RESEARCH ARTICLE

Microglial Contribution to Glioma Progression: an Immunohistochemical Study in Eastern India

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Abstract

Human glioma, arising from glial cells of the central nervous system, accounts for almost 30% of all brain tumours , neoplasms with a poor prognosis and high mortality rates worldwide. In the present study we assessed tissue architectural modifications associated with macrophage lineage cells, controversial major immune effector cells within the brain, in human glioma tissue samples from eastern India. Ethically cleared post-operative human glioma samples from our collaborative neurosurgery unit with respective CT/MRI and patient history were collected from the Nodal Centre of Neurosciences in Kolkata, over 9 months. Along with conventional histopathology, samples were subjected to silver-gold staining and fluorescence tagged immunophenotyping for the detection of electron dense brain macrophage/microglia cells in glioma tissue, followed by immune-phenotyping of cells. With higher grades, CD11b+/Iba-1+ macrophage/microglia architecture with de-structured boundaries of glioma lesions indicated malfunction and invasive effector state. Present study documented a contribution of microglia to glioma progression in Eastern India.

Keywords: Glioma - astrocytoma - silver-gold staining - CD11b - Iba1 - microglia

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Introduction

The occurrence of glioma, one of the most dreadful malignancies with fatal consequences, was reported for about 60% cases among all intracranial malignancies in and around Kolkata, where majority were among astrocytic grades (Ghosh et al., 2004). However, several meta-population based distributional and occurrence variation among different ethnicities (roughly whites and blacks in USA) and the population of this area of eastern India, particularly, in case of IR (Incidence Rate) of astrocytoma and (Glioblastoma multiforme) was observed in the studies (Ghosh et al., 2004; CBTRUS, 2011). As glioma IRs varies with histology, a more detailed molecular epidemiology, physiological and etiological profiling has initiated to figure out global trends and local variants (CBTRUS, 2011; Efird, 2011; Ng et al., 2011). Even In that first prominent survey report of the region though several epidemiological trends of the disease were shown, some patho-immunological assessment of the glioma samples were found in a different follow-up study where investigators showed that the immune-efficacy of blood borne lymphocytes and macrophages decreases severely with higher glioma grades irrespective of conventional therapeutic interventions (Bhattacharjee et al., 2006). This peripheral immune suppression was evident in different ways in studies of patient blood samples due to suppressing factors released by glioma, CD4+ and CD8+ T cell and cytokine imbalance, impairment of receptor expression and immune-effector signalling pathways etc (Elliot et al., 1990; Dix et al., 1999). In contrary, the presence of macrophage/microglia in glioma showed a different picture in patient samples in several studies since 1990's. Different macrophage/monocyte lineage markers, lectin histochemistry etc showed presence of glioma associated microglia/macrophage (GAM) somewhere forming bands surrounding glioma mass or 'trapped' in tumour mass in considerable numbers (Morioka, et al., 1992; Roggendorf et al., 1996).Overall, increasing GAM with ascending glioma grades interestingly not able to restrict glioma carnage and shows variable histological and immune-phenotypic features in the neoplastic tissue.

There are several hypothesis and attempt for explaining this apparent controversy of decreasing peripheral immune performances but increasing presence of microglia in high grade gliomas (Ghosh and Chaudhuri, 2010; Wei et al., 2013), which remains to be concluded so far. However, the primary evidences of presence and distribution of microglia in human glioma and associated variations, if any, in the population in Indian subcontinent is still

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not recorded. That influences us to document the GAM profile in glioma grades among the patients undergone for surgical resections in the meta-population around Kolkata, the largest city and population hotspot of eastern India (22º 34'N, 88º 24'E). The samples were taken from the nodal centre of neurosciences of the region catering patients from mostly southern West Bengal and some even referred patients from north-eastern provinces of India as well as neighbouring countries around the region. With normal histological study, special staining and immune-phenotyping of the sample tissue shows us the interplay between glioma-microglia associations with changing grades among the patients in the region for the first time.

Materials and Methods

Legislative approval for working with human sample

As the entire protocol is dependent on post-operative human glioma samples, with primary permission from Bangur Institute of Neurosciences (BIN) necessary human ethical clearance is obtained from the Institutional Ethical Committee (IEC) of hosting institute of BIN, namely, Institute of Post Graduate Medical Education and Research (IPGME&R), Kolkata, West Bengal, India vide memo no. Inst/IEC/553 dated 15.01.2014 supplemented with patient consent form which legally fortifies World Medical Association (WMA) declaration of Helsinki (last amended on 64th WMA General Assembly, Fortaleza, Brazil, October 2013).

Collection of human glioma tissue sample with allied metadata

A small portion of post-operative human glioma tissues were procured from patients undergoing surgical resection for the time spanning of April 2014 to January 2015 from our collaborative neurosurgery team at the Bangur Institute of Neurosciences (BIN) in the Institute of Post Graduate Medical Educationand Research (IPGME&R), Kolkata, West Bengal, India along with tabulated neurological case histories. Under advised condition, part of freshly collected tumour tissue samples were collected in 10% buffered formalin solution ('formol') for their histopathological examinations and rest of the parts were taken in chilled 4% parafoamldehyde (MERCK, India) solution for their immunophenotypic studies. Identification and gradation of the collected tumour biopsy samples were done by collaborative pathological expertise abiding by the World Health Organization (WHO) grading system (Louis et al., 2007). The respective Computed Tomography (CT)/ Magnetic Resonance Imaging (MRI) data were taken to draw radiological-histopathological correlations. A total of 25 samples with stringent histopathological screening towards astrocytic glial tumours were selected within the mentioned time period for further study among which 8 were grade II astrocytoma (n=8), 3 grade III astrocytoma (n=3), 9 grade IV astrocytoma or glioblastoma/GBM (n=9) and 5 oligoastrocytoma grade II (n=5). Astrocytoma grade I and oligodendroglioma grade I, being extremely rare due to their poor prognosis, had not been found. Ependymoma, mostly found as spinal Space Occupying Lesion (SOL) termed myxopappilary ependymoma were not considered in this regard as we were primarily focused on the intra-cranial neoplastic lesion of astroglial origin. We significantly rejected secondary or accessory glioma and recurrent glioma samples from our count. 2 normal non-malignant human brain tissues (n=2) from freshly dissected cadaver died in non-malignant brain lesion related disease that had been used as control, was a kind gift from Dr Nirjhar Bhattacharyya, Department of General Surgery, Sir Nil Ratan Sircar Medical College and Hospital, Kolkata, West Bengal, India abiding by requisite legal norms.

Histopathology with conventional Hematoxylin-Eosin (HE) staining

Formol fixed tumour tissues were dehydrated through ascending alcohol gradation (30%, 50%,70%, 95% and absolute respectively) followed by paraffin (MERCK, India) embedding under crucially monitored temperature cycles. From the prepared paraffin blocks, tissue section ribbons of 7 μm of thickness were cut by laboratory bench top microtome (WESWOX, India), taken in clean grease free Mayer's affixative treated glass slides, stretched and fixed. The sections were then stained with hematoxylin (NICE, India) solution followed by eosin (NICE, India) counter-staining to identify the histopathological features of the tumour tissues. Selected fields were visualized under bright-field microscope (Nikon, Model TS 100-F Ecplise, Nikon Corp., Japan), photographs were taken with CCD camera (DS-Fi2-U3) and presented through NIS Element-BR Software (Nikon Corp., Japan).

Specialized Silver-Gold (SG) staining

10% buffered formalin fixed tumour samples underwent paraffin blocking, tissue sectioning (10μm) and embedding in grease free slides. After that they were passed through silver staining first introduced by Rio Hortega (1918) and gold toning by Penfield (Penfield and Cone, 1937) and later modified by Mc Carter.As previously described, briefly, the staining process includes passing of slides through freshly prepared light sensitive Ammoniacal Silver Carbonate (Ag_2CO_3) solution in dark for staining, rinsing through 10% formalin solution as the reducing agent (NICE, India) followed by Gold Chloride solution (HAuCl4) for gold toning and finally fixing by sodium thiosulphate (MERCK, India) solution (Ghosh et al., 2015). For best staining result, slides were passed through Globus' 10% hydrobromic acid (MERCK, India) solution before staining with Ag_2CO_3. After mounting with DPX, the slides were visualized in bright field by Nikon Microscope (TS 100-F Eclipse, Nikon Corp., Japan), photographed with CCD Camera (DS-Fi2-U3) and documented with NIS Element-BR Software (Nikon Corp., Japan) to detect the distribution of electron dense macrophage / microglia as they tend to take deep stain (silver impregnation) and can be demarcated from other cells. Staining of normal human brain tissue had been implicated as control.

Immunohistochemistry (IHC) and fluorescence microscopy

Part of post-operative glioma tissue samples were taken in ice-cold 4% paraformaldehyde (MERCK, India)

solution, kept for 24 hours, then thoroughly washed and stored in 1X PBS at 4˚C for future use. Thereafter slides were prepared from paraffin embedded blocks having tissue thickness of 10µm. The overnight heat-fixed tissue slides of both tumour and normal human brain as control were stained separately with Alexa Fluor® 488 primary conjugated human reactive mouse monoclonal anti-Glial Fibrillary Acidic Protein (GFAP) antibody (BD Pharmingen, New Jersy, USA) at 1:500 dilution to identify distribution of astrocytic lineage cells and FITC primary conjugated human reactive mouse monoclonal anti-CD11b antibody (BioLegend, San Diego, CA, USA) at 1:500 dilution to identify common monocyte/ macrophage lineage cells separately in different slides. Moreover, to detect brain macrophage / microglia, heat fixed tissue slides of both tumour and normal human brain tissue as control were stained separately with primary non-conjugated human reactive mouse monoclonal anti-Ionized calcium-binding adapter molecule 1 (Iba 1) antibody (Abcam, Cambridge, MA, USA) and counteracted by PE-conjugated anti-mouse goat secondary antibody (Abcam, Cambridge, MA, USA). All the primary and secondary antibodies were incubated in dark under humid chamber at 4ºC. 5% Foetal Bovine Serum (GIBCO, Life Technology, Grand Island, NY, USA) in 1X PBS solution had been used to block nonspecific bindings and all the antibodies were diluted in 1% Foetal Bovine Serum (GIBCO, Life Technology, Grand Island, NY, USA) in 1X PBS solution. After mounting in DPX, the slides were viewed through Nikon TS 100-F Eclipse Microscope with Epifluorescence attachment ((Nikon Corp., Japan) using Epi-FL filter Block B-2A Green Channel (Nikon Corp., Japan) for Alexa Fluor® 488 / FITC and Epi-FL filter Block G-2A Red Channel for PE (Nikon Corp., Japan). Photographs of fluorescence stained cells were captured with CCD camera DS-Fi2-U3 (Nikon Corp., Japan), processed, analysed and documented with NIS Element-BR SoftwareVersion 4.20 (Nikon Corp., Japan).

Results

GFAP immunophenotyping does not show direct correlation with astrocytoma grades while MRI shows positional propensity

Astrocytic glioma, the outcome of abnormally proliferated astro-glial cells, has been assessed for specific astrocytic marker GFAP in astrocytoma grade II, III, GBM and oligodendroglioma grade II. The corresponding MRI were corroborated with the expressional analysis with Alexa Fluor® 488 (green emitter) conjugated anti human GFAP antibody as depicted in Figure 1. The T1 weighted MRI showing iso- to hypo-intense signals in astrocytoma grade II / diffuse or fibrillary astrocytoma (Figure 1A) and astrocytoma grade III / anaplastic astrocytoma (Figure 1B) whereas hypo-intense central heterogenous signals are visible in T1 with surrounding necrotic mass in astrocytoma grade IV / glioblastoma (Figure 1C). The low heterogenous mass in MRI T1 with increased GFAP expression confirms the outcome of oligoastrocytoma grade II as we have considered it as a case of mixed astrocytoma / oligoastrocytoma (Figure 1D). With the increasing grades of astrocytic tumour (1A-1C) though the spatial increment of GFAP positive astrocytic cells were not much prominent or correlated, but the tissue matrix gets highly degraded and heterogeneous, seemed to be churned in a graded manner indicating the increased invasiveness and destruction of tissue matrix particularly in astrocytoma grade III and GBM (Figure 1B and 1C).

Hematoxylin-Eosin (HE) staining under bright field microscope confers histopathological characteristics of glioma tissue

In course of the study, we have considered the non-malignant human brain tissue as the glioma control in which distinct integrity morphology of brain parenchyma with neuronal integrity, restricted blood vasculature, moderate number of requisite glial cells with non-proliferative mitotic index and normal neuropil structure has been documented (Figure 2A, blue arrow head). Giving stress on these histological features glioma tissue samples have been graded according to WHO guideline (Louis et al., 2007). Moderate to high cellular nuclear atypia with graded hyper cellularity have been seen in accordance to the grades of astrocytic tumours in case of astrocytoma grade II (Figure 2C-2D, white arrow head), astrocytoma grade III (Figure 2E-2F, whitearrow head). Unlike the grade II and grade III, loads ofnecrotic tissues have been seen in astrocytoma grade IV (Figure 2H, orange tissue patch). Overall, increasing tissue dis-integrity appears as prominent feature. Features like hyper micro-

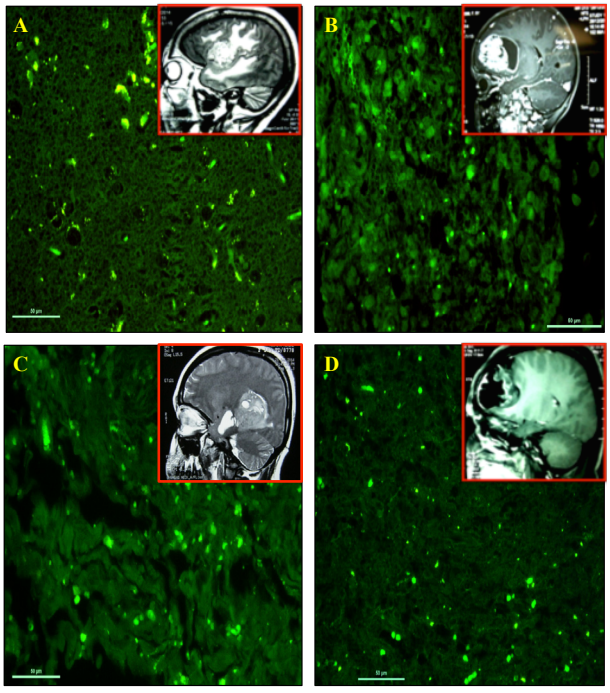

Figure 1. T1 Weighted MRI Records with Alexa Fluor® 488-Conjugated GFAP Expression. Note increased expression of GFAP (glowing green) along with visible heterogenous tissue matrix at growing levels in (A) astrocytoma II (B) astrocytoma III (C) astrocytoma IV and (D) oligoastrocytoma grade II. Magnification 40X

calcification and evasive neo-angiogenesis have also been witnessed along the increasing grades of astrocytoma. On the other hand, hyper-proliferated monomorphic cells having typical "fried egg appearance" intermingled with astrocytic cells clearly signify oligoastrocytoma, a mixed type tumour of astrocytic origin (Figure 2B, white arrow head). The striking cellular character witnessed in glioma is that it hosts round, deeply stained cells which increase in numerical and spatial orientation in accordance with the ascending grades. The number of these cells are handful in astrocytoma grade II and oligoastrocytoma grade II (Figure 2C-2D, green arrow head and Figure 2B, green arrow head respectively) to moderate in astrocytoma grade III (Figure 2E-2F, green arrow head) to evasive in astrocytoma grade IV (Figure 2G-2H, green arrow head). The distributionof these cells weregenerally concentrated near the ruptured blood vessels in low grade glioma and entirely diffused in anarbitrary pattern throughout the tumourtissues of high grade glioma. However these kinds of cells have hardly been foundin normal brain tissue sections (Figure 2A, green arrow head). Those cells might be macrophage or microglia that infiltrates within the malignant tissue in a rapid proportion, although its confirmationrequires more convincing experimental approaches that are followed subsequently.

Silver-gold (SG) staining detect brain macrophage/ microglia affirmed by immunophenotyping showing assimilation in higher grades leading to tissue demolition in GBM

Thus far we have stated that from the histopathological studies some deeply stained cells have been found in an increasing rate along with the increasing glioma grades. Silver ions ($Ag2^{+}$) bind with arzyrophilic macrophagic cell structure by the phemomena known as "silver impregnation" and later replaced by more electropositive gold ion giving off deeply stained cells vivid than the background pale stained cytoplasm. Indeed we have recorded a steep increase of these deeply stained cells from astrocytoma grade II and oligoastrocytoma grade II (Figure 3B, blue rectangle and Figure 3E, blue rectangle respectively) to astrocytoma grade III (Figure 3C, blue rectangle) and being affluent in astrocytoma grade IV (Figure 3D, blue rectangle). An easily comparable diminished amount has been seen in normal brain tissue (Figure 3A, blue rectangle area).

To be more stringent in the course of our finding, we have treated the tissues with FITC conjugated (green emitter) human reactive CD11b antibody by immunohistochemistry that implemented the actuality of the data obtained from SG staining giving a signific antincrease of CD11b expression from least in normal brain tissue to moderate increase in low grade astrocytic (Figure 3G, 3H respectively) and oligoastrocytic (Figure

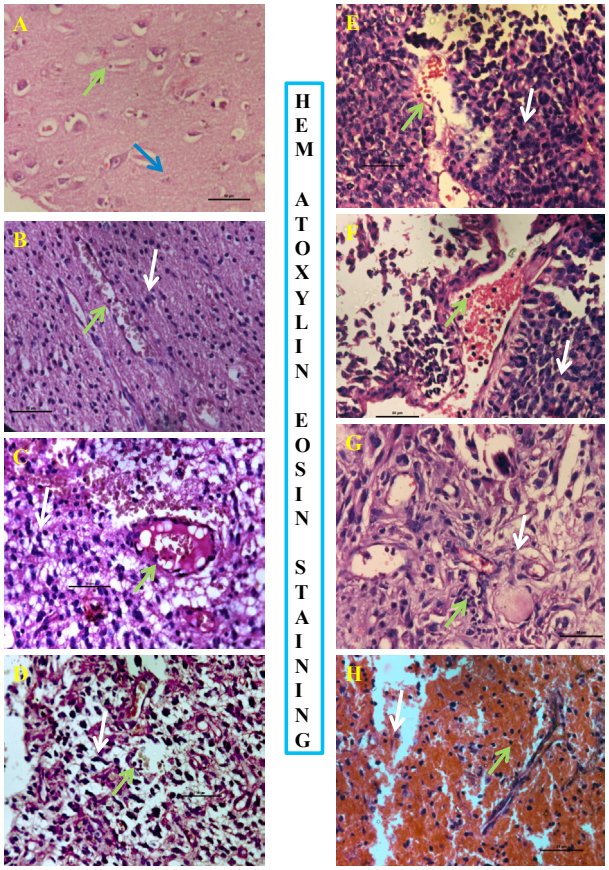

Figure 2. Histopathological Graded Analysis of Tumour and Normal Human Brain Tissue. Note hypercellularity, nuclear atypia, and presence of some deeply stained cells that might be brain macrophage in (A) normal human brain tissue, (B) oligoastrocytoma, (C-D) astrocytoma II, (E-F) astrocytoma III, and (G-H) astrocytoma IV. Magnification 40X

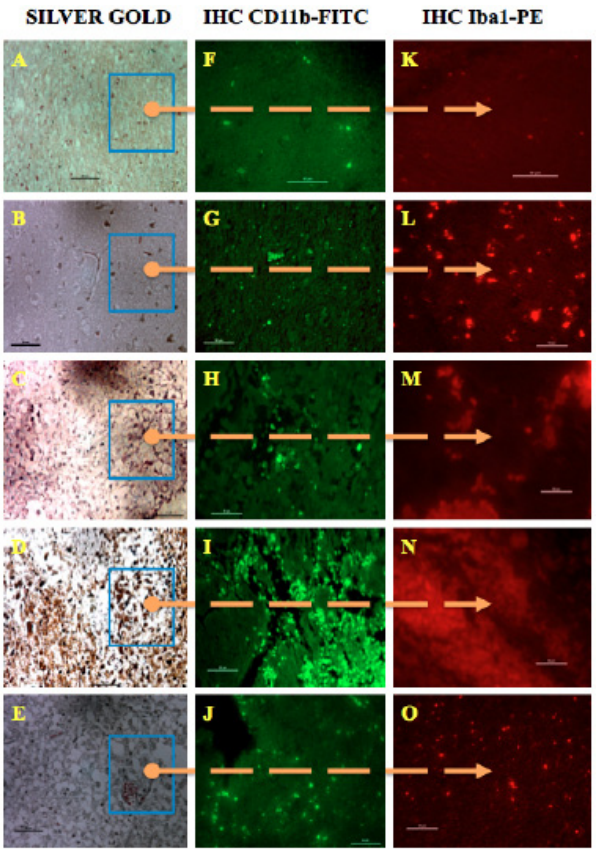

Figure 3. Comparative Analysis of Monocytic Macrophages by Silver-Gold Staining and Immunophenotyping with FITC Conjugated CD11b and PE Texas Red Conjugated Iba1. (A, F, K) normal brain tissue, (B, G, L) astrocytoma II, (G, H, M) astrocytoma III, (D, I, N) astrocytoma IV and (E, J, O) oligoastrocytoma grade II. See text for details. Magnification 40X

3J) tumour to prolific in high grade invasive glioblastoma (Figure 3I, marked region). Huge tissue disruption and matrix degradation with considerable necrotic areas, where macrophage lineage cells smeared found in high grade astrocytomas indicate a probable disarrayed contribution of macrophagic cells in the orientation of severity.

To check ultimately that if these macrophage were residential (i.e, microglia) to central nervous system (CNS), we further performed IHC with PE-Texas Red conjugated (red emitter) human reactive Iba1 antibody, a well-known pan marker for microglia, that ultimately fetched the same expressional outcome as previously found in IHC with CD11b. The radical increase in microglial expression in astrocytoma grade II (Figure 3L), grade III (Figure 3M) and oligoastocytoma (Figure 3O) into astrocytoma grade IV (Figure 3N) aided with profuse necrotic tissue cluster and devastated tissue matrix with rapidity resolve the hypothesis regarding a nexus between glial tumours and brain resident microglia/macrophage or infiltrated macrophages. However, in the normal brain tissue, expression of CD11b (Figure 3F) and Iba1 (Figure 3K) has been recorded in a pitifully small amount referring that the number of monocytic lineage cells or active microglia in non-malignant CNS is much smaller and restricted than the malignant counterparts, probably reinforcing the thought under discussion that steady increase in the numerical and expressional diversity of macrophage or microglia is directly proportional to the invasive severity of the glioma with ascending grades.

To quantifying our findings, we graphically interpolated the total area versus total sum intensity of the expression of monocytic lineage cells or microglia as depicted in three types of classical astrocytoma (i.e, grade II, III and IV or GBM) in respect to the normal brain tissue as we depicted in the Silver-gold staining and immunohistochemistry with PE conjugated Iba1 separately (Figure. 4A) by using NIS Element-BR Software Version 4.20 (Nikon Corp., Japan). We have further elucidated the graphical comparison of total sum intensity found in the Silver-gold staining and immunohistochemistry with PE conjugated Iba-1of three types of classical astrocytic tumours in relation to the non-malignant human brain tissue (Figure. 4B), considering Iba-1 as microglial cell marker. All of these graphical representations showed clear steep increase in the total sum intensity of macrophagic/microglial cell population along with the progressive grades of tumour compared to a basal level expression found in non-malignant tissue reflecting their prominent quantitative increase. While having these intensity profiling, oligoastrocytoma has been categorically rejected being a mixed oligodendroglioma grade II in nature as we primarily focused on the intensity wise distribution of monocytic lineage cells/microglia associated with classical astrocytic glioma grades.

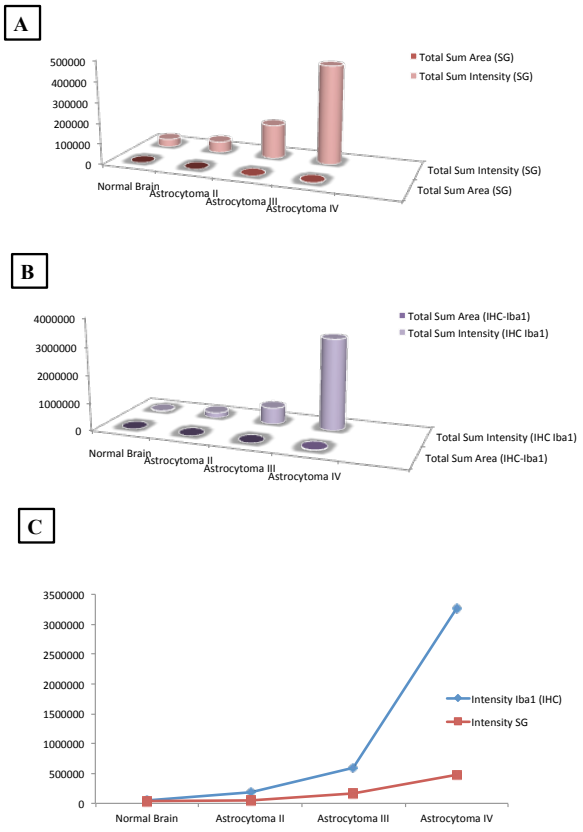

Figure 4. Graphical Interpolation of Total Sum Area and Intensity of Brain Macrophage/Microglia. Association in accordance with the ascending grades of glioma after being individually treated with (A) Silver-Gold (SG) staining and (B) immunohistochemistry (IHC) by PE-Conjugated Iba1 (C) Correlated graphical representation.

Discussion

The incidence of glioma is increasing with leaps and bounds globally as well as in India also. In West Bengal alone, among primary neuro-epithelial tumour, about 46.8% are astrocytoma among which 7.9% are GBM with medial survival time is almost a year in GBM (Khan et al., 2009 and Preusser et al., 2011). There are several intrinsic and extrinsic inducing factors including recent lifestyle hazards like mobile phone uses (Barchana et al., 2012) and their occurrence in mid-age group and low median survival has been proved repeatedly in different populations from different parts of world (Ghosh et al., 2004; Ng et al., 2011; Trabelsi et al., 2014). Several diagnostic factors in local populations like Ki67 or IBH etc in corroboration of global incidences has been figured out (Das et al., 2013, Kerkhof and Vecht, 2013; Xavier-Magalhães et al., 2013; Chen et al., 2015). Our present study was an attempt to characterize the distributional and cellular profile of the chief immunomodulatory cell of brain, i.e., microglia in grades of gliomas, particularly in higher grade of astrocytomas in patient population of the said region of Indian subcontinent. The collaborative neurosurgery unit at IPGME&R, Kolkata serve as a super speciality demographical medical hub encircling the various parts of West Bengal, the North Eastern region and even SAARC (South Asian Association for Regional Cooperation) associated neighbouring countries like Bangladesh, Bhutan and Nepal partially. Thus we have studied on the samples collected from the meta-population of Eastern Indian subcontinent primarily for astrocytic brain tumours to view the inflicting presence and distribution of microglia and macrophages.

As this glioma associated microglia/macrophages

and their role in glioma progression and invasiveness has already evoked pronounced debates and discussion, this documentation in the said meta-population was the necessity to visualize the actual scenario. In our present study we have also established this fact as we got increased expressional outcome of CD11b+ monocytic lineage cells that were also Iba1+ microglia with increased grades of glioma (Figure 3, F-O) deriving the fact that the invasiveness is highly mediated by GAM. Small numbers of brain macrophage cells are pressed into action having a brawl with the hyper proliferating astrocytes in low grade glioma as expressionally documented in our findings. In the pathway of invasiveness, the huge disarrayed growths of astrocytes become the releasing site of chemoattracting substances that recruits large number of macrophagic cells. These macrophagic cells, by their inherent nature, colonize more with the uncontrolled dividing astrocytes. Their massive infiltrative property stimulates profuse necrosis as we have showed in GBM both by HE as well as SG staining method. This necrosis might act as a dual selective pressure in the tumour microenvironment. In one way the random cellular necrosis boost up the mitotic index of the astrocytes at a greater extent and on the other hand, on a wild killing spree, the proinflammatory substances released by GAM degrade the tissue matrix in such a way that the entire tissue architecture get busted making an ease in the tumour cell metastasis on other locations in the CNS enhancing the fatality at per. Our findings of increased gradualism of hyper cellularity, nuclear atypia, matrix degradation and tissue necrosis from the histopathology can in turn be directly correlated with the numerical and expressional upliftment of monocytic lineage cells/microglia as obtained vide silver-gold staining and immunophenotyping.

From the evidences of the increased presence of microglia/macrophages in glioma it was concluded earlier that the cells directly contribute in glioma progression (Morioka et al., 1992; Roggendorf et al., 1996; Badie and Schartner, 2001). This one sided liability on the monocytic lineage cells present in the neoplastic lesions was tamed down with the counterargument that those defenders rushes to the glioma site in urgency to gain control over the situation, but in turn, help to dissolute matrix paving glioma invasiveness (Ghosh and Chaudhuri, 2010).This made the observations of involvement of GAM secreted (Membrane-type 1 matrix metalloproteinase) that activate pro (matrix metalloproteinase 2) which along with Transforming Growth Factor β (TGFβ) is associated with brain tissue matrix remodelling causing high level of invasion more explainable (Markovic et al., 2009; Coniglio and Segall, 2013). Our observation of inclining monocytic lineage cells in astrocytoma grades to GBM, both in SG staining and CD11b+/Iba-1+ immunophenotyping with severely degraded tissue organization in higher grades indicate the fact of GAM involvement in glioma invasion where TGFβ, IL-6, proteases, EGF/EGFR, CSF-1 and other factors, their molecular interaction are playing the key roles (Ye et al., 2012; Coniglio and Segall, 2013). Our repeated observations show marked deterioration of glioma tissue organization and GAM architecture from astrocytoma grade III and becomes remarkably prominent in GBM with considerable necrotic mass.

In the presence of glioma cells in the CNS, microglia furnishes two different kinds of phenotypic attributes: M1 phenotypes serving against the glioma invasion and M2 phenotypes, also regarded as 'neoplastic macrophages' serving in favour of glioma (Wei et al., 2013). Findings have also suggested that glioma associated microglia having M2 phenotype unwillingly helps glioma stem cells in the progress of metastatic invasion by TGF-β1 signalling pathway (Ye et al., 2012; Coniglio and Segall, 2013). In our concerned study, correlation with the number of CD11b+ and Iba1+ cells have been drawn in which the increasing grades of astrocytic tumour is directly proportional to the amount of macrophage infiltrations show the remodelling of matrix structure in a graded condition (Figure 1, A-C) that aid the invasiveness of the glioma also. M2 like GAM also trigger the synthesis of neovascularisation factors like Vascular endothelial Growth Factor (VEGF) mediated by Receptor for Advanced Glycation End Products (RAGE) that result into angiogenesis by supplying nutrition and aiding gaseous exchange uplift the growth and proliferation of the glioma tissue (Chen et al., 2014). However, the recent study has demonstrated different phenotype of GAM from M1 and M2 polarized state which is still to be evaluated in human glioma (Wei et al., 2013; Szulzewsky et al., 2015).

Altogether we have commemorated the association of brain macrophage/microglia in glioma which is opportunistically advantageous for the invasiveness and progression of glioma, this is the first ever demonstration of the kind in the region from human glioma samples. The fatality increases at a direct proportion with the increasing tumour associated microglia. Silver impregnated gold toned slides show deformed cellular architecture of assembled monocytic cells with distorted tissue organization. The feature is more prominent in astrocytoma III and GBM. That dissociated tissue backs glioma cells to expedite around crossing boundaries causing evasion, where microglia/macrophages with CD11b+/Iba-1+ show assemblage with deformed appearance indicating their disturbed effector state in GBM. Indeed the dreadfulness of this very disease has gone from strength to strength as its being aided by our very own immunological cell of our most immune-privileged part of the body, the CNS. Henceforth, not only proper anti neoplastic therapeutics are needed to curb down the threat of glioma, our 'manipulated' guardian should be 'remanipulated' so that a better way of curing with longer viability of patients can be given as recently validated in primary stage (Sarkar et al., 2014). A thorough understanding of the biology of glioma-microglia interaction might be the only way for some benevolent therapeutic approach.

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References

ABadie B, Schartner J (2001). Role of microglia in glioma biology. *Microsc Res Tech*, **54**, 106-11.

Barchana M, Margaliot M, Liphshitz I (2012). Changes in brain glioma incidence and laterality correlates with use of mobile phones – a nationwide population based study in Israel. *Asian Pac J Cancer Prev*, **13**, 5857-63.

Bhattacharjee M, Bose I, Sarkar P, et al (2006). A sequential scanning of the immune efficacy in astrocytoma (grade i – grade iii), meningioma and secondary glioma patients with and without therapeutic scheduling. *Cancer Invest*, **24**, 502-13.

CBTRUS statistical report (2011).Primary brain and central nervous system tumors diagnosed in the United States in 2004-2007. source: central brain tumour registry of the united states, hinsdale, IL. Website: www.cbtrus.org.

Chen WJ, He DS, Tang RX, et al (2015). Ki-67 is a valuable prognostic factor in gliomas: evidence from systematic review and meta-analysis. *Asian Pac J Cancer Prev*, **16**, 411-20.

Chen X, Zhang L, Zhang IY, et al (2014). RAGE Expression in tumor-associated macrophages promotes angiogenesis in glioma. *Cancer Res*, **74**, 7285-97.

Coniglio SJ, Segall JE (2013). Molecular mechanism of microglia stimulated glioma invasion. *Matrix Biol*, **32**, 372–80.

Das BR, Tangri R, Ahmad F, et al (2013). Molecular investigation of isocitrate dehydrogenase gene (IDH) mutations in gliomas: first report of IDH2 mutations in indian patients. *Asian Pac J Cancer Prev*, **14**, 7261-4.

Dix AR, Brooks WH, Roszman TL, et al (1999). Immune defects observed in patients with primary malignant brain tumours. *J Neuroimmunol*, **100**, 216-32.

Efird JT (2011). Epidemiology of glioma. in 'glioma: exploring its biology and practical relevance', Ed. Ghosh A. InTech, Rijeka, Croatia, 3-24.

Elliott LH, Brooks WH, Roszman TL (1990). Inability of mitogen activated lymphocytes obtained from patients with malignant primary intracranial tumors to express high affinity interleukin 2 receptors. *J Clin Invest*, **86**, 80-6.

Ghosh A, Chaudhuri S (2010). Microglial Action in Glioma: A Boon Turns Bane. *Immunol Lett*, **131**, 3-9.

Ghosh A, Sarkar S, Dutta S, et al (2004). The First Cross-sectional survey on intracranial malignancy in Kolkata, India: reflection of the state of art in southern west bengal. *Asian Pac J Cancer Prev*, **5**, 259-67.

Ghosh P, Mukherjee N, Ghosh K, et al (2015). Prospective microglia and brain macrophage distribution pattern in normal rat brain shows age sensitive dispersal and stabilization with development. *Ind J Exp Biol*, **53**, 561-7.

Khan MK, Hunter GK, Vogelbaum M, et al (2009). Evidence-based adjuvant therapy for gliomas: current concepts and newer developments. *Ind J Cancer*, **46**, 96–107.

Louis DN, Ohgaki H, Wiestler OD, et al (2007). The 2007 WHO Classification of Tumours of the Central Nervous System. *Acta Neuropathol*, **114**, 97-109.

Markovic DS, Vinnakota K, Chirasani S, et al (2009). Glioma induce and exploit microglial MT1-MMP expression for tumor expansion. *PNAS*, **106**, 12530-5.

Morioka T, Baba T, Black KL, et al (1992). Inflammatory cell infiltrates vary in experimental primary and metastatic brain tumours. *Neurosurgery*, **30**, 891-6.

Ng K, Kesari S, Carter B, et al (2011). Molecular etiology of glioblastomas: implication of genomic profiling from the cancer genome atlas project. in 'glioma: exploring its biology and practical relevance', Ed. Ghosh A. InTech, Rijeka, Croatia, 25-36.

Penfield W, Cone W (1937). Neuroglia and microglia (the metallic methods). In: Handbook of Microscopical Techniques, Eds. McClung CE, Paul B. Hoeber Inc, New York, 489-521.

Preusser M, de Ribaupierre S, Wöhrer A, et al (2011). Current concepts and management of glioblastoma. *Ann Neurol*, **70**, 9-21;

Roggendorf W, Strupp S, Paulus W, et al (1996). Distribution and characterization of microglia/macrophages in human brain tumours. *Acta Neuropathol*, **92**, 288-93.

Sarkar S, Döring A, Zemp FJ, et al (2014). Therapeutic activation of macrophages and microglia to suppress brain tumor-initiating cells. *Nat Neurosci*, **17**, 46-55.

Szulzewsky F, Pelz A, Feng X, et al (2015). Glioma-associated microglia/macrophages display an expression profile different from M1 and M2 polarization and highly express gpnmb and spp1. *PLoS ONE*, **10**.

Trabelsi S, Brahim DH, Ladib M, et al (2014). Glioma epidemiology in the Central Tunisian Population: 1993-2012. *Asian Pac J Cancer Prev*, **15**, 8753-7.

Wei J, Gabrusiewicz K, Heimberger A (2013). The controversial role of microglia in malignant gliomas. *Clin Dev Immunol,* **2013**, 285246.

Xavier-Magalhães A, Nandhabalan M, Jones C, et al (2013). Molecular prognostic factors in glioblastoma: state of the art and future challenges. *CNS Oncol*, **2**, 495-510.

Ye XZ, Xu SL, Xin YH, et al (2012). Tumor-associated microglia/macrophages enhance the invasion of glioma stem-like cells via TGF-beta1 signaling pathway. *J Immunol*, **189**, 444–53.