

ABSTRACT

Introduction: Glioma, a prevalent primary brain malignancy, involves multiple signaling pathways crucial for its progression. This study aimed to investigate the clinical significance of Notch1 in patients with glioma.

Methods and Material: In this study, 48 histopathologically confirmed astrocytoma tumors were comprehensively incorporated for the assessment of Notch1 gene expression through Real-time PCR. Following $\Delta\Delta CT$ computation, Notch1 expression was systematically assessed in correlation with clinicopathological parameters, progression-free survival (PFS), and overall survival (OS) in glioma patients using SPSS software. Significance was determined at P values ≤ 0.05 .

Results: The study found abnormal Notch1 expression in 90% of patients, among 70% patients showed up-regulated Notch-1. The up-regulated Notch1 demonstrated significant positive correlation with higher KPS (Karnofsky Performance Status) score ($P = .006$) and trends towards significance with grade of tumors ($P = 0.07$). Kaplan-Meier univariate survival analysis for 24 months PFS and OS demonstrated that a part from high KPS score (PFS: $P = <.001$, OS: $P = <.001$) and patients with up-regulated Notch1 had significantly increase PFS ($P = .009$) and better OS ($P = <.001$) compared with patients with down-regulated Notch1, indicating favorable prognosticator. Also, grade 4 IDH wild-type showed inversed correlation with worse OS ($P = .005$), however, patients who had tumors in frontal lobes, showed significantly better OS than their respective counterparts. In multivariate

survival analysis, only low KPS score emerged as significant independent parameter for predicting shorter PFS and poorer OS. Including Notch1 other parameters lost its significance.

Conclusion: Our study revealed up-regulated Notch1 in low-grade gliomas, correlating with extended PFS and OS. Consequently, we conclude that high Notch1 expression serves as a positive prognostic indicator for glioma patients, however, further studies are needed for validation in larger sample size.

Keywords: Notch1, Glioma, Up-regulation, PFS, OS, Multivariate analysis

INTRODUCTION

Gliomas, the predominant primary malignant brain tumors, exhibit extensive infiltration, significant morbidity, and a high relapse rate, contributing to an unfavorable prognosis. World Health Organization's classification (2021) incorporates molecular biomarkers such as IDH gene mutation, ATRX mutation, p53 mutation, 1p/19q co-deletion, among others.^[1] The global occurrence of primary malignant brain and Central Nervous System (CNS) tumors is 3.5 per 100,000 individuals, and despite notable advancements in standard treatment, the 5-year survival rate is around 5%.^[2] Glioma tumors, being highly heterogeneous and characterized by invasive proliferation, present challenges in therapeutic interventions. This complexity may stem from an incomplete comprehension of gene expression patterns crucial to glioma development.^[3] Thus, unravelling molecular pathways and identifying specific markers, alongside histological observations and understanding the tumor microenvironment, is imperative for enhancing diagnostic precision, refining therapeutic strategies, and optimizing patient prognosis. Multiple signaling pathways are deregulated in various astrocytic tumors.^[4] The Notch signalling pathway is essential for cell fate determination, development, and tissue homeostasis, and remains active in adult brains despite being traditionally seen as a developmental pathway.^[5] Aberrantly activation of Notch signaling is implicated in tumor initiation, progression, angiogenesis, and therapy resistance. Notch1 can behaves as an oncogene or tumor suppressor gene depending on the biological setting. Dysregulation of

Notch1 signaling is linked to various cancers, including glioma, breast cancer, and leukemia, among others.^[6] Hence, this research aims to examine the expression of the Notch1 gene in various grades of astrocytic glioma tumors and establish connections between its expression, clinicopathological factors, and the outcomes of glioma patients.

2. MATERIAL AND METHOD

2.1 Patients

This retrospective study included 48 adult patients with astrocytic glioma tumors treated at the Gujarat Cancer & Research Institute (GCRI) between July 2016 and February 2022. The Institute's Ethics Committee approved, and all participants provided written consent. Demographic data retrieved from the institute's Medical Record Department (Table 1) revealed a median age of 40 years (range: 18-69 years). Notably, 65% of tumors were situated in the frontal lobe. The median KPS score cut-off was 65, with 52% of patients scoring <65 KPS score. Out of 48 patients, 50% (24/50) patients had IDH mutant glioma tumors. Based on 5S WHO (2021) CNS classification [7], Louise 2021), 48%, 21%, and 4% patients showed grade 2, 3 and 4 IDH mutant astrocytoma tumors, whereas, 23% patients were diagnosed as glioblastoma (GBM) with histologically grade 4 and IDH wild-type tumors. Surgery served as the primary treatment for all patients, followed by adjuvant therapy in the form of radiotherapy and/or chemotherapy. PFS and OS analysis was performed in 48 patients with available follow-up data across a minimum of 24 months. Over the 24-months observation period, 25% (12/48) patients experienced disease relapse, while 37% (18/48) died of the condition after surgery (Table 1).

Table 1. Patient and Tumors Characteristics

Characteristic	N = 48	%
Age (Range: 18-69 years; Median 40 years)		
≤ 40 years	24	50
> 40 years	24	50
Gender		
Female	18	38
Male	30	62
Location of tumors		
Frontal	31	65
Parietal	11	23
Temporal	05	10
Occipital	01	02
KPS* Score (Range: 10-95, Median: 65)		
High >65	23	48
Low ≤65	25	52
Grades of tumors		
2	23	48
3	10	21
4 (IDH mutant)	04	08
4 (GBM)	11	23
Treatment		
Surgery (S) or surgery followed by	23	48
S + Radiotherapy (RT)	04	08
S + Chemotherapy (CT)	04	08
S + RT + CT	17	36

Progression-free survival (n=37)		
GC Well	35	95
Recurrence	02	05
Overall survival (n=37)		
Alive	29	78
Died	08	22
<i>IDH1/2</i> Mutations		
Absent	24	50
Present	24	50

* KPS (Karnofsky Performance Status)

FFPE blocks from the same patients were employed for concurrent extraction of DNA and RNA using the QIAamp DNA FFPE Tissue Kit (Qiagen) and High Pure FFPE RNA Isolation Kit (Roche Life Science). The DNA extraction focused on detecting IDH1/2 mutations, while RNA extraction was carried out for RT-PCR analysis of Notch1 gene expression. Subsequently, the Qubit Fluorometer (Invitrogen, USA) was employed to measure DNA and RNA concentrations, and DNA integrity was assessed via electrophoresis on agarose gel.

2.2 ARMS RT-PCR for IDH1/2 mutation detection

IDH1/2 gene mutations were detected using ARMS PCR with the theascreen IDH1/2 RGQ PCR kit as described previously.^[8]

2.3 Notch1 gene expression using real-time PCR

For Notch1 gene expression detection, cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit with 1 µg RNA.^[9] Rotor-Gene Q 5-plex HRM instrument (Qiagen) was utilized for relative quantification. Amplification involved QuantiTect SYBR Green RT-PCR Kit, with 0.4 µl Notch1 forward primers

“TGAATGGCGGGAAGTGTGAAG” and reverse primers “GGTTGGGGTCCTGGCATCG”, 18s RNA forward primers “GGAGTATGGTTGCAAAGCTGA” and reverse primers “ATCTGTCAATCCTGTCCGTGT”, and 10 µl SYBR Green RT-PCR Master Mix in a total 20 µl reaction volume. The protocol included an initial denaturation step at 95 °C for 15 min, followed by 45 cycles at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The 18s RNA gene served as a housekeeping gene, and the commercially available normal brain (Takara Bio Inc. US) served as a calibrator. Relative quantification employed the $2^{-\Delta\Delta C_t}$ method,^[10] presenting data as a fold change of gene expression. In this study, normal Notch1 gene expression fell within a fold change range of 0.5-2, while a fold change exceeding 2 was deemed indicative of up-regulated Notch1 expression.^[11]

2.3 Statistical Analysis:

Statistical analyses conducted in SPSS version 20.0 included a two-tailed chi-square (χ^2) test for parameter relationships. Spearman's correlation coefficient (r) assessed correlations with Notch1 gene expression. Kaplan-Meier survival analysis evaluated PFS and OS. Multivariate survival analysis utilized the Cox Regression Step-wise Forward LR model, with significance set at $P \leq 0.05$.

3.0 RESULT

3.1 Distribution of Notch1 Gene Expression in Glioma Tumor Patients

Among 48 astrocytoma patients, 10% (05/48) exhibited normal Notch1 expression (fold change 0.5-2), while abnormal expression was seen in 90% (43/48) [Figure 1].

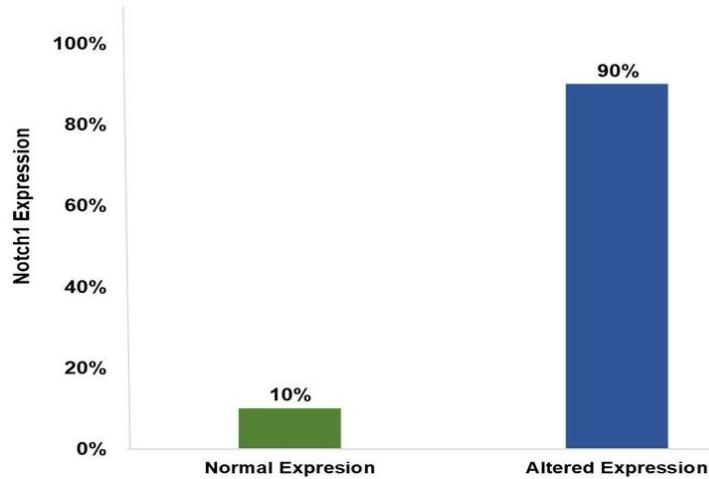


Figure 1. Notch1 gene expression in glioma patients

Within the latter group, 30% (13/43) displayed down-regulation, and 70% (30/43) exhibited up-regulated Notch1 gene expression [Figure 2].

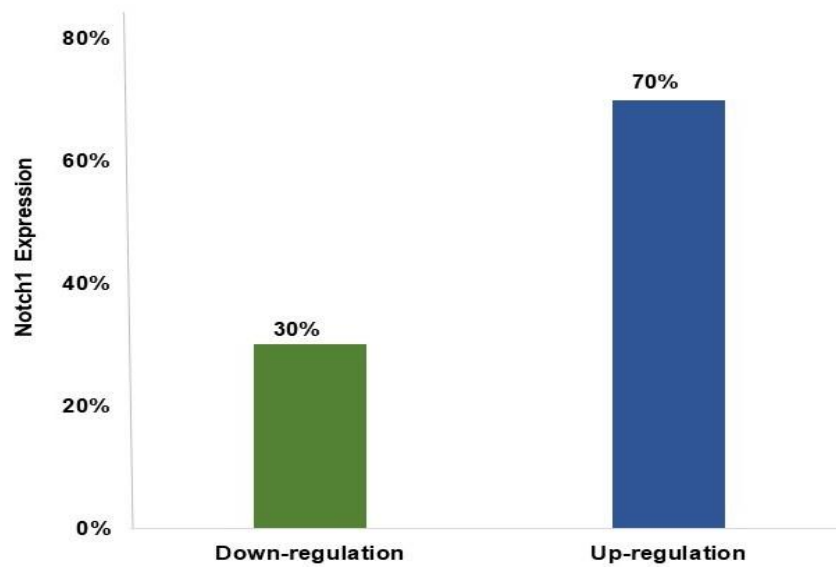


Figure 2. Altered expression of Notch1 in glioma patients

3.2 Associating Notch1 Gene Expression with Clinical Features in Glioma Tumors

Notch1 gene expression correlation with clinicopathological parameters is detailed in Table 2, excluding patients with normal Notch1 expression.

Table 2: Correlation between clinicopathological parameters and altered Notch1 gene

	N	Notch1 gene expression		χ^2	r	P-value
		Down-regulation N = 13	Up-regulation N = 30			
KPS Score						
High >65	20	02 (10)	18 (90)	7.25	-0.41	0.006
Low ≤65	23	11 (48)	12 (52)			
Grade of Tumors						
2	20	04(20)	16 (80)	3.86	-0.27	0.07
3	09	02(22)	07 (78)			
4	14	07(50)	07 (50)			

A significantly inverse correlation emerged with KPS score. In patients with high KPS scores, Notch1 gene up-regulation was notably higher (90%) compared to those with ≤65 KPS score (52%) ($\chi^2 = 7.25$, $r = -0.411$, $P = .006$) (Table 2 Figure 3).

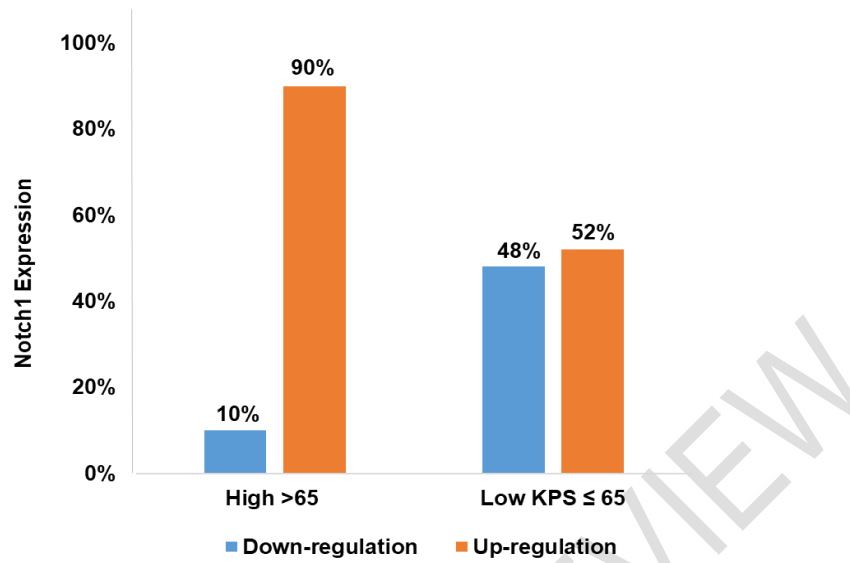


Figure 3. Correlation between Notch1 gene expression and KPS score

Beyond KPS, a trend toward significance was observed between Notch1 gene expression and glioma tumor grade. Up-regulation incidence significantly decreased with higher tumor grades (Table 2). Up-regulation of Notch1 gene was noted in 80% of grade 2 glioma tumors, followed by 78% and 50% in grade 3 and 4 glioma tumors, respectively ($\chi^2 = 3.86$, $r = -0.271$, $P = .07$) (Figure 4).

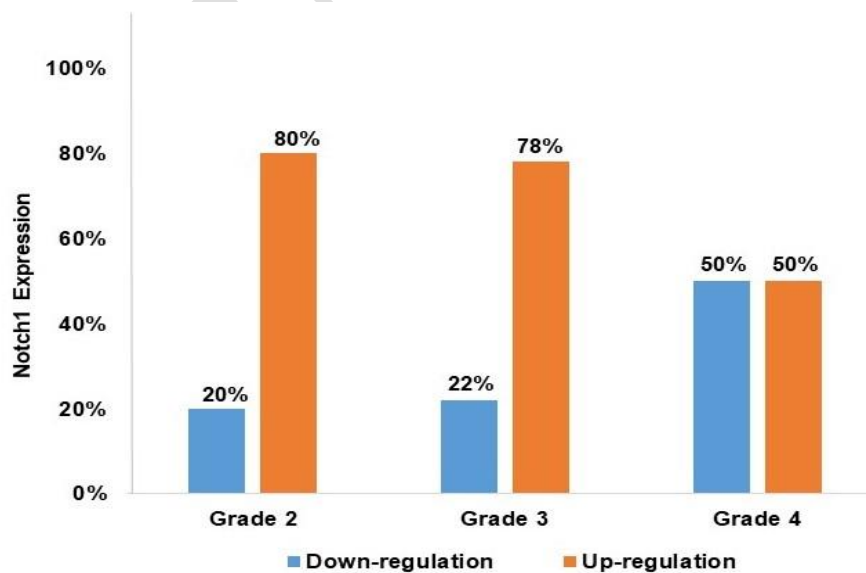


Figure 4. Notch1 expression association with grade of glioma tumors

3.3 Survival analysis

To assess the relevance of Notch1 gene expression to disease outcome, univariate and multivariate survival analysis performed using the Kaplan-Meier survival analysis and Cox Proportion Hazard Regression Step-wise Model for 24 months PFS and OS.

3.4 Exploring PFS and OS through Univariate Kaplan-Meier Survival Analysis

Progression-free survival

The Kaplan-Meier Survival curve for PFS indicated that patients with up-regulated Notch1 expression had significantly lower incidence (17%) of relapse within 24 months compared with down-regulated Notch1 expression (54%) (Log rank = 9.389, $P = .009$, Figure 5a). Similarly, patients with high KPS score (>60) was found to be significantly associated with longer PFS (Log rank = 13.843, $P = <.001$, Figure 5b). Also, the relapse incidence was significantly higher (50%) in patients with grade 4 IDH mutant tumors compared to grade 3 (30%) and grade 2 tumors (9%) (Log rank = 8.300, $P = .04$, Figure 5c).

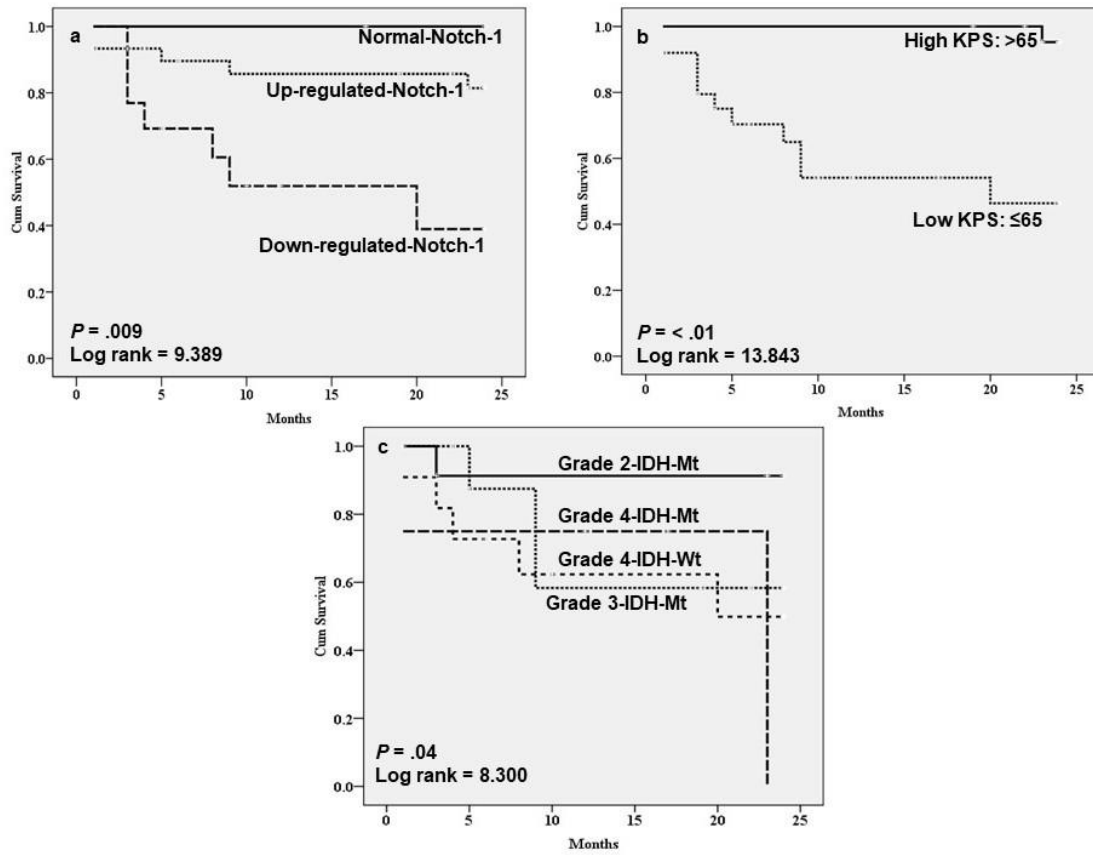


Figure 5. Kaplan-Meier Survival curves for PFS. **a.** Patients with different Notch1 gene expression **b.** Patients with KPS score **c.** Patients with different grade of tumors.

UNDER REVIEW

Overall Survival

In the univariate Kaplan-Meier survival analysis for OS, the incidence of death was significantly lower (20%) in patients whose tumors showed up-regulated Notch1 expression compared with patients with down-regulated Notch1 expression (92%) (Figure 6a), this indicates Notch1 act as a good prognosticator with better 24 OS. Similar result we noted for high KPS score (>60 KPS) that showed longer OS as compared with lower KPS score (Figure 5b). Also, patients with grade 4 astrocytoma with IDH wild-type tumors showed high incidence (64%) of death compared with patients with IDH mutant with either grade 3 (50%) or grade 2 (13%) tumors (Figure 5c). Interestingly, tumor location was found to be significantly associated with disease outcome. Patients whose tumors were located in frontal lobe had significantly better OS compared with patients with either in parietal, occipital or temporal lobes (Figure 5d). With age and gender, no such significant association with PFS was detected.

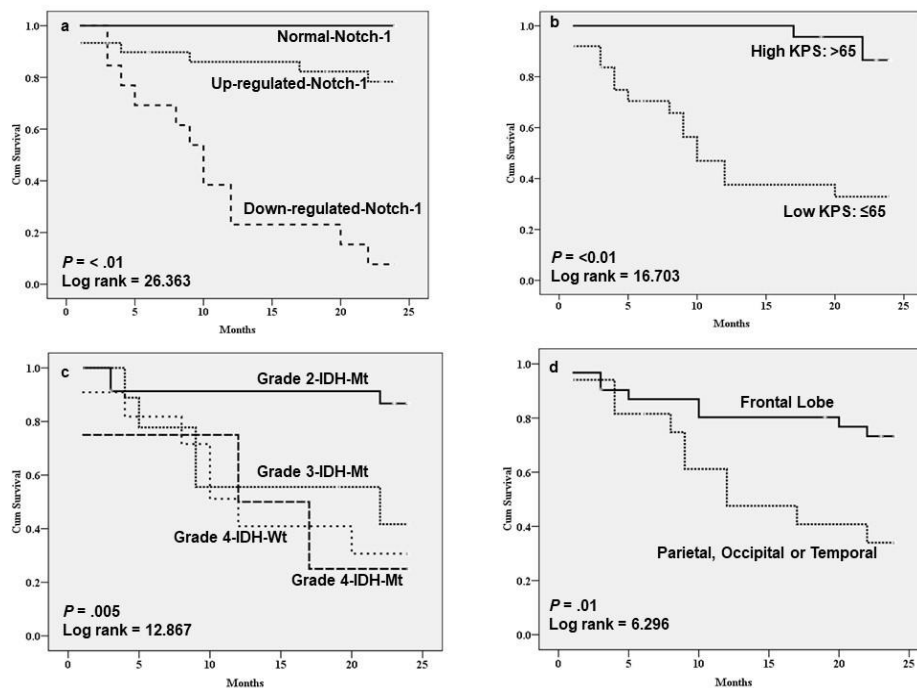


Figure 6. Kaplan-Meier Survival curves for OS. **a.** Patients with different Notch1 gene expression, **b.** Patients with KPS score, **c.** Patients with different grade of tumor **d.** Location of tumors

Multivariate survival analysis using Cox Proportional Hazard Step-wise Regression

Model for PFS and OS:

Multivariate survival analysis for PFS and OS demonstrated that only KPS score emerged as significant independent parameter at step 1 (PFS; HR = 17.911, 95% CI = 2.270-141.315, $P = .006$) (OS; HR = 8.78, 95% CI = 2.510-30.759, $P = .001$), whereas, Notch1 and grade of tumors failed to predict either PFS or OS of glioma patients. Using “enter” method of multivariate survival analysis, significance of Notch1 for PFS and OS was evaluated after adjusting for potential co-factors such as KPS score and grade of tumors. However, Notch1 did not reach at the statistically significant for independently predicting either PFS or OS, probably due to less sample size.

Table 3: Multivariate survival analysis for PFS and OS using Cox Proportion Hazard Step-wise Forward LR Method

	Step	HR*	95% CI [@]		P-value
Progression-free survival			Low	High	
KPS Score	1	17.91	2.270	141.31	0.006
Overall survival					
KPS Score	1	8.78	2.510	30.759	0.001

*Hazard ratio, [@] Confidential Interval

4. DISCUSSION:

In this study, we assessed Notch1 gene expression in glioma patients and explored its impact on disease outcomes. Our findings suggest that up-regulated Notch1 may serve as a positive prognostic indicator for glioma patients. Notch1 fold change expression was correlated with clinicopathological parameters and 24 months PFS and OS.

4.1 Up-regulation of Notch1 in glioma patients

In the overall assessment, 90% of glioma patients exhibited altered Notch1 gene expression, comprising 70% up-regulation and 30% down-regulation. A similar trend in Notch1 gene alteration was reported by Narayanappa et al. (2016) in GBM tumors; however, they did not observe down-regulation of this gene. It is important to note that, in addition to Notch1 gene, they found down-regulation of Notch 2, 3, and 4. This discrepancy may be attributed to their specific focus on GBM patients, emphasizing exclusive Notch1 up-regulation.

4.2 KPS score, tumor grade and location of tumors are significant prognosticators for glioma patients

KPS score proves to be a significant prognostic factor for glioma patients. In our study, a significant inverse association between Notch1 expression and KPS score was observed. Corroborate to our findings, Li et al (2018)^[12] have shown an inverse association between Notch1 expression and KPS score in a study of 33 glioma patients. Patients with a high KPS score at diagnosis exhibited significantly increased Notch1 gene expression, indicative of up-regulation. In contrast, Jiang et al. (2011)^[13] reported a notable rise in Notch1 expression among patients with a low KPS score. Additionally, Li et al. (2011)^[14] presented conflicting results, associating heightened Notch1 protein expression notably with a KPS score below 80 rather than exceeding 80. Reduced KPS scores often correlate with diminished survival rates in severe illnesses. Our study established a correlation between elevated Notch1 gene expression and higher KPS scores, suggesting heightened Notch1 expression as a potential predictive factor for enhanced OS. This signifies its potential as a favorable prognostic indicator for glioma patients.

Our investigation revealed an increased prevalence of up-regulated Notch1 gene expression in low- grade gliomas compared to high-grade tumors. Notably, with the advancement of tumor grade, the incidence of up-regulated Notch1 gene expression decreased. This aligns

with Purow et al.'s (2005)^[15] findings, demonstrating elevated Notch1 mRNA expression in low-grade gliomas compared to GBM. Conversely, Cheung H et al. (2006)^[16] observed abnormal Notch1 expression across all glioma grades, absent in a subset of grade 4 gliomas. While some studies reported Notch1 overexpression in GBMs^[17], Alternative theories suggest that the activation of Notch induces apoptosis in neural progenitor cells via a pathway dependent on p53.^[18] The reason for reduced Notch1 expression in highly malignant GBM tumors compared to other primary human gliomas remains unclear. The findings indicate the involvement of Notch1 in sustaining the undifferentiated state of glioma cells, and its inhibition facilitates the maturation of these cells into a less aggressive phenotype.^[19]

Our findings underscore the crucial prognostic significance of Notch1 and KPS score in predicting longer PFS and better OS for glioma patients. The Kaplan-Meier survival curves for both, PFS and OS indicated that patients with higher Notch1 expression and high KPS score had significantly longer PFS and better OS than their respective counterparts. Thus, Notch1 and KPS score remained positive prognosticators for glioma patients. Additionally, particularly notable is patients with grade 4 IDH mutant gliomas, they found to be associated with significantly higher incidence of relapse within 24 months. Grade 4 glioma tumors are most aggressive glioma tumors phenotypically and molecularly also, irrespective of IDH status. They are more heterogeneous and molecularly complex. Therefore, patients having grade 4 tumors showed higher incidence of relapse and worse prognosis.

This study identifies key prognostic indicators influencing OS, including KPS score, grade of tumors, tumor locations and Notch1 expression. A significantly elevated incidence of death was associated with a high KPS score ($p = <0.001$). Consistent with multiple studies, lower KPS scores have been consistently linked to poorer outcomes in glioma patients. Schröder et al. (2022),^[20] reported a significantly shorter median OS in GBM patients with KPS scores of 70 or less compared to those with scores above 70. Further, tumor location proves to be a critical determinant in astrocytic glioma patients. Our investigation reveals that patients with tumors in

the frontal lobe exhibit a significantly lower incidence of death within 24 months compared to those with tumors in the parietal and occipital lobes ($p = 0.012$). This underscores the tumor heterogeneity across different brain regions. Previous studies, such as the one study by Roux et al. (2019)^[21] have also reported increased survival with periventricular involvement and suggested a potential survival distinction between left- and right-sided tumors. While gliomas in the parietal lobe is generally linked to a poorer prognosis, individual outcomes can vary due to unique patient factors. Even small gliomas in crucial brain regions pose challenges in treatment, potentially resulting in significant neurological deficits that impact both the patient's quality of life and survival.

4.3 Notch1 might act as good prognostic marker for glioma patients

Apart from conventional prognostic parameters, in the present study, we found a significant association between Notch1 gene up-regulated status with improved PFS and OS of glioma patients (Figure 5a and Figure 6a), suggesting that Notch1 up-regulation could serve as a valuable positive prognostic indicator. Patients with Notch1 down-regulation exhibited poorer OS compared to those with up-regulation of the Notch1 gene, in which 44% of patients in our study demonstrated Notch1 up-regulation, correlating with superior 24-month OS compared to patients with down-regulated Notch1. The results of the present study indorsed a tumor suppressive role of Notch1 in glioma tumors. This result was essentially in agreement with Giachino et al. (2015).^[22] They patients found that in glioma, the high expression of specific NOTCH target genes positively correlates with a better prognosis in human glioma tumors. In line of this, in head and neck squamous cell carcinoma, Wirth et al (2018)^[23] have reported that high expression of Notch1 was associated with better OS and disease-free survival. This association attributed to the role of Notch1 signaling in regulating the proliferation, survival, and migration of astrocytoma cells. Studies by Luistro et al. (2009),^[24] and Purow et al. (2005),^[15] have highlighted the involvement of Notch1 signaling in angiogenesis, a critical process for

tumor growth and progression. Additionally, Wang J et al. (2010),^[25] underscored that Notch1 down-regulation is associated with increased resistance to chemotherapy and radiation therapy in astrocytoma, implicating Notch1 signaling in the regulation of genes involved in DNA damage repair and apoptosis. However, in multivariate survival analysis, Notch1 failed to predict PFS or OS due to potential conventional prognosticator, KPS score. Also, the small sample size could be the possible reason for not reaching Notch1 as independent prognostic marker in multivariate analysis.

Further, differences in the outcome of Notch modulation likely relate to the stage of disease progression, crosstalk with other signaling pathways, and intra-tumoral cell heterogeneity, and this inter-tumoral heterogeneity could be one critical factor underlying the observed discrepancies in Notch function (Parmigini et al. (2020)).^[26] Overall, the effects of the Notch signaling heavily rely on the cellular environment and involve intricate crosstalk with other signaling pathways. Therefore, targeting the Notch pathway may intervene in these processes and potentially bring better therapeutic effects for patients with glioma.

However, there are several limitations of the current study including small sample size, single center study which could be a result bias or a possible reason for not to reach Notch1 at statically significant using multivariate survival analysis. Also, due to significance of potential conventional prognostic factors such as grade of tumors and KPS score hinder Notch1 to reach at statistically significant level. Overall future studies including more number of glioma patients are warranted.

5. CONCLUSION:

In conclusion, the study's findings highlight the potential prognostic significance of Notch1 gene in glioma, particularly, its correlation with patient survival and grade of tumors. These results contribute to the understanding of the molecular mechanisms underlying Notch signaling pathway associated with glioma pathogenesis that may have implications for the development of prognostic markers and potential therapeutic strategies. However, further studies are needed

to elucidate the precise molecular mechanisms of the Notch signaling pathway in glioma tumors that will be beneficial for developing novel therapeutic strategies. Also, validation of Notch1 in larger patients' cohort is necessary for targeting Notch1 molecule as targeted therapy for glioma patients.

CONSENT

Written consent were taken from all patients included in this study.

ETHICAL APPROVAL

This study was approved by the Institutional Review Board and Institutional Ethics Review Committee) (IRC Approval No.: IRC/2024/P-20)

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