Co-occurrence of ATRX and IDH Mutations Identify Subgroup of Glioma Patients for Better Survival

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Summary

In gliomas, with IDH mutational status, ATRX loss of protein classified diffuse astrocytic low grade and primary and secondary glioblastoma (GBM). The current study we sought to explore the clinical impact of ATRX and IDH in glioma patients. A total of 47 astrocytoma tumors of glioma were included and loss of ATRX protein expression and IDH1/2 mutations were detected using immunohistochemistry and real-time PCR, respectively. Data was evaluated by SPSS software. Loss of ATRX protein was noted in 46.7% and mutation in IDH1/2 was detected in 42% of glioma tumors. Further, significantly high incidence of loss of ATRX protein was noted in patients with frontal lobe of tumors compared to temporal and parietal locations. ($\chi 2$=10.473, r=+0.482, p=0.003). This is indicating that the incidence of ATRX mutation is significantly varied based on location of the glioma tumors. With marginal statistical significance, we observed a positive correlation between ATRX loss and IDH mutation in astrocytoma lineage tumors ($\chi 2$= 3.59, r = +0.283, p=0.060). In survival analysis, multivariate survival analysis demonstrated that patients whose tumors showed co-occurrence of mutations of ATRX and IDH together have significantly better progression free (HR=0.234, 95% CI=0.085-0.641, p=0.005) and overall survival (HR=0.447, 95% CI=30.224-0.897, p=0.024) compared to patients with either absent of both genes mutations and/or presence of any one mutation. Thus, our results indicated that though glioma is single entity, ATRX behaves biologically different in different location of glioma tumors. Also, co-detection of ATRX and IDH has more clinical impact in predicting for better progression free survival and longer overall survival than analyzing any one marker mutational status.

Keywords: Glioma, ATRX, IDH, Co-detection, Multivariate analysis

Introduction

Gliomas are the most frequent primary malignant brain tumors, characterized by complex biological behavior with a heterogeneous molecular background. Fortunately, for this devastating neoplasm, more and more research on the intra-tumoral heterogeneity has been specified over the years. Currently, mutational classification of brain tumors have led to a more targeted management of gliomas tailored to individual patients' mutations[1] , however, the molecular picture is still not clear and therefore, till date the targeted therapy didn't reach to the clinic for glioma patients management!

The revised version of 2016 updated WHO classification of CNS tumors has had a particular impact on the diffuse low and high grade astrocytoma tumors. Accordingly, the three molecular markers utilized for diffuse astrocytomas are absence/presence of isocitrated dehydrogenase (IDH) mutations, TP53 mutation, and α thalassemia/mental retardation syndrome X-linked (ATRX) loss.[2] ATRX, is a transcriptional regulator, originally it was discovered in patients with the X-linked mental retardation syndrome (ATRX syndrome).[3] However, since two decades, the significance in cancer is rising. In gliomas, with IDH mutational status, ATRX loss of protein classified diffuse astrocytic low grade and primary and secondary glioblastoma (GBM). Thus, a tight bonding between IDH mutations and ATRX mutations has been noted.[4] They differentiate astrocytic lineage of glioma tumors. For glioma patients, though, IDH is one of the most common only molecular prognostic factor; no novel therapeutic targeted therapy still translated at clinic, based on IDH status. For ATRX also, deciphering a comprehensive role in gliomas is still in its infancy. Therefore, in the current study, we aim to evaluate the clinical impact of ATRX and IDH mutations for glioma patients using immunohistochemistry and real-time PCR, respectively.

Material and Method

Patients

A total 47 untreated histologically confirmed glioma patients with astrocytoma tumors registered at The Gujarat Cancer & Research Institute from January 2017 to January 2020 were enrolled in the current study. The study was approved by the institute's ethics committee board and written consent forms were obtained from all the patients prior to treatment administration. Detailed clinical and pathological history of the patients was obtained from the case files maintained at the Medical Record

Table 1: Patient and Tumor Characteristics

Characteristics	N	%
Total Patients	**47**	
Age		
≤45	26	55.3
>45	21	44.7
Gender		
Female	22	46.8
Male	25	53.2
Tumor Location		
Frontal	23	49.0
Temporal	11	23.4
Parietal	9	19.1
Occipital	4	8.5
Treatment		
Only Surgery	23	48.9
Followed by		
Radiotherapy and/or Chemotherapy	24	51.1
Histological Grade		
Grade II	16	34.0
Grade III	8	17.1
Grade IV	23	48.9
GBM-based on IDH status		
Primary GBM	26	57.8
Secondary GBM	19	42.2
Progression free survival (n=37)		
Norecurrence	16	43.2
Recurrence	21	56.8
Overall survival (n=37)		
Alive	20	54.1
Died	17	45.9

Table 2: IDH1/2 Mutations Detection using Therascreen Mutation Detection Assay

Gene	Mutation	Amino Acid Change
IDH1	c.395G>A	R132H
Codon132	c.394C>T	R132C
	c.394C>A	R132S
	c.394C>G	R132G
	c.394G>T	R132L
	c.394_395CG>GT	R132V
Codon100	c.299G>A	R100Q
IDH2	c.515G>A	R172K
Codon172	c.515G>T	R172M
	c.514A>T	R172W
	c.516G>T	R172S
	c.514A>G	R172G

Table 3: Lab Established ΔCT Cut-Off value for Mutation Detection

Mutations	Lab established Cut-off ΔCT
ΔCTIDH1R132Mut	≤4.25
ΔCTIDH1MutR132H	≤4.40
ΔCTIDH1MutR132C	≤5.80
ΔCTIDH1R100Mut	≤4.22
ΔCTIDH2R172Mut	≤4.00
ΔCTIDH2MutR172K	≤5.70

Department of our institute. The clinico-pathological characteristics of the enrolled patients are enlisted in Table 1.

In the present study, more than 50% of patients were <45 years. All patients underwent for surgery as primary treatment. Fifty-one percent of patients had taken radiotherapy and/or chemotherapy as adjuvant therapy. Out of a total of 47 patients, 37 patients could be followed for a minimum period of 24 months or until their death within that period. Progression-free survival (PFS) and overall survival (OS) was evaluated. Within 24 months, 56.8% (21/37) patients had developed recurrent disease and 45.9% (17/37) of patients died within 24 months. (Table 1)

Also, according to tumor location site, majority of patients had tumors in temporal (49%) and frontal (23%) sites of the brain. According to histological grade of tumors, 34% patients had grade II tumors and 17% patients had grade III tumors and 49% of patients had grade IV astrocytoma tumors. Based on IDH mutational status, Glioblastoma patients were categorized into primary and secondary GBM. In the present study, 57.8% patients had primary GBM, whereas, 42.2% patients had secondary GBM tumors. (Table 1)

Immunohistochemistry

ATRX protein expression was studied using immunohistochemistry described previously (5). Formalin-fixed paraffin embedded tissue blocks retrieved from the tissue repository of our institute's Pathology Department. The blocks were cut into 4 µm sections and mounted on 3-amino propyl triethoxy silane (APES)-coated slides. The staining was performed using HRP/DAB (ABC) Detection IHC kit (Abcam, Cambridge, UK) according to manufacturer's protocol. Briefly, antigen retrieval treatment was given by heating the sections in 10 mM sodium citrate buffer (pH-6.0) in a pressure cooker. Then after, sections were incubated overnight at 4^0 C with the primary monoclonal antibody from Boster Bio; anti-ATRX clone RAD54 with 1:100 dilution in TBS. Similarly, for IDH1 R132H, the primary antibody used was anti-human IDH1 R132H mouse monoclonal antibody DIA clone H09 (Dianova, Germany) at a dilution of 1:100. The stained sections were mounted with DPX and observed under the light microscope. Sections with intense staining for IDH1 R132H were used as positive control, whereas negative control was obtained by omission of primary antibody. IDH R132H using mutation specific clone of DO9 is recommended method for detection of IDH mutational status for brain tumors. Therefore, here for validation of real-time PCR method, we evaluated cytoplasmic staining pattern of IDH1 R132H in more than 10% of patients.

Assessment of ATRX expression

For ATRX, only nuclear staining was considered for evaluation. Loss and retention of nuclear expression was noted for each patient. If nuclear staining was present in >10% of area, then considered retention for ATRX (no loss of expression) (Liu et al 2019).

DNA Extraction

Genomic DNA was extracted from histopathology confirmed astrocytoma FFPE blocks retrieved from histopathology department of our institute. DNA isolation was done using the AuPreP GENbt DNA extraction Kit, according to the manufacturer's instructions. The concentration, purity and quality of the extracted DNA were determined by Qubit 2.0 Fluorometer (Invitrogen, USA) and 0.8% gel agarose electrophoresis, respectively.

Real-time PCR for IDH1/2 mutation detection

IDH1/2 mutations was detected using ARMS PCR using therascreen IDH1/2 RGQ PCR kit following manufacturer's instructions (Qiagen). Qualitative detection of 6 mutations within IDH1 codon 132, one within IDH1 codon 100 (R100Q) and 5 within IDH2 codon 172 was noted (Table 2). PCR was performed using the Rotor-Gene Q 5-plex HRM instrument (Qiagen). Quality control was seen using CT values of controls. With each assay, we run positive, negative and no template control to ensure that acceptable Ct values were met and that the reactions were performed correctly. The PCR condition used was: 95°C Time: 10 min Cycling 40 times 95°C for 15 sec 60°C for 60 sec with an acquisition of FAM™ fluorescence in channel Green: Single. Sample ΔCt values were calculated as the difference between the mutation assay Ct and respective total assay Ct from the same sample. Samples were classified as mutation positive if the ΔCt value was less than or equal to the ΔCt cut-off value of the respective mutation assay. (Table 3)

Statistical Analysis

The data was analyzed statistically using SPSS Inc. version 20 software. The correlation between the loss or retention of ATRX protein with clinicopathological parameters of glioma patients was determined by two-tailed chi square test ($\chi 2$) and spearman's correlation. Survival analysis was performed using Kaplan-Meier survival function and the differences in survival were tested for statistical significance using log-rank statistic. Multivariate survival analysis was performed using Cox forward stepwise proportional hazard regression model. $p \leq 0.05$ was considered to be statistically significant.

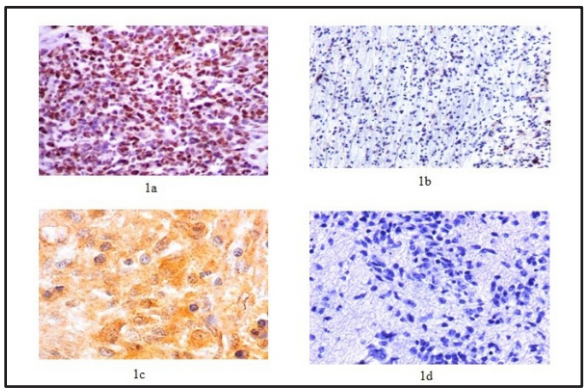

Figure1: Representative immunohistochemical staining of ATRX and IDH1 R132H in glioma patients

Figure 1a: Retention of ATRX in astrocytoma tumors
Figure 1b: Loss of ATRX expression in astrocytoma tumors
Figure 1c: Cytoplasmic expression of IDH1 R132H (clone: HO9) in astrocytoma tumors
Figure 1d: Negative control for IDH1 R132H

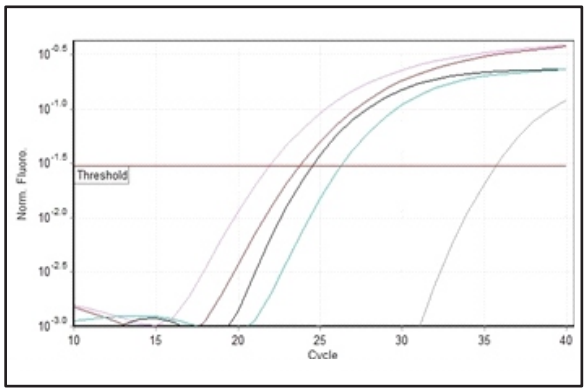

Figure 2: Representative real-time PCR threshold values curve of IDH1/2 mutation of glioma patients

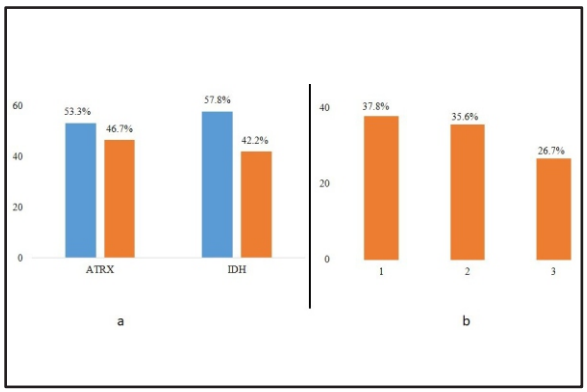

Figure 3: Representative image of incidence of ATRX and IDH mutations in glioma patients

a. ATRX: Retention and loss of expression of ATRX protein
IDH: Absent and present of IDH mutations in glioma tumors
b. Absence of mutations in ATRX and IDH1/2 genes
Mutations present in any one gene (ATRX or IDH)
Mutations are present in ATRX and IDH1/2 genes

Table 4: Correlation between ATRX and Tumor Location

Variables	N	ATRX Expression		p value	r	$\chi 2$
TumorLocation	**45**	**Retention N(%)**	**Loss N(%)**	**0.003**	**-0.482**	**10.473**
Frontal	22	8(36.4)	14(63.6)			
Temporal	10	5(50)	5(50)			
Parietal	9	9(100)	0(0)			
Glioblastoma						
Primary	26	17(65.2)	9(34.6)	0.060	+0.283	3.59
Secondary	19	7(36.8)	12(63.2)			

Table 5: Univariate survival analysis for PFS and OS using Kaplan-Meier Analysis

Univariate survival		PFS			OS		
Parameters		**Patients relapsed**	**p value**	**Log rank**	**Patients died**	**p value**	**Log rank**
	N	**N(%)**			**N(%)**		
Grade of tumors							
Grade II	10	03(30)	NS	-	02(20)	0.063	5.53
Grade III	07	04(57)			01(14)		
Grade IV	20	14(70)			14(70)		
ATRX expression							
Retention	20	16(80)	0.002	9.99	12(60)	0.024	5.10
Loss	17	05(29)			05(29)		
IDH1/2mutations							
Absent	22	03(14)	0.062	3.49	14(64)	0.035	4.44
Present	15	05(33)			03(20)		
ATRX and IDHmutations							
Bothare absent	16	12(75)	0.003	11.75	10(63)	10(63)	6.97
Anyone present	10	08(80)			06(60)		
Bothare present	11	01(09)			01(09)		

Table 6: Multivariate survival analysis using all parameters for PFS and OS

Survival	Step	Parameter	HR	Lower	Upper	p value
PFS	1	ATRXmutation	0.234	0.085	0.641	0.005
OS	1	ATRX & IDH mutations together	0.447	0.224	0.897	0.024

Results

Incidence of ATRX and IDH ½ mutations in glioma patients

Loss of nuclear staining of ATRX indicates the presence of ATRX mutation phenotype in glial tumors. The incidence of loss of ATRX protein in glioma tumors was 46.7%, (21/45) and retention of ATRX was observed in 53.3% (24/45) of tumors (Figure 1). The IDH1/2 mutations using qPCR was detected in 42% (20/47) of glioma tumors (Figure 2). Mutation of either ATRX or IDH1/2 was noted in 36% of patients. We also observed mutations of ATRX and IDH1/2 both together in 27% of patients. (Figure 3)

Relation of ATRX loss with clinicopathological parameters

A significantly high incidence of loss of nuclear ATRX was observed in tumors from frontal lobe of brain compared to tumors from temporal and parietal locations. ($\chi 2$=10.473, r=-0.482, p=0.003). This is indicating that the incidence of ATRX mutation is significantly varied based on location of the glioma tumors. Also, this result indicates different biological behavior of glioma tumors based on sites of brain from where they are located (Table 4).

Similar difference in ATRX protein expression we observed between primary and secondary GBM tumors. The incidence of loss of

ATRX protein was significantly high in patients having presence of IDH mutation (secondary GBM) as compared to patients with absence of IDH mutation (primary GBM), however, we found marginal statistical significance in this correlation ($\chi 2 = 3.59$, r = +0.283, p= 0.060). (Table 4)

Univariate Survival Analysis

Univariate survival analysis for PFS and OS was performed using Kaplan-Meier survival analysis for all clinicopathological parameters and ATRX and IDH mutational status.

Progression free survival

Univariate Kaplan-Meier survival analysis for PFS demonstrated that patients with retention of ATRX protein in their tumors showed significantly high incidence of relapsed in comparison to patients with loss of ATRX expression (p=0.002, df=1, Log rank=9.99). This finding indicates that patients with presence of ATRX mutation had significantly longer PFS compared to their respective counterparts. However, with IDH mutational status, marginal significance was observed with PFS (p=0.06, df=1, Log rank=3.49). In line of this, most striking result we noted was that patients with presence of both mutations in their tumors had significantly low incidence of relapsed in comparison to patients with either any one mutation present or absent of both mutations together in their tumors (p=0.003, df=2, Log rank=11.75). (Table 5)

Overall survival

Univariate Kaplan-Meier survival analysis for OS indicated that patients with retention of ATRX protein in their tumors showed significantly high incidence of death in comparison to patients with loss of ATRX expression (p=0.024, df=1, Log rank=5.10). This finding indicates that patients with presence of ATRX mutation had significantly better OS compared to their respective counterparts. Similar result we noted for IDH mutation status. The incidence of death was significantly high in patients whose tumors showed absent of IDH mutations in comparison to patients with presence of IDH mutations (p=0.035, df=1, Log rank=4.44). Also, we noted significantly low incidence in death rate in those patients whose tumors showed presence of ATRX and IDH mutations together as compared to patients with either any one mutation present or absent of both mutations (p=0.037, df=2, Log rank=6.97). (Table 5)

Multivariate Survival Analysis

Progression-free survival

To assess the dependence of the predictive value of ATRX and IDH on other known prognostic factors (age, gender, location of tumors, histologic grade), a multivariate Cox forward stepwise proportional hazard regression analysis was performed. We observed that for PFS, only presence of ATRX mutation that is loss of ATRX nuclear protein expression entered the equation at step 1. Thus, ATRX remained a significant risk factor for recurrence of disease (HR=0.234, 95% CI=0.085-0.641, p=0.005; Table 6). This indicated that loss of nuclear ATRX protein could serve as an independent prognostic factor for predicting progression-free survival.

Overall survival

Multivariate survival analysis using the Cox forward stepwise proportional hazard regression model demonstrated that for OS, presence of both ATRX and IDH mutations together entered the equation at step one (HR=0.447, 95% CI=30.224-0.897, p=0.024; Table 6). Thus, for glioma patients, overall survival remains better if their tumors showed presence of ATRX and IDH both mutations together.

In addition, we would like to add that in our study, out of 37 patients, only 50% of patients had completed planned treatment. Therefore, PFS and OS analysis based treatment subgroups was not done due to small sample size.

Discussion

Currently, the updated 2016 WHO classification for CNS tumors has incorporated molecular aberrations that might help to resolve the discrepancy between classification and clinical outcome of astrocytic glioma tumors. For glioma patients, IDH mutation is emerged as prognostic and predictive parameter independent of the WHO grade of tumors.[1] However, based on IDH status, till date, no novel therapeutic targeted therapy is translated at clinic. On the contrary, in many cases, WHO grade II or III IDH-wild-type infiltrating astrocytoma patients have worse outcomes than IDH-mutant glioblastomas (grade IV), reflecting that their tumors are likely to behave in a manner similar to IDH-wild-type glioblastoma. This is creating a significant problem in the current grading criteria. Moreover, in addition to IDH mutations, ATRX mutation has just been discovered in gliomas and have been the subject of numerous studies on the classification and prognosis of glioma. Keeping this in mind, in the current study, we evaluated the clinical significance of ATRX alongwith IDH mutations for glioma patients with astrocytic tumors.

Many types of tumor cells, including glioma tumor cells maintain telomere length via telomere activation, while some types of tumors elongate telomere length by telomere independent manner, which is known as "ALT" and this ALT phenotype was significantly correlated with ATRX loss.[8, 9] To

detect ATRX mutation phenotype in glial tumors, loss of nuclear expression of ATRX protein using immunohistochemistry is used.[10] This loss of nuclear protein may occur due to mutations, deletions, or gene fusions and correlates with ALT phenotype. In the current study, loss of ATRX protein expression was observed in 46.7% of glioma tumors indicating presence of ATRX gene mutations. In grade II astrocytoma tumors, the loss of ATRX was noted in 60% and in secondary GBM it was found in 43%. There are many reports demonstrating ATRX mutation or loss in multiple tumors, including low and high grade astrocytomas. This is indicating an imminent "driver" role of ATRX in cancer. Also, Wiestler et al (2013) have found 41% ATRX loss in astrocytoma tumors. However, Jiao et al (2012) described a significantly higher mutation rate of ATRX mutation with 73% anaplastic astrocytomas tumors. This difference of percentage in ATRX mutation and loss of expression, is probably due to the different techniques used.

In the present study, we found statistically significant difference in incidence of loss of ATRX protein with different locations of glioma tumors. We noted that patients with frontal glioma tumors had significantly high incidence of ATRX protein loss compared to patients with tumor in temporal followed by parietal tumors. Similar to our findings, Ebrahini et al (2016) have observed significant loss of ATRX in frontal lobe tumors. This is further corroborated by the relatively high frequency of seizures in the frontal lobe attributed to IDH mutant gliomas.[12] In addition, Debajyoti et al (2018) also noted ATRX loss of expression most frequently seen in frontal region of the brain. This is indicating that the incidence of ATRX mutation is varied based on location of the glioma tumors. Thus, ours and others findings demonstrated that though glioma is a single entity, the biological behavior may differed based on location of primary sites.

In addition, we also noted a significant difference in incidence of loss of ATRX protein between primary and secondary GBM. With marginal significance, a high incidence of loss of ATRX protein in patients having secondary GBM than patients with primary GBM. This is indicating that mutation of ATRX is more frequent in secondary GBM compared to primary GBM. Similarly, Ebrahimi et al (2016) also noted frequent loss of ATRX expression in secondary GBM compared to primary GBM. In addition, Haase et al (2018) have noted that expression of ATRX is varied with respect to GBM. According to the mutator hypothesis of oncogenesis, early mutations in "caretaker genes" can drive further tumor development.[15] ATRX has role for NHEJ DNA repair pathway. It is possible that the genetic instability in ATRX-deficient GBM drives proliferation by affecting cell cycle control or differentiation, as has been shown in other genetically unstable tumor models. Additionally, impaired apoptotic signaling through defective DNA-PKcs phosphorylation and/or concurrent TP53 mutations could provide an additional proliferative advantage to ATRX-mutated tumors.[15]

Recently, Hu et al (2020) have shown significant correlation between ATRX loss and presence of IDH1/2 mutations in grade II gliomas. Also, Mukherjee et al (2018) have shown how expression of mutant IDH1 initiates telomeric dysfunction and alters DNA repair pathway preferences at telomeres, cooperating with ATRX loss to defeat a key barrier to gliomagenesis. This is suggesting new therapeutic options to treat low-grade gliomas. In the current study, we also have noted positive correlation between ATRX loss of protein and presence of IDH with marginal statistical significance (p=0.066), probably due to less sample size. However, most striking result we noted when we analysed univariate and multivariate survival analysis using co-detection of ATRX and IDH mutations. Patients with presence of both genes mutations together emerged at step 1 for PFS and OS indicating their significance in predicting survival and early recurrence for glioma patients. In astrocytoma tumors of glioma patients, presence of both mutations together showed better OS than any one mutation or absent of both mutations. This invariable co-occurrence of ATRX with IDH mutations support a cooperative pathogenic mechanism by which dysfunction in both proteins is required for oncogenesis in a large subset of diffuse glioma tumors. Also, overlap of IDH1/2 mutations and ATRX alterations argues for a specific role of ATRX in IDH-driven gliomagenesis. Additionally, multiple studies have shown that as a consequences of ATRX loss, genomic instability caused, and these same functional relationships recapitulate in IDH-mutant glioma tumors too! Also, Kanan et al (2012) have reported high frequency of ATRX gene mutation which was entirely restricted to IDH-mutant low grade gliomas of astrocytic lineage-astrocytoma. Further, a better prognosis for patients with ATRX mutations has been suggested in a retrospective cohort by Noushmehr et al (2010). Further, Jiao et al (2012) have experimentally proven that loss of ATRX caused by siRNA induced apoptotic cells increasing, reduced tumor cell proliferation and repressed the cell migration in glioma cells. Moreover, Cai et al (2015) reported that decreased expression of ATRX can cause inhibition of migration, promotion of apoptosis and reducing of proliferation in glioma cells.

Conclusion

ATRX loss of protein expression is present in glioma patients having tumors of astrocytic lineage.

We concluded that co-occurrence of ATRX and IDH mutations in glioma tumors has more clinical impact in predicting PFS and OS of glioma patients than studying any one molecular marker. Thus co-detection of ATRX and IDH mutations could identify subgroup of glioma patients with better clinical outcome. However, as only half of our patients completed planned treatment and due to overall low number of patients studied, we cannot conclusively confirm that. Therefore, validation of this data is recommended in larger sample size.

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