ratio; hTERT: Human telomerase reverse transcriptase; KYN: Kynurenine; IL-6: Interleukin-6; NA: Not assessed; NLR: Neutrophil-lymphocytes ratio; TIMP-1: Tissue inhibitor metalloproteases-1.

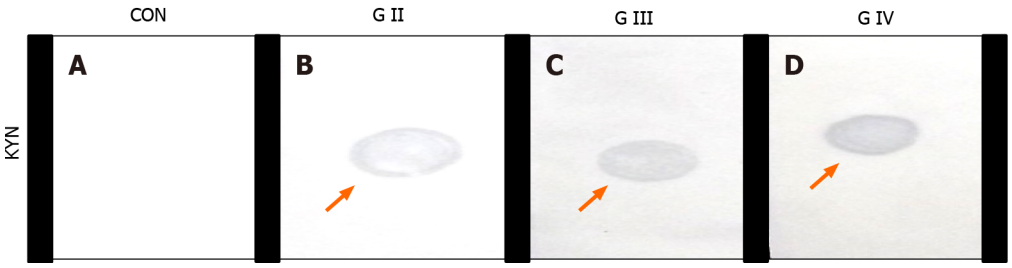

Figure 1 Qualitative screening of kynurenine by dot–immune binding assay in control, grade II, grade III, and grade IV samples; red arrow indicates the presence of immune-complex. A: Control; B: Grade II; C: Grade III; D: Grade IV. KYN: Kynurenine; CON: Control; G: Grade.

HR 0.25 with 95%CI: 0.1318-0.4741, $P = 0.0008$), and hTERT (18 mo *vs* 48 mo, HR 0.21 with 95%CI: 0.1178-0.4741, $P = 0.0006$).

On follow-up, paired t-test between pre-and 3-mo post-surgery levels of KYN ($P = 0.0148$), 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 debulking of the tumor (Table 5).

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 area under the curve of 0.983 as seen in combination VII (Figure 2F).

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

Table 5 Difference in preoperative and postoperative concentrations of markers in blood samples collected in a 3-mo follow-up

No	Markers	Preoperative concentration	3-mo postoperative concentration	*P* value	rs value
1	KYN (ng/mL)	126.1 (19.410-387.440)	75.405 (16.340-227.400)	0.0148[a]	0.4509
2	IL-6 (pg/mL)	75.65 (23.96-209.73)	60.03 (21.85-140.77)	0.0107[a]	0.1850
3	NLR	4.30 (1.43-15.00)	2.93 (0.80-6.90)	0.0038[b]	0.7260
4	hTERT (ng/L)	2.27 (1.16-6.86)	1.33 (0.78-4.78)	0.0016[b]	0.6574

[a]$P < 0.05$.
[b]$P < 0.01$.
Concentration presented as median with ranges. hTERT: Human telomerase reverse transcriptase; IL-6: Interleukin-6; KYN: Kynurenine; NLR: Neutrophil-lymphocytes ratio.

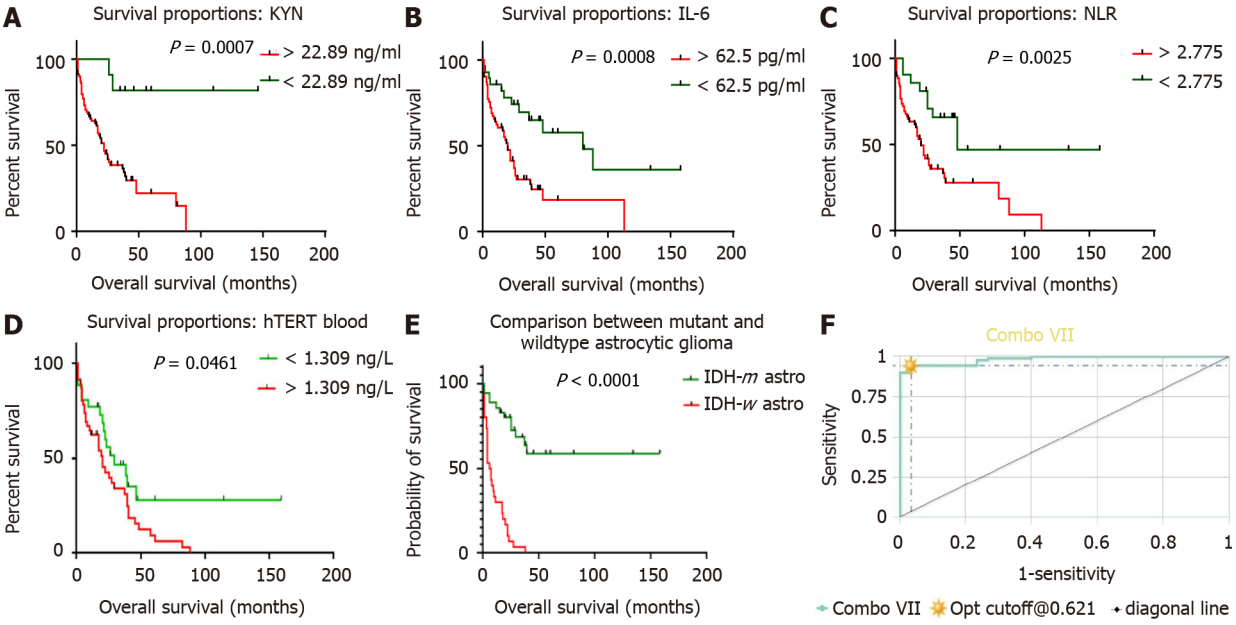

Figure 2 Survival curves of circulatory markers kynurenine, interleukin-6, neutrophil-lymphocyte ratio and human telomerase reverse transcriptase(red indicates worse prognosis while green represents favorable prognosis), differential survival between astrocytic isocitrate dehydrogenase-*mutant* and isocitrate dehydrogenase-*wildtype* gliomas, combination receiver operating characteristic of the panel of kynurenine, interleukin-6, neutrophil-lymphocyte ratio, and human telomerase reverse transcriptase to achieve increased sensitivity and specificity for predicting overall survival. A: Kynurenine; B: Interleukin-6; C: Neutrophil-lymphocyte ratio; D: Human telomerase reverse transcriptase; E: Differential survival between astrocytic isocitrate dehydrogenase-mutant and wildtype gliomas; F: Combination receiver operating characteristic of the panel of kynurenine, interleukin-6, neutrophil-lymphocyte ratio, and human telomerase reverse transcriptase to achieve increased sensitivity and specificity for predicting overall survival. KYN: Kynurenine; IL-6: Interleukin-6; hTERT: Human telomerase reverse transcriptase; NLR: Neutrophil- lymphocyte ratio; IDH-*m* astro: Isocitrate dehydrogenase mutant astrocytic; IDH-*w* astro: Isocitrate dehydrogenase wildtype astrocytic.

DISCUSSION

The link between a tumor and inflammation is a well-established fact and is one of the major attributes of malignancy[19]. Inflammation in the TE mediates all aspects of glial oncogenesis, including *in situ* progressive development of vasculature and tissue remodeling[20]. Thus, by and large, the inflammatory molecules orchestrate the extrinsic and intrinsic stimuli, thereby initiating and contributing to tumor progression [21].

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

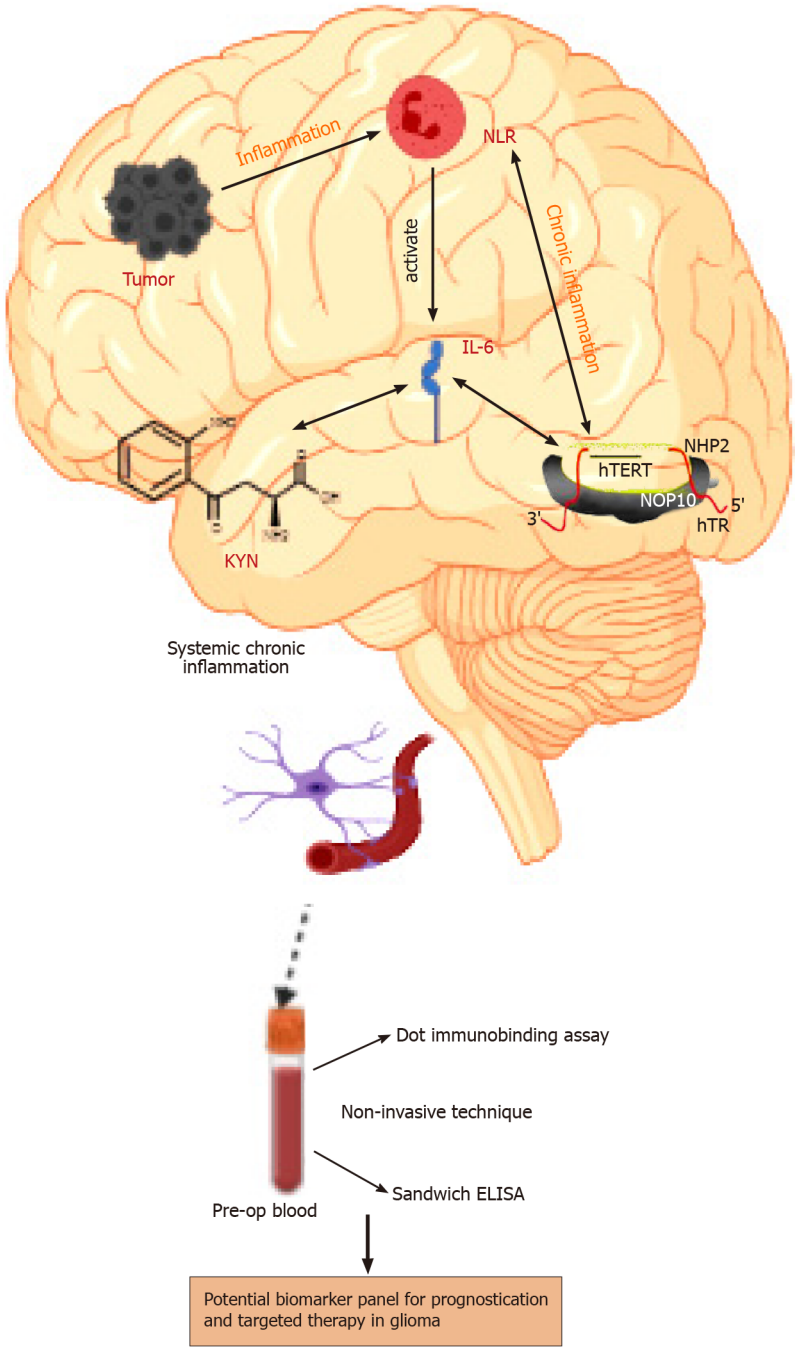

Figure 3 A non-invasive model for evaluating chronic systemic inflammation and overall survival in glioma. KYN: Kynurenine; IL-6: Interleukin-6; NLR: Neutrophil lymphocytes ratio; hTERT: Human telomerase reverse transcriptase; ELISA: Enzyme-linked immunosorbent assay.

surgical intervention, as seen in 10 GB samples[22]. However, the research by Lenzen *et al*[23] documented significantly decreased serological values of KYN after 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 (Figures 1 and 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 IL-6 in our enrolled glioma patients. Similarly, limited systemic studies available 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,

24,25]. These studies suggest that the 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 in 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 of this marker among grades, IDH-*m*/*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[31,32] which show that there is a significant association of NLR with OS[33-36]. 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[18].

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 TIMP-1 by ELISA[39]. Similarly, while working with 36 patients, it was suggested by Crocker *et al*[40] that serum TIMP-1 levels may serve as an independent predictor of survival in glioma subtypes[40]. In an extensive study on low-grade glioma patients, Zeng *et al*[41] established that plasma TIMP-1 expression was significantly correlated with OS and relapse-free survival 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.

CONCLUSION

The systemic levels of target molecules discussed herein 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 is able to differentiate between histological grades, IDH-*m/w*, in terms of patient survival, along with IL-6, hTERT, 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. The study needs to be replicated in a bigger cohort and conducted as a multicentric study to establish clinical utility of this panel.

ARTICLE HIGHLIGHTS

Research background

Chronic persistent inflammation is a hallmark of glioma and a major contributor to the disease progression. Currently there are no serological or molecular markers that are routinely evaluated before deciding treatment and in the follow-up period for monitoring survival and therapeutic efficacy.

Research motivation

A non-invasive inflammatory marker panel is essential to define survival and plan for a better clinical outcome.

Research objectives

The objective of this investigation was to assess the utility of the non-invasive biomarker panel to estimate systemic inflammation and whether it can define and differentiate survival in glioma subgroups.

Research methods

Dot-immune assay for screening of the expression of molecular makers followed by estimation of circulatory levels by enzyme-linked immunosorbent assay are easy, cost-effective and sensitive methods even in resource limited settings. The expression of marker kynurenine (KYN) has been validated by immunofluorescence-immunohisto-chemistry *in situ*.

Research results

The molecular marker panel of KYN, interleukin-6, human telomerase reverse transcriptase and neutrophil-lymphocyte ratio were negatively correlated with mortality ($P < 0.0001$) and achieved higher sensitivity and specificity (> 90%) than stand-alone markers, to define survival. It could discriminate between WHO-grades, isocitrate-dehydrogenase-*mutant/wildtype* and define differential survival between astrocytic isocitrate-dehydrogenase-*mutant/wildtype*. Association of KYN with neutrophil-lymphocyte ratio, interleukin-6, and human telomerase reverse transcriptase was significant. Cox-regression described KYN, interleukin-6, neutrophil-lymphocyte ratio, and human telomerase reverse transcriptase, as good prognostic markers, independent of confounders. Multivariate linear-regression analysis confirmed a concomitant significant decrease in the levels of the markers, in a 3-mo follow-up.

Research conclusions

This is a first of its kind evidence-based study of a non-invasive panel that can estimate chronic systemic inflammation, wherein the identified over-expressed molecular markers can be targeted to design a personalized therapy.

Research perspectives

The study model when replicated in a bigger sized multicentric cohort can pave way for the use of the inflammatory molecular screen for routine patient care.

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