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RESEARCH ARTICLE

CATHEPSIN B AS FUTURE PROGNOSTIC MARKER IN CASES OF GLIOMA

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Abstract

Introduction:-

Gliomas are the most common primary malignant brain tumor in adults. They can occur anywhere in the central nervous system but primarily occur in the brain and arise in the glial tissue [2]. Gliomas are either astrocytic, oligodendrocytic, ependymal or a mix of these 2 cell types and are typically categorized according to the International Classification of Diseases– Oncology, version 3 (ICD-O-3) and World Health Organization (WHO) grade [1].

Malignant gliomas are among the most challenging of all cancers to treat successfully. The tumor cells vigorously invade surrounding tissue, which renders complete surgical resection difficult and contributes to the high incidence of the recurrence [3]. Invasion of glioma cells into adjacent brain tissue is dependent on their interaction with the extracellular matrix (ECM) and possible destruction of matrix barriers [4]. These processes are mediated by multiple degradative activities in an enzymatic cascade [5]. Members of all five classes of endopeptidases (matrix metalloproteinases, serine proteases, aspartic proteases, threonine proteases, and cysteine proteases such as cathepsin B) have been implicated in the progression of tumors [6-12].

Of all the proteolytic enzymes, studies have shown that Cathepsin B (Cat B) is of significant importance as it is involved in various pathologies and oncogenic processes. It can function as an endopeptidase, cleaving internal peptide bonds, as well as an exopeptidase (carboxydipeptidase activity) [13, 14]. Cat B is regulated at multiple levels from transcription through posttranslational processing and trafficking to activation and inhibition. High levels of expression of Cat B at both transcriptional and protein levels have been observed in cancers, for example, esophageal [15], gastric [16], prostate [17], glioblastoma [18, 19], breast [20]. Multiple promoters for the gene have been identified, including in glioma [21]. Numerous studies have shown that Cat B overexpression is correlated with invasive and metastatic cancers [22–24]. Various therapeutic strategies have been developed to suppress proteolytic activity of proteases in an attempt to curb metastatic infiltration mediated by proteases. Among the strategies developed, chemical inhibitors, antibodies, and gene therapy approaches have shown promising developments. Inhibitors of Cat B have been isolated from various sources such as the marine bacteria Pseudomonas, marine sponges and other organisms [25]. Hence localizing and targeting the expression of Cat B could have significant therapeutic implications.

Prompted by these observations we have examined the expression of Cat B in gliomas and correlated the results with the morphological grading and overall survival of patients. We have also compared the expression of Cat B

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with another well-established marker of biological aggressiveness in malignancies i.e. Ki-67[26, 27] to prove its role in oncogenic process of gliomas.

Material and Methods:-

Type of Study:
Observational study.

Tissue samples and Processing:
This study includes a series of glioma specimens operated between September 2015 to August 2017 from the Department of Neurosurgery of our institute which were taken on the basis of clinicoradiological findings with adequate patient's clinical information and proper consent. Both the histopathological diagnosis and immunohistochemistry (IHC) was performed against antibodies to Cathespin B and Ki-67. Total 140 cases were studied which included 62 cases of Low grade glioma and 73 cases of High grade glioma along with five normal brain specimen (Autopsy) as control. Specimens were processed according to the standard protocols and grading was done after staining with Hematoxylin and Eosin stain (H & E) based on World Health Organization (WHO) grade [1]. However due to lack of IDH1 expression status or other molecular markers all the tumors were categorised as "Not otherwise specified (NOS)". The cases of WHO Grade I and Grade II tumors were included in the category of low grade glioma and the cases with WHO Grade III and Grade IV were included in the category of high grade glioma.

Immunohistochemistry analysis and Interpretation:
Immunohistochemistry was performed with antibodies to Cathepsin B and Ki-67. Cathepsin B antibody was manufactured by Abcam® (Rabbit monoclonal to cathepsin B diluted in PBS, ph 7.6, in a dilution of 1:100) and Anti Ki-67 was manufactured by Biogenex® (Pre-diluted ready–to-use Mouse monoclonal antibody to Ki-67 antigen).IHCstaining was performed using the standard technique.

Immunostaining for Cathepsin B and Ki-67 were scored separately for tumor cells. Sections of tumors of various grades were stained and compared with the expression pattern observed in normal brain. The frequency of Cathepsin B immunostaining in tissue sections was evaluated as negative when no positive cells were observed within the tumor, weak(1+) when < 30% of the tumor cells were positive, moderate (2+) when 30–59% of the tumor cells were positive and strong (3+) when ≥ 60% of tumor cells were positive. The intensity of staining was evaluated as 0 for no staining, 1+ for weak staining [Fig 1], 2+ for medium staining [Fig 2], and 3+ for strong staining [Fig 3], IHC score was determined as the sum of the frequency and intensity score for tumor cells.The results of staining were subdivided into two groups. The group with scores 0 to 4+ were taken as weak positive staining and the group with score 5+ & 6+ as strong positive staining. The frequency of Ki-67 immunoreactivity in tissue sections was evaluated as the percentage of positively stained tumor cell nuclei out of the total tumor cells counted (n=1000) and the scoring was done as ≤4% Labelling index (Score 1), 5-10% Labelling index (score 2), >10% Labelling index (score 3).

Statistical Analysis:
The statistical analysis was done using SPSS (Statistical Package for Social Sciences) Version 15.0 statistical Analysis Software. The values were represented in Number (%) and Mean±SD. The level of significance was calculated using chi-square test. Survival of the patients was estimated using the Kaplan-Meier method. The Kaplan-Meier method was used to obtain survival curves, survival medians, and probabilities at different time points (1, 3, and 12months). Log-rank test was used to assess the association between survival and the variables.

Results:-

The study was conducted to evaluate expression of Cathepsin B in Glioma and correlate immunohistochemistry status with the histomorphological grading, Ki-67 labelling index and overall survival rate.

Demographic profile of the cases:
Out of 135 cases of glioma, 62 (45.92%) were graded as Low grade and rest 73 (54.07%) as high grade glioma. Among low grade glioma most common diagnosis was Pilocytic astrocytoma (37.10%) followed by Ependymoma (20.97%) and Diffuse Astrocytoma (19.35%). Among High Grade Glioma most common diagnosis was Glioblastoma (61.64%) followed by Anaplastic astrocytoma (12.33%). Out of 135 cases of glioma, two-third of cases were male(66.67%) and rest one-third cases were female (33.33%).

Clinical Parameters:
In overall 62 low grade glioma cases the population with highest number of cases 31 (50.0%) is ≤ 20 yrs. In overall 73 high grade glioma cases the population with highest number of cases 32 (43.84%) is 40-60 yrs. Association of age with histological diagnosis among High grade glioma cases was found to be statistically significant. Higher duration of symptoms was observed in cases of Low grade of glioma while lower duration of symptoms was observed in cases of High grade of glioma. Mean duration of complaints among patients of Low grade glioma (8.78±10.08 months) was found to be higher as compared to High grade glioma (4.51±9.51 months).

Correlation between Cat B and Ki-67 Immunostaining with Histological Parameters of Brain Tumors:
All the cases having Cathepsin B score 5-6 were High grade gliomas while majority of cases having Cathepsin B score 0-4 were Low grade gliomas (64.58%) and rest 35.42% were High grade gliomas. Association of Cat B score and grade of gliomas was found to be statistically significant (p<0.001) [Table 1].Majority of patients having Ki67 score 1 were low grade (68.67%) while proportion of patients of high grade glioma were higher as compared to low grade having Ki67 score 2 (60.00% vs. 40.00%) and Ki67 score 3 (97.62% vs. 2.38%). Association of Ki67 score and grade of gliomas was also found to be statistically significant (p<0.001). Majority of patients (70) having weak positive CatB (score 0-4) also had low Ki67 (score1) while majority of patients (23) having strong positive CatB (5-6) had high Ki67 (score 3).This association was found to be statistically significant (p<0.001) [Table 2].

Table 1:- Association of Cat B Score and Grade of Glioma.

Cat B Score	**Low Grade Glioma(n=62)**		**High Grade Glioma(n=73)**		**Total**	
	No.	**%**	**No.**	**%**	**No.**	**%**
CatB score 0-4	62	64.58	34	35.42	96	71.11
CatB score 5-6	0	0.00	39	100.00	39	28.89
Total	62	45.93	73	54.07	135	100.00
			χ^2=46.580(df=1); p<0.001 (Sig)			

Table 2:- Association of Ki67 score and Cathepsin B Score.

Ki67 Score	**CatB 0-4**		**CatB 5-6**		**Total**	
	No.	**%**	**No.**	**%**	**No.**	**%**
Ki67 Score 1	70	84.33	13	15.67	83	61.48
Ki67 Score 2	7	70.00	3	30.00	10	7.41
Ki67 Score 3	19	45.24	23	54.76	42	31.11
Total	96	71.11	39	28.89	135	100.00
			χ^2=20.760(df=2); p<0.001 (Sig)			

Prognostic Relevance of Histological Parameters:
Out of 135 cases enrolled in the study 27 (20.00%) were lost to follow up, 9 (6.67%) patients expired due to post-op complications, 46 (34.07%) expired due to Glioma and only 53 (39.26%) patients survived during the follow up period. The patients who were lost to follow up and the patients who died due to post-op complications were excluded from the survival studies and the data of 99 patients were included in the survival studies. Overall survival time was 13.54±1.19 months among study population. Mean survival of Low grade of glioma patients (20.87±1.41 months) was significantly higher as compared to High grade of glioma patients (6.94±1.00 months). This difference was found to be statistically significant (p=0.001).

Prognostic Relevance of Cathepsin B and Ki-67:
Mean survival of patients with CatB score 0-4 (16.18±1.40 months) was significantly higher as compared to patients with CatB score 5-6 (6.50±1.38 months). This difference was found to be statistically significant (p<0.001). [Table 3].Mean survival of Ki67 score 1 patients (18.08±1.41 months) was significantly higher as compared to patients with Ki67 score 2 (7.50±2.42 months) and Ki67 score 3 (5.76±0.87 months). This difference was found to be statistically significant (p<0.001) [Table 4].

Table 3:- Comparison of Survival among of Low & High Grade Glioma with different Cat B Score.

CatB Score	**Total N**	**No. of Mortalities**	**%**	**Mean survival time±SE**

CatB score 0-4	70	24	34.29	16.18±1.40
CatB score 5-6	29	22	75.86	6.50±1.38
Overall	99	46	46.46	13.54±1.19

Log Rank (Mantel Cox)$\chi 2=18.310$; $p<0.001$

Table 4:- Survival Analysis for different Ki67 scores.

Ki67 Score	**Total N**	**No. of Mortalities**	**%**	**Mean survival time±SE**
Ki67 score 1	61	17	27.87	18.08±1.41
Ki67 score 2	7	5	71.43	7.50±2.42
Ki67 score 3	31	24	77.42	5.76±0.87
Overall	99	46	46.46	13.54±1.19

Log Rank (Mantel Cox)$\chi 2=20.627$; $p<0.001$

Discussion:-

Gliomas are the most common form of brain tumors, contributing to more than half of the incidence of brain tumors. Despite recent advances in imaging, surgical resection techniques and the development of novel adjuvant therapies, the long-term survival of patients suffering from malignant gliomas remains low. It is presumed that the processes of disordered adhesion, motility, and proteolysis are involved in glioma tumor invasion. The present study correlates the expression of Cat B with the increasing grade of glioma hence providing information about its use as a future prognostic marker; also the inhibitors of Cat B could be used as a potent strategy for treating cancer as its upregulation is seen in malignancies. In this study we have shown that Cat B is expressed in glial tumor cells. Significantly more cases with high Cat B IHC score in tumor were observed in high grade gliomas as compared to low grade gliomas. Cat B staining was also observed around the areas of endothelial cell proliferation depicting its role in the process of angiogenesis [Fig 4]. In our study we also found heterogeneity in Cat B staining intensity and distribution in cases of Glioblastoma in which staining intensity ranged from 10-80%. Another observation is that the expression of Cat B was higher in the invading edge of [Fig 5], at the interface between normal brain and tumor and between necrosis and tumor [Fig 6]. This is one of the few clinical study on prognostic impact of Cat B in tumors of the CNS and shows that the survival time is significantly longer in patients with low Cathepsin B immunostaining score, as compared with patients with strong staining.These observations were in concordance with the study done by **Mikkelsen et al. (1995)[28]** who found that glioblastoma multiforme showed highest (3+ score) Cat B score in comparison with anaplastic astrocytoma and normal brain. In low grade astrocytoma they found that majority of cases (68.8%) did not show staining for cat B. Moreover, they reported a heterogeneity in the staining intensity and its regional distribution, with the proliferative tumor margin staining more intensely than the tumor core which is also a concurrent finding in our study.Similar results were found in the study by **Tadej Strojnik et al (1999) [29]**who found that Cat B is expressed in glial tumor cells, proliferative endothelial cells, and macrophages near vessels adjacent to necrotic areas and high Cat B score in tumor and endothelial cells were observed in malignant compared with benign tumors. They also found that patients having higher Cat B score had significantly shorter survival than patients with lower scores. These results were similar to the survival analysis of our study. With the best of our knowledge till date apart from our study this is the only research article that had shown prognostic impact of Cat B immunostaining on tumors of central nervous system. Other studies that studied Cat B expression in brain tumors (**Rempel et al. (1994)[18]**, **Tang JJ et al. (2006)[30], Sivaparvathi et al.(1995)[31]**)showed the similar results. There is, thus, general agreement that brain tumor progression is associated with increased expression of Cat B in tumor cells. A similar association has been observed for higher score of Cat B in tumor cells of lung [32], colon carcinoma [33] pancreas [34] and prostate [35].

In our study the majority of cases with Ki-67 score were low grade , while cases with Ki-67 score 3 were mostly high grade and Ki-67 score 2 shows an overlap of cases with majority being of high grade glioma. Thus, Ki-67/MIB-1 is useful for differentiating between high and low-grade gliomas, but the overlap of Ki-67 score 2 is a main limitation of this immunostaining. On survival analysis we found that the survival time decreased significantly for patients with Ki67 score 1 to score 3. This shows that with increasing Ki-67 score the survival of patients decreases and that there is a negative correlation between Ki-67 score and survival. However our study also proves that survival not only depends on Ki67 score but also on the histological grade. Hence Ki67 alone cannot be used as a single prognostic marker. Similar resuts were obtained in the study done by **David W Ellison et al.(1995)[36]**.

Fig 1:- Cathepsin B weak expression (1+) in glial tumor cells.

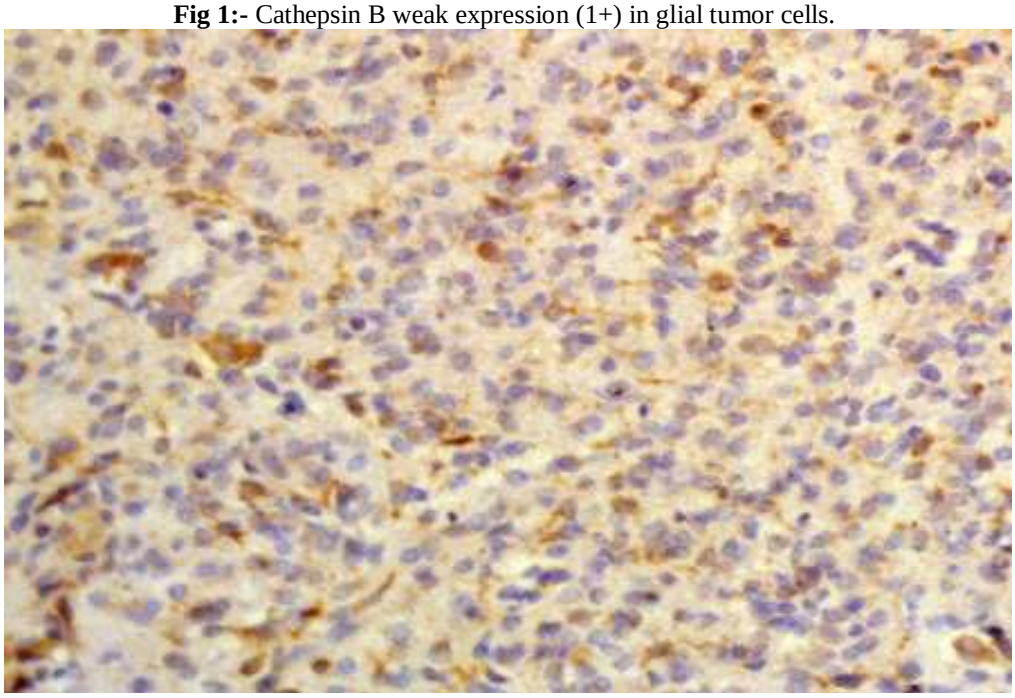


Fig 2:- Cathepsin B medium expression (2+) in tumor cells.

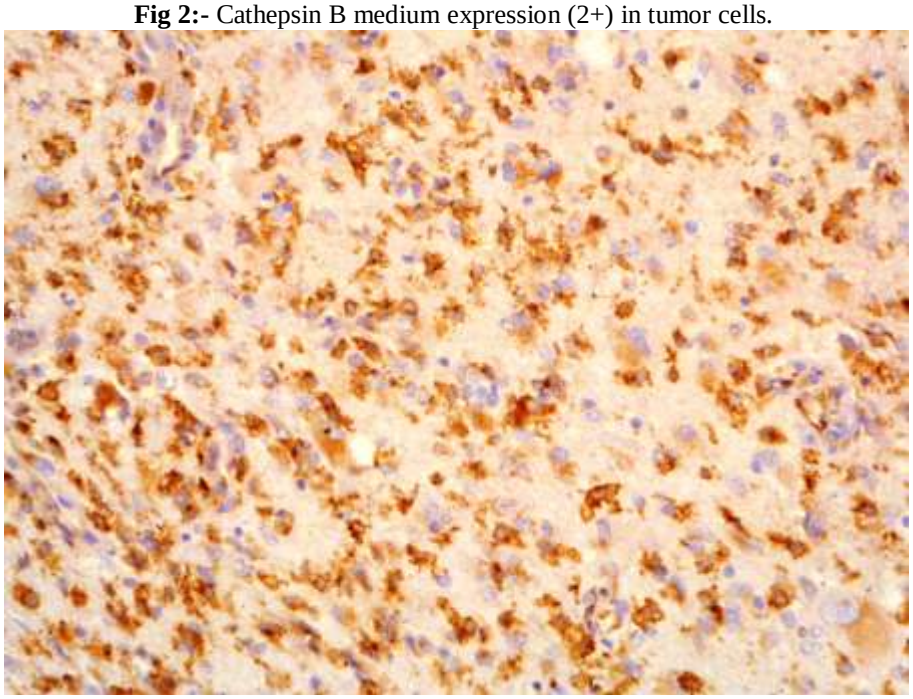


Fig 3:- Cathepsin B strong (3+) expression in tumor cells.

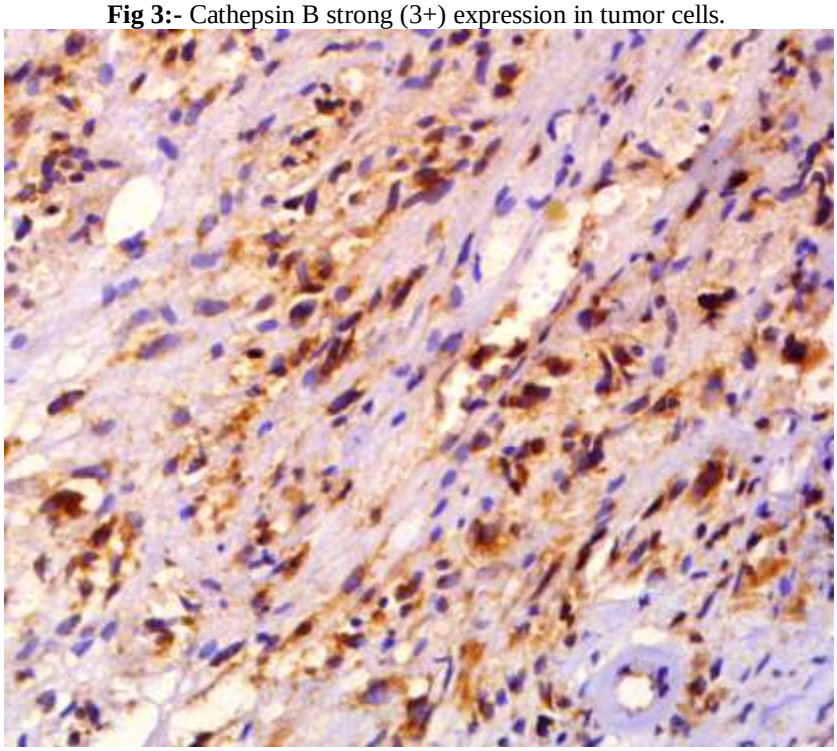


Fig 4:- Cathepsin B strong (3+) expression around cells near the vessel wall.

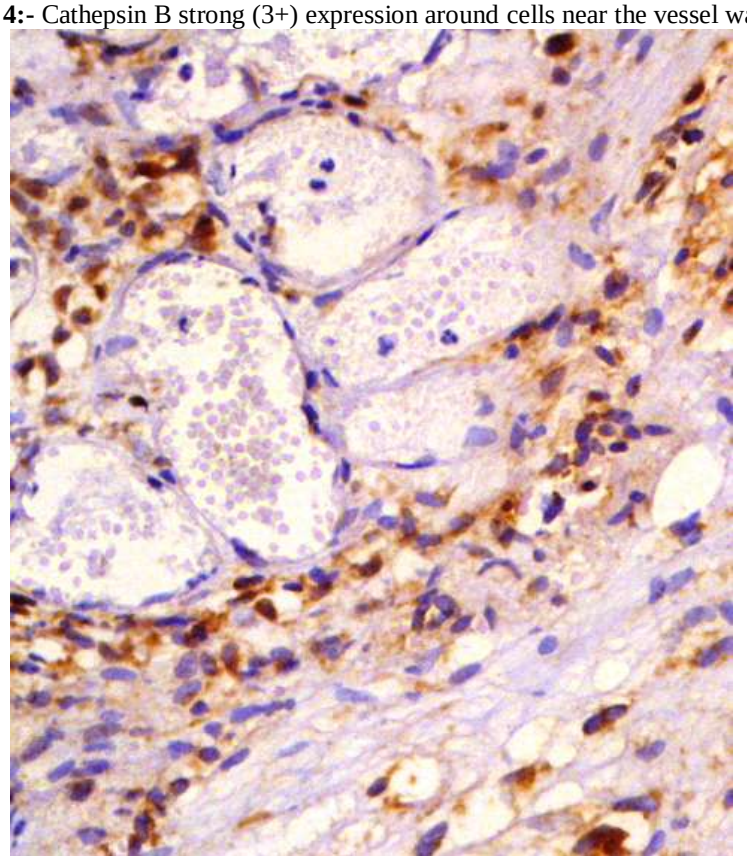


Fig 5:- Cathepsin B strong expression (3+) at the invading edge of tumor.

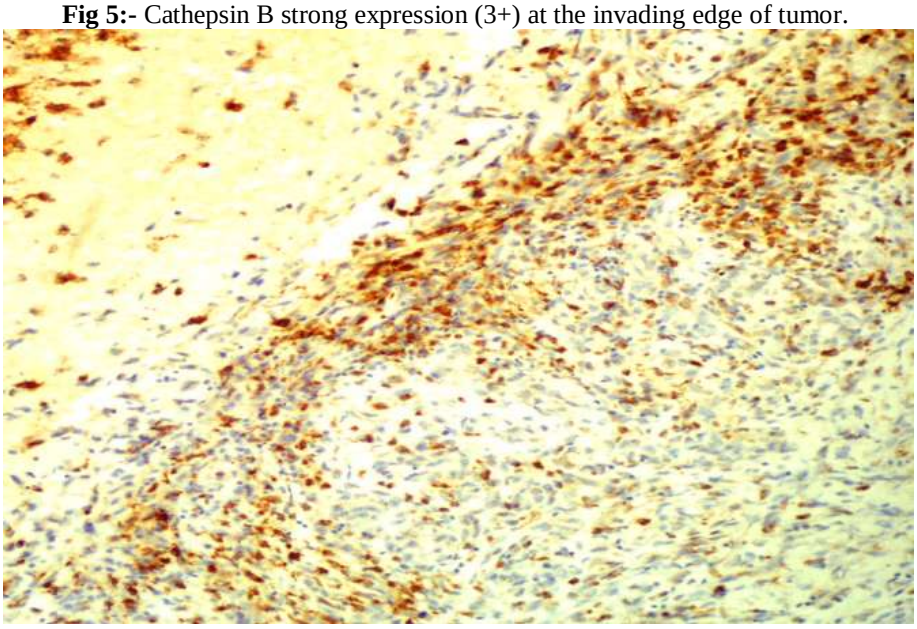
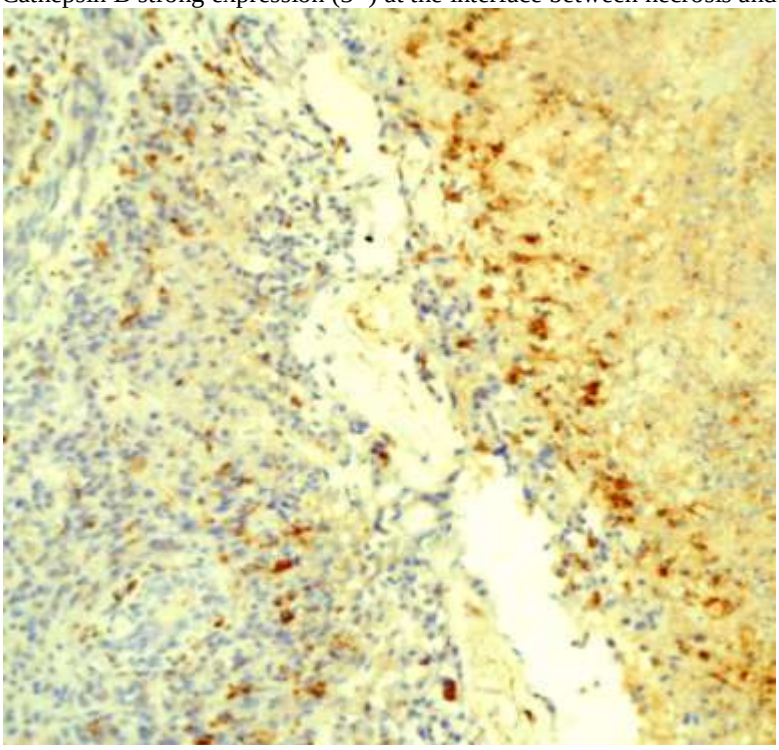


Fig 6:- Cathepsin B strong expression (3+) at the interface between necrosis and tumor.



Conclusion:-

We have demonstrated that Cathepsin B is localized in tumor cells and in endothelial cells of primary tumors of the CNS. The immunostaining of Cathepsin B correlated with high histological score and was significantly associated with poor survival. The level of expression of Cathepsin B in tumor cells is a strong prognostic marker for primary tumors of the CNS. Intense immunostaining of Cat B in endothelial cells may be used to predict the survival of glioma patients and, in addition, it indicates the involvement of Cat B in tumor-associated angiogenesis. These results suggest that Cathepsin B positivity increase with increasing grade of glioma which in future may serve as an important cancer target therapy and may improve the survival of patients.

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