Master's Thesis of Pathology

Immunohistochemical analysis of ATRX, p53 and IDH Gene mutations in glioblastoma patients and their co-relations with patient survival in Seoul National University Hospital, Seoul, South Korea

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Seoul National University
Department of Pathology
College of Medicine

Ajay Chaurasia

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By

Ajay Chaurasia

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Thesis Committee:

Professor Sung Hye Park Chairperson

Professor Jung Wook Seo Vice Chairperson

Professor Kyungkuk Park

ABSTRACT

IMMUNOHISTOCHEMICAL ANALYSIS OF ATRX, p53 AND IDH GENE MUTATIONS IN GLIOBLASTOMA PATIENTS AND THEIR CO-RELATIONS WITH PATIENT SURVIVAL

Ajay Chaurasia

Department of Pathology

Graduate School of Medicine

Seoul National University

Introduction- Glioblastoma (GBM) is the most common malignancy of central nervous system and is classified according to morphologic criteria established by the World Health Organization (WHO) into grade IV astrocytoma. With the most advanced treatments they have a very poor patient prognosis with average overall survival in the range of about 12 months. Recently molecular studies revealed that some molecular variants are associated with better survival outcomes as compared to others and may be used as prognostic markers. Mutations of ATRX, p53 and IDH genes are frequently reported to be present as well as associated with survival outcome in GBM.

Material and method- We evaluated the mutation status of ATRX, p53 and IDH genes immunohistochemically in 156 unselected GBM patients and analyzed their association with overall patient survival in order to find out if they can be considered for distinguishing clinically distinct prognostic subgroups of GBM. We compared the immunohistochemical characteristics of ATRX, p53 and IDH genes in adult and pediatric GBM. We evaluated the survival outcome of GBM cases based on different treatment modalities used.

Results- We found that 62.2% unselected GBM tumors showed loss of ATRX expression, 72.4% tumors had overexpression of p53 protein and 10.2% tumors were mutant for IDH1. We found that loss of ATRX expression and IDH1 mutations are associated with better patient survival while the p53 wild type is associated with a better patient survival as compared to overexpression of p53 protein. Patients with loss of ATRX expression had median overall survival of 20.2 months as compared to 13.5 months for patients who showed ATRX expression; similarly patients having IDH1 mutant GBM had a better median overall survival of 28.6 months as compared to 15.5 months for GBM patients with wild type IDH1. However, patients showing mutant expression of p53 had a poor overall survival of 15.5 months as compared to 21.6 months for patients who had negative expression of p53. We also found that there were statistically significant survival differences when these genes were analyzes in different two and three gene combinations. While carrying out immunohistochemical and survival outcome analysis in pediatric and adult GBM cases separately we found that

immunohistochemically pediatric GBM are different from adult GBM in genetic signature expressions. Survival analysis of adult GBM cases showed similar results as of unselected GBM survival outcome results..

Conclusion- Aberrant expressions of ATRX, p53, and IDH encoded by different tumor regulatory genes frequently occur in GBM and also, that mutant ATRX, mutant IDH and negative expression of p53 protein individually and their combinations are associated with statistically better patient outcome, which may be used as prognostic factors in GBM.

Key Words: Glioblastoma, Loss of ATRX expression, p53 over expression, IDH mutation.

Student number: 2012-24050

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1. INTRODUCTION

1.1 GLIOBLASTOMA

BACKGROUND

Glioblastoma (GBM) is the most common diffuse glioma of astrocytic lineage and corresponds to grade IV of WHO classification (1). It is the most common central nervous system malignancy, accounting for 45.2% of malignant primary brain and CNS tumors, 54% of all gliomas, and 16% of all primary brain and CNS tumors (2). Among all human cancers GBM is associated with one of the worst 5-year survival rates. In spite of most advanced treatment approaches, comprising surgery, radiotherapy and chemotherapy; the median overall survival time is still approximately 12 months (3). Population-based survival studies for GBM indicate still shorter median overall survival (4). However, a very small fraction of GBM patients survive more than 36 months. These patients surviving for more than 36 months are referred to as "long-term survivors". Most GBM tumors originate in a sporadic fashion without any known genetic predisposition (5), with the exception of rare instances of GBM tumor in patients with a hereditary tumor conditions, such as Turcot's syndrome (6), Li–Fraumeni syndrome (7). Studies are done to evaluate the effects of various external factors, such as ionizing radiation, diet, smoking, socioeconomic status, cellular phones, electromagnetic fields, and education level, as well as medical risk factors such as immunological status, allergy, viral infections etc. (8), but, no clear evidence linking specific risk-factors

to GBM has emerged from these studies, except for an association with the exposure to ionizing radiation (9). In addition, a good Karnofsky performance score (KPS), and younger age at the time of diagnosis are established prognostic factors associated with longer median survival (10). In spite of the huge development in the understanding of the genes and genetic aberrations in GBMs, useful clinical molecular markers which can help to predict survival prognosis and response to therapy are still rare (8). About 50% of gliomas are GBMs. They are most common in adults ages 45–65, and affect more men than women. GBMs arise from normal brain tissue, they may invade and migrate away from the main tumor within the brain; however, GBM will rarely spread elsewhere in the body.

GBMs comprise of primary and secondary subtypes, which evolve through different genetic pathways, affect patients at different ages, and have differences in survival outcomes (11). Primary (de novo) GBMs account for 90-95% of GBMs, have a very meager survival and occur in older patients (mean age, 62 years). Secondary GBMs develop from lower-grade astrocytoma or oligodendrogliomas that have been genetically programmed to eventually transform into malignant, rapidly growing GBMs; have relatively better survival outcome as compared to primary GBM and occur in younger patients (mean age, 45 years) (12, 13).

Glioblastoma (Grade IV astrocytoma) accounts for 80% of malignant astrocytomas and is marked by an extremely poor prognosis: half of all patients die within 1 year of diagnosis. Key histopathological features of GBM, such as necrosis and endothelial proliferation, distinguish these tumors from lower-grade astrocytic

tumors that have a much better prognosis. The mechanisms that underlie the correlation between high grade and poor prognosis are unknown, because all grades of astrocytoma are characterized by inappropriate proliferation, invasion of normal brain tissue, and disruption of normal brain functions. As indicated in the name of the tumor, GBM is characterized by marked cytologic and histologic variation and displays extensive genetic and biological variability (14). GBMs are more commonly located in the supratentorial region (frontal, temporal, parietal, and occipital lobes), are rarely seen in the cerebellum, and are very rare in the spinal cord (15, 16). WHO has classified gliomas into four grades, grade I to grade IV. The most violent grade IV GBM attributes of being the "most common" brain tumor. Because of their aggressive behavior and frequent occurrence these tumors have drawn significant attention and are being studied extensively. Most GBM patients die of their disease in less than a year and half and rarely have long-term survival. Any of the therapeutic intervention for GBM has not shown promising results. One of the reasons GBM do not responds well to therapeutic intervention is the complex nature of the tumor itself. As the name GBM itself says, GBM is. It is multiforme grossly, showing regions of necrosis and hemorrhage. It is multiforme microscopically showing presence of microvascular proliferation, pleomorphic nuclei and cells, with regions of pseudopalisading necrosis. Genetically it is multiforme, with a variety of amplifications, deletions, and point mutations (17). However, the terminology of 'multiforme" does not use anymore in the name of disease because of redundancy.

The standard of care for treatment of GBM has essentially been the same for many decades; safe surgical removal of as much of the tumor, followed by radiotherapy and chemotherapy which damages DNA or otherwise inhibits DNA replication of the malignant cells. Even under the best conditions, in which basically all of the tumor apparent on MRI scan can be removed surgically and the patients are fully treated with chemotherapy and radiotherapy, the average survival of the GBM patients is only extended fractionally from 2-3 months to 1 year (17). Patients surviving past 2 years from diagnosis have a relatively favorable conditional probability of survival into the future compared with newly diagnosed patients (18, 19). GBM is an aggressive neoplasm, which has a median survival of 3 months if untreated (20, 21). Several variables affect the prognosis of patients with GBM, including age, preoperative performance status, tumor location, preoperative imaging characteristics of the tumor, and extent of resection (4, 22, 23).

Based on the 2013 Central Brain Tumor Registry of the United States (CBTRUS) report, the average annual age adjusted incidence rate (IR) of GBM is 3.19/100,000 population (2). A higher incidence of GBM has been reported in men as compared with women (2). The IR of GBM is 1.6 times higher in males as compared with females (3.97 vs. 2.53) (2) with a male-to-female ratio 1.56:1(24).

In this new era, molecular markers have been intensively explored to overcome the limitation in the histopathological diagnosis of gliomas. Gene expression profiling has given rise to new molecular classification schemes. This classification by gene expression profiling has also revealed molecular classes not detected by traditional

methods of looking at tumor samples under a microscope. Some molecular expressions/gene mutations are constantly associated with specific types of tumors and are well correlated with the patient's survival outcomes and may be used as prognostic markers. These molecular studies may be very useful tools for the diagnosis, prognosis, treatment, and investigation of GBM. Many molecular markers are studied in GBM cases; most common examples are amplification of EGFR, (approximately 36% cases), loss of heterozygosity 10q (70% cases), deletions of p16INK4a (approximately 31% cases), and mutations of PTEN (25% cases), in primary glioblastoma, and over-expression of TP53 (70% cases) IDH mutations (80% cases) and recently ATRX gene mutations/ loss of ATRX protein on immunostaining (60% cases) in secondary GBMs. Association with survival and constant presence of these molecular markers in GBM cases may help distinguish clinically distinct prognostic subgroups of GBM and name these molecular markers as markers of prognosis. Studies of IDH, p53 and ATRX mutations have shown promising relationships with survival outcomes in GBM patients. This study is focused on analysis of these three genes by immunohistochemical staining.

1.2 PURPOSE OF THE STUDY

This is an unselected GBM series study. Purposes of this study were to 1) determine the prognostic value of loss of ATRX protein expression in establishing prognostically distinct GBM subgroups along with other relevant gene mutations

such as IDH and p53. 2) confirm the hypothesis that IDH, and ATRX mutations are associated with a better survival outcome in GBM cases. 3) to see the effects of p53 overexpression on survival in our GBM cohort. In this study, gene mutation studies were carried out for ATRX, p53, and IDH individually and in different two and there gene combinations by immunohistochemical analysis in 156 GBM patients to determine if their mutations are association with survival outcome in GBM patients and as such to define prognostically distinct molecular subgroups of GBM irrespective of histopathological diagnosis.

2. LITERATURE REVIEW

It is now well established that the characteristic mutations of secondary GBMs are p53, a thalassemia/mental retardation syndrome X-linked (ATRX) and IDH mutations, while primary GBMs shows mutations of EGFR (over expression), phosphatase and tensin homolog gene (PTEN) mutations, loss of heterozygosity (LOH) 10q, p16 deletions, high frequency of telomerase reverse transcriptase (hTERT) promoter mutations, and absence of IDH mutation (4, 12, 13, 25).

In unselected GBMs series IDH mutations are found in approximately 8.8% GBM, but selectively IDH mutations are detected in roughly 80-90 % of secondary GBM and are uncommon in primary GBM (26-28). As they are frequently and contently present in secondary GBM they are considered as molecular marker of secondary GBMs (27, 29, 30). IDH gene mutations are associated with better survival

outcome in GBM patients as compared to their wild type counterparts (26, 27, 31). IDH gene mutations prevent isocitrate dehydrogenase 1 from carrying conversion of isocitrate to 2-ketoglutarate. Instead, the altered enzyme takes on a new, abnormal function: the production of a compound called D-2-hydroxyglutarate. D-2-hydroxyglutarate likely blocks the maturation of cells, resulting in overproduction of immature cells and tumor formation.

Mutant p53 gene has been found in more than 60% - 70% of secondary GBMs, 25% - 30% of primary GBMs, and frequently occurs in young patients (4, 13, 26). Association of p53 with survival outcome in GBM patients has not been consistent; some studies reported better survival with p53 gene mutations (32, 33) while others have not found any such association (24). In study by Elizabeth W. Newcomb p53 mutations were found to be related to survival in an age specific manner being favorable if mutations are found in younger population (22-40 age group) but unfavorable in 41-60 and 61-80 age groups.

In the cell, p53 protein binds to the DNA, which consequently stimulates another gene to produce yet another protein called p21 which interacts with a cell division-control protein called cdk2. overexpression of p53 protein cannot bind to DNA in an efficient manner, and as a result the protein p21 is not produced adequately and so it cannot perform its function of ‘stopping cell division of damaged cells’. The damaged cells continue to grow and divide in an unregulated way, which can lead to tumor formation.

Lately, mutation of the ATRX (thalassemia/mental retardation syndrome X-linked) gene and loss of expression of ATRX protein detected by immunohistochemistry have been observed in GBMs. ATRX alterations are present mainly in tumors of an astrocytic lineage and are relatively specific to astrocytic tumors carrying IDH and p53 mutations (32). ATRX is frequently mutated in secondary GBMs (57%), but is infrequent in primary (4%) (32, 34, 35). In secondary GBMs ATRX mutation is usually accompanied by IDH and p53 mutations (34). Studies suggest that mutations in ATRX causes telomere maintenance by a mechanism called alternative lengthening of telomeres (ALT), a presumed precursor to genomic instability. This protein enables the incorporation of the variant histone 3.3 into heterochromatin, giving rise to changes in telomere length and genomic instability. Loss of ATRX function leads to numerous cellular aberrations, including abnormal methylation and gene expression patterns, as well as chromosome missegregation which results in a telomerase-independent telomere maintenance mechanism leading to cancer formation (36, 37).

3. MATERIALS AND METHODS

3.1 PATIENT POPULATION AND TISSUE MICROARRAY

A group of 156 patients with diagnosis of GBM (n = 156), who had surgery and were treated for GBM at Seoul National University Hospital (SNUH) between

1999 and 2014 forms the basis of this study. Archived slides and paraffin blocks were obtained for these patients from pathology department of Seoul National University Hospital. We studied total of 156 GBM cases (10 pediatric GBM, 146 adult GBM) using tissue microarray (TMA). Age range for the included patients was; Adults 19-79 years, pediatric and adolescence 4-18years. Cohort included 93 males and 63 females. We took one section from each patient. Average core size of 3-mm was cut out from formalin-fixed and paraffin embedded tissue blocks and stained using standard methods. Total 21 TMA (seven sets consisting of each of ATRX, p53, and IDH immunostaining) were prepared with average 22 sections per TMA. Patient's clinical details were taken from the SNUH online portal. Archival Hematoxylin and eosin stained slides were available for all cases and were re-evaluated to define the representative tumor regions for inclusion in the TMA. Tumors slides were reevaluated by our neuropathologist Professor Park Sung Hye.

3.2 HISTOLOGICAL EVALUATION

Histological evaluation was performed in Hematoxylin and Eosin stained archival slides in all 156 cases. All cases were reviewed by our neuropathologist Professor Park Sung Hye and confirmed as WHO grade IV astrocytomas according to the WHO classification scheme. The accepted histological criteria for a GBM included a malignant glial neoplasm consisting of cells of astrocytic lineage or totally undifferentiated cells together with the presence of vascular and endothelial

proliferation, as well as necrosis. All paraffin-embedded hematoxylin and eosin–stained sections of tissue submitted to the laboratory were re-evaluated. Several features were graded as either present or absent (necrosis, increased vascularity, and lymphocytic infiltrates), and others were qualitatively graded (endothelial proliferation, nuclear pleomorphism, and size) with presence of microvascular proliferation and tumor necrosis as the important features. When necessary, additional stains were used in few GBM samples, to confirm diagnosis and classification as WHO grade IV astrocytomas.

3.3 IMMUNOHISTOCHEMICAL STUDIES

Immunohistochemical staining was performed according to the manufacturer's protocol Ventana autostainer. Representative sections of 3 μm thickness were rehydrated after deparaffinization by xylene. Antigen retrieval was performed, after which the sections were washed with citrate buffer. Then, the sections were immersed in 3% H_2O_2 for 10 minutes to inhibit endogenous peroxidase activity. After having been washed three times by phosphate-buffered saline (PBS) over the course of 5 minutes, the sections were incubated with various primary antibodies. Immunohistochemistry was performed using antibodies against ATRX (rabbit polyclonal, Sigma-Aldrich, St. Louis, MO, USA, 1:600), p53 (mouse monoclonal, Clone: DO-7, Dako, Glostrup, Denmark; 1:50) and IDH (mouse monoclonal, clone H09, Dianova, Hamburg, Germany; 1:50) mutant protein on

TMA tissue sections. IDH was scored as positive or negative. p53 and ATRX were scored using a four tiered scale. The specimen were evaluated for the percentage of stained nuclei and grouped into one of the four classes: (-), < 10% of the cells stained, means absent immunoreactivity; (l+), 10 to 25% of the cells stained; (2+), 26 to 50% of the cells stained; (3+), >50% of the cells stained (24, 33, 38). Multiple readings from the representative sections were taken and the results were averaged to arrive at a final score in each tumor core. Immunoreactivity analyses were performed using Aperio image scope software using Aperio nuclear v9 algorithm.

3.4 CONTROLS

In all TMA series, positive and negative controls were included. Positive controls were the sections from known mutation positive and immunoreactive GBM tumors. Negative controls consisted of sections incubated with normal rabbit serum instead of the primary antibody. Samples were coded and the immunostaining was jointly evaluated at a multi-head microscope.

3.5 STATISTICAL ANALYSIS

3.5.1 SURVIVAL ANALYSIS

To determine the clinical significance of the ATRX, p53 and IDH mutation in GBM with patient survival, we evaluated ATRX, p53 and IDH mutation positive and ATRX, p53 and IDH mutation negative tumor cores with respect to survival. Analysis included survival from time of initial surgery up to the endpoint. Survival analysis was performed using the Kaplan–Meier estimator and log rank test to assess the importance of association of ATRX, p53 and IDH protein staining (mutation positive versus negative) with length of survival. Proportions in the pair wise assessment of gene expression were evaluated by Pearson Chi-square analysis or Spearman correlation coefficient analysis. Log rank test p value < 0.05 was considered statistically significant. Analyses were carried out using IBM Statistical Package for Social Science (SPSS) software version 22.0.

3.5.2 PATIENT FOLLOW-UP

Patient follow up was defined as the interval between initial brain surgery and death of the patient or last official contact until 31 December 2014. It was obtained by calculating the difference between the initial surgery date and the death date (or the last hospital visit date; in cases where patient was still alive as of 31 December 2014).

3.5.3 OVERALL SURVIVAL (OS)

It is defined as the time elapsed between patient first surgery and patient death due to any cause or last official contact till 31st December 2014 if the patient was still alive. OS, the primary endpoint, was obtained by calculating the difference between the initial surgery date and the death date (or the last hospital visit date; in cases where patient was still alive as of 31December 2014). Median OS time for the entire cohort was 16.3 months.

3.5.4 PROGRESSION-FREE SURVIVAL (PFS)

Progression-free survival can be defined as the time elapsed between treatment initiation and tumor progression or death from any cause, with censoring of patients who are lost to follow-up. In this study it was obtained by calculating the difference between the initial surgery date and the second operation date (or death date, if patient didn't have second surgery). Median PFS time for the entire cohort was 12.8 months.

3.5.5 STUDY APPROVAL

Clinical data were obtained from Seoul National University Hospital (SNUH) online medical records in accordance with Institutional Review Board (IRB)-approved protocols from the institution (1307-093-505).

4. RESULTS

Clinical database - The survival and demographic characters of the study cohort with GBM are summarized in Table 1. This study cohort consisted of 156 GBM patients with a median age at diagnosis of 46 years (Age range: Adults 19-79 years, pediatric 4-18years). There were 93 males and 63 females in our cohort.

Table 1 Demographic summary of patient cohort with GBM

Parameter	Value
Total number of GBM cases	156
Adults (19-79years)	146
Pediatric And Adolescence (4-18years)	10
Males	93
Females	63
Males: Female Ratio	1.48:1
Overall Median Survival (OS)	16.3
Progression Free Survival (PFS)	12.8

Length of Survival - The median overall survival (OS) for all patients from the time of index surgery was 16.3 months. The median progression free survival (PFS) duration for all patients from the time of index surgery was 12.8 months. In our cohort, we found a 1.48:1 ratio of GBM tumors for men: women comparable to the ratio of 1.5:1 reported in previous studies (24).

4.1 INDIVIDUAL ATRX, p53 AND IDH MUTATION ANALYSIS

The immunohistochemical characters of the GBM population are summarized in Table 2.

In the study population of 156, Overall, 97 (62.2%) of the GBM tumors showed defective ATRX expression, 113 (72.4%) defective p53 expression and 16 (10.2%) defective IDH expression.

Among GBM samples that stained positive for ATRX stain, (i.e. preserved ATRX or No loss of ATRX), 37% samples were 3+, 23% samples were 2+, and 38% samples were 1+.

Among GBM samples stained positive for p53 stain, (i.e. over expression of p53), 40% samples were 3+, 27% samples were 2+, and 33% samples were 1+.

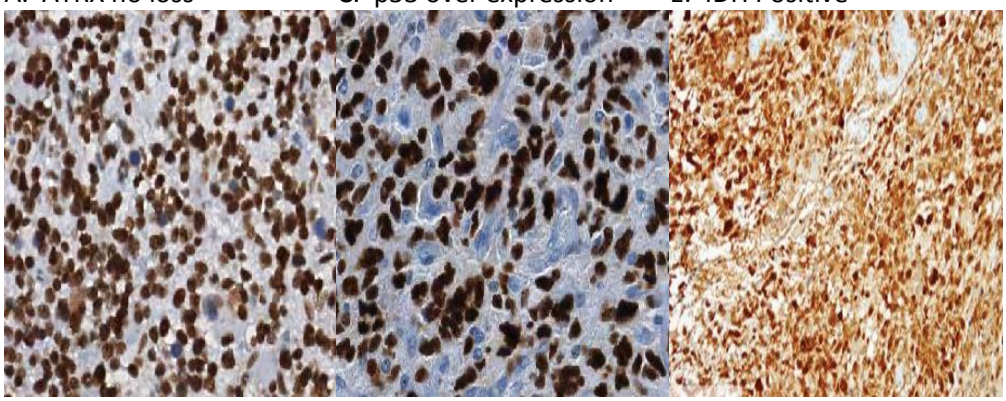
Table 2 ATRX, p53, IDH mutation and immunohistochemical staining characters

Gene	Number of mutation +ve	Number of mutation -ve	3+	2+	1+
ATRX	97 (62.2%)	59 (37.8%)	37%	23%	38%
p53	113 (72.4%)	43 (27.6%)	40%	27%	33%
IDH	16 (10.2%)	140 (89.7%)			

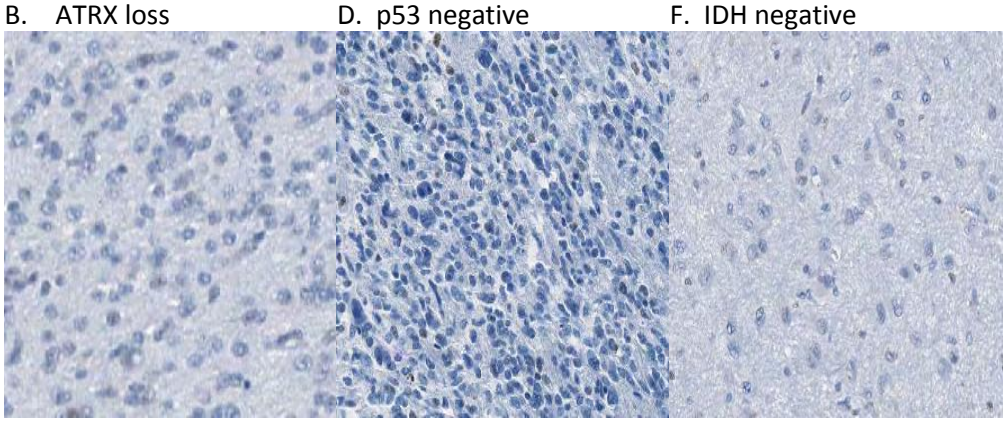
Table 3 ATRX, p53, IDH gene specific mutation and survival outcome (Months)

Gene	Median OS mutation +ve	Median OS mutation -ve	P value
ATRX	20.2	13.5	0.001
p53	15.5	21.6	0.002
IDH	28.6	15.5	0.008

A. ATRX no loss C. p53 over expression E. IDH Positive



B. ATRX loss D. p53 negative F. IDH negative



Images of immunohistochemical evaluation of ATRX, p53 and IDH. A. Nuclear expression of ATRX is detected in >10% of tumor cells; no loss (mutation negative). B. ATRX loss (mutation positive). C. Nuclear p53 accumulation can be observed in > 10% of tumor cells; over expression (mutation positive). D. p53 no accumulation (mutation negative). E. Immunohistochemical staining with antibodies against mutated IDH shows a strong cytoplasmic immunoreactivity in the tumor cells (mutation positive). F. IDH negative (mutation negative).

4.2 IMMUNOHISTOCHEMICAL ANALYSIS OF GENE COMBINATIONS

Combined two gene analysis for ATRX and p53 genes revealed that 41% (64/156) tumors showed ATRX loss and p53 over expression (mutation positive for both ATRX and p53) while 6.4% (10/156) tumors showed ATRX no loss and negative p53 expression (mutation negative for both ATRX and p53). 21.1% (33/156) samples showed ATRX loss and negative p53 expression, while 31.4% (49/156) samples showed ATRX no loss and over expression of p53.

Table 4 Combined ATRX-p53 gene mutation outcome

	Number	Median OS	p value
ATRX-/P53+	64	18.5	
ATRX+/ P53-	10	13.5	0.001
ATRX-/P53-	33	33.5	
ATRX+/ P53+	49	13.2	

While examining the combined immunohistochemical results for ATRX and IDH, 5.8% (9/156) tumors showed ATRX Loss and positive IDH, while 33.3% (52/156) tumors showed ATRX no loss and negative IDH. 56.4% (88/156) samples showed ATRX loss and were negative for IDH, while 4.5% (7/156) samples showed ATRX no loss but were positive for IDH mutations.

Table 5 Combined ATRX-IDH mutation outcome

	Number	Median OS	p value
ATRX-/IDH+	9	44.5	
ATRX+/IDH+	7	20.7	0.001
ATRX+/IDH-	52	12.0	
ATRX-/IDH-	88	17.2	

Analysis of combined immunohistochemical characters of p53 and IDH showed, 8.3% (13/156) samples were positive for both IDH and p53 mutations, 25.6% (40/156) samples were negative for both IDH and p53 mutation. 2.0% (3/156) samples were positive for IDH but negative for p53 mutation, while 64.1% (100/156) samples were negative for IDH but positive for p53 mutation.

Table 6 Combined p53-IDH mutation outcome

	Number	Median OS	p value
IDH+/P53+	13	25.7	
IDH-/ P53+	100	14.0	0.001
IDH-/ P53-	40	20.1	
IDH+/P53-	3	54.4	

Combined three gene mutation analysis revealed eight different categories of GBM. 4.5% (7/156) samples were positive for mutations of all three ATRX, p53,

and IDH genes, while 5.8% (9/156) samples were negative for mutations of all three ATRX, p53, and IDH genes. ATRX-/p53+/IDH- was the major category with 36.5% (57 /156) samples. Smallest number of samples 0.6% (1/156) fell in ATRX+/p53-/IDH+ category.

Eight different molecular variants of GBM were found on the basis of different combinations of the mutation status of three genes. Figure 1 shows the Distribution of ATRX, p53, IDH mutations, and their correlation to each other in this GBM cohort (n=156). Each line represents one GBM patients and the relative immunohistochemical results of ATRX, p53, and IDH gene are represented together with the histological diagnosis of the tumor. In this cohort the histological diagnosis was GBM for all the cases and so the corresponding grade for all the cases was WHO grade IV.

Table 7 Combined ATRX-p53-IDH mutation outcome

	Number	Median OS	p value
ATRX-/P53+/IDH+	7	28.6	
ATRX+/P53-/IDH+	1	20.7	
ATRX-/P53-/IDH-	31	29.3	
ATRX+/ P53-/IDH-	9	13.5	0.001
ATRX+/P53+/IDH-	43	12.0	
ATRX-/P53-/IDH+	2	54.4	
ATRX+/P53+/IDH+	6	15.9	
ATRX-/P53+/IDH-	57	15.7	

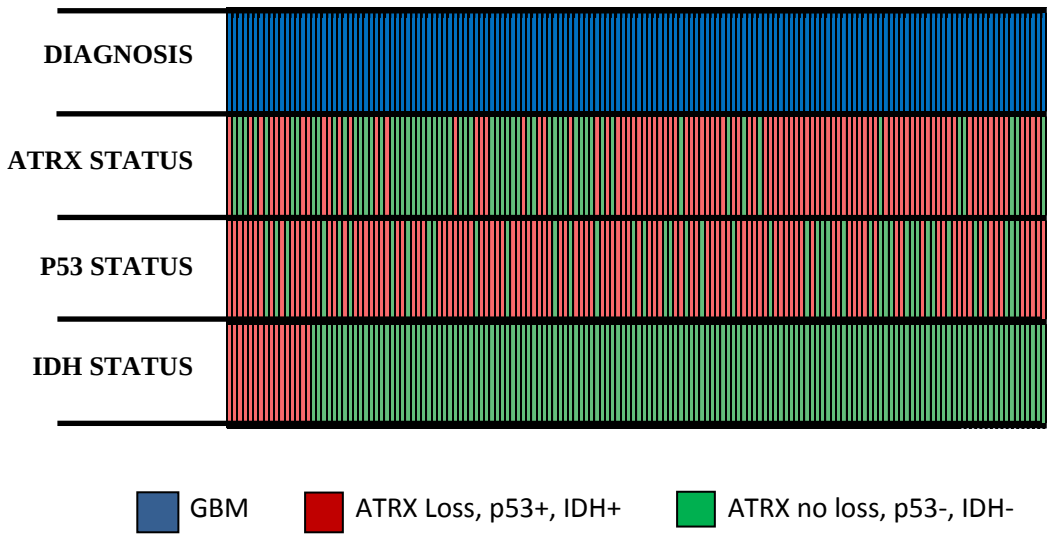


Figure 1 Overview of reference histology, molecular aberrations and molecular diagnosis in GBM patients

Every column represents a patient. Distribution of ATRX, p53 and IDH mutations in GBM. Data are from 156 GBMs. Diagnosis (all GBM in this study) is indicated in the top row by blue color. Second, third and fourth rows are indicating mutation status of ATRX, p53 and IDH gene respectively, with red color indicating respectively the loss of ATRX, over expression of p53 and IDH positivity while green color indicating respectively ATRX no loss, no expression of p53, and IDH negativity.

4.3 SURVIVAL ANALYSIS BY GENE EXPRESSION PROFILING IN GLIOBLASTOMA

To establish if mutation status of these three genes are of prognostic importance in determining survival outcome among patients with GBM, we compared the median overall survival time between mutation positive and mutation negative patients for each of the three genes. Table 3 contains the results of this comparison and Figures

2-8 show the Kaplan-Meier plot of survival for mutation positive versus mutation negative GBM for each of the 3 genes, individually and in different combinations.

4.3.1 ATRX LOSS IS A FAVORABLE PROGNOSTIC MARKER; ATRX MUTATION IS ASSOCIATED WITH BETTER SURVIVAL IN GBM

Loss of immunopositivity for ATRX was seen in 97 of 156 (62.2%) patients with GBM. Among these GBM patients, longer median survival was noticed with ATRX mutations/ loss of ATRX protein expression as compared to preserved ATRX protein expression. The 97 GBM showing loss of immunopositivity for ATRX had a median OS of 20.2 months as compare to the 13.5 months in 59 GBM patients with normal ATRX expressions. The difference in survival was statistically significance (p=0.001). Kaplan- Meier survival curve for ATRX immunopositive and immunonegative GBM cases is shown in figure 2.

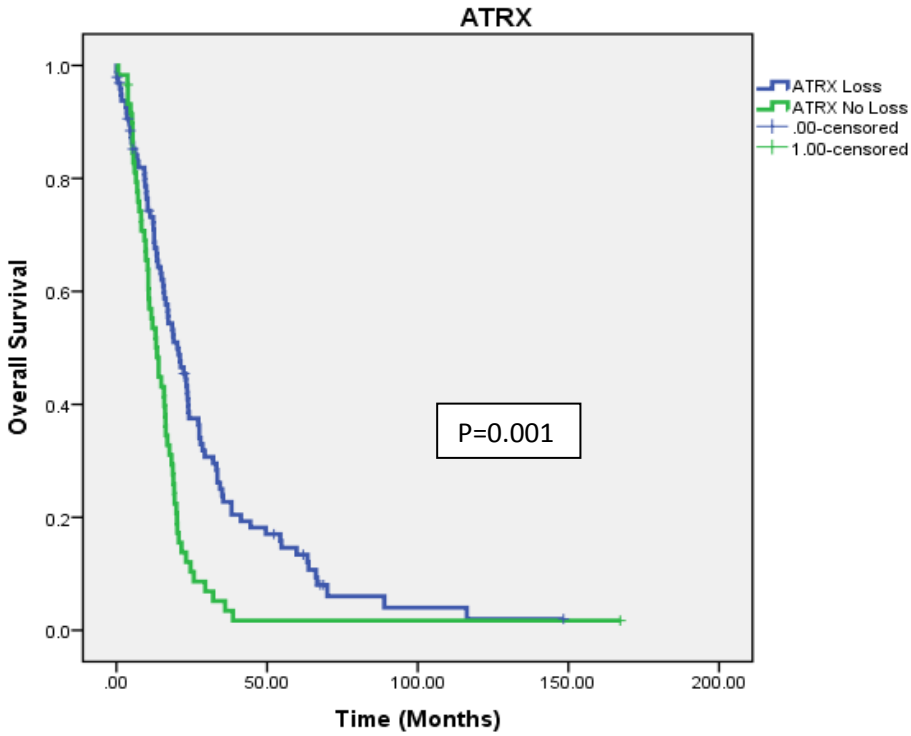


Figure 2 GBM patient survival curve (Kaplan-Meier curve) by ATRX gene mutation status

Survival of GBM patients with ATRX mutation (n=97) and without ATRX mutation (n=59). Patient survival with ATRX mutation is better than patients with no ATRX Mutation, with p value reaching statistical significance (p=0.001)

4.3.2 p53 MUTATION DO NOT LEAD TO BETTER SURVIVAL IN GBM IN ALL AGE GROUPS

In our cohort p53 protein accumulation was detected in 113 of 156 (72.4%) tumors. Amongst these GBM patients, a propensity for shorter median overall survival was observed with p53 mutations compared to GBM patients with no p53 mutation or absence of protein accumulation. The survival outcome difference was statistically significant (p=0.002). Kaplan- Meier survival curve for p53 mutation positive

versus mutation negative GBM patients is shown in figure 3. Patients with normal expression of p53 had a median survival of 21.6 months, compared to 15.5 months for those showing p53 protein accumulation, and this difference was statistically significant (p=0.002).

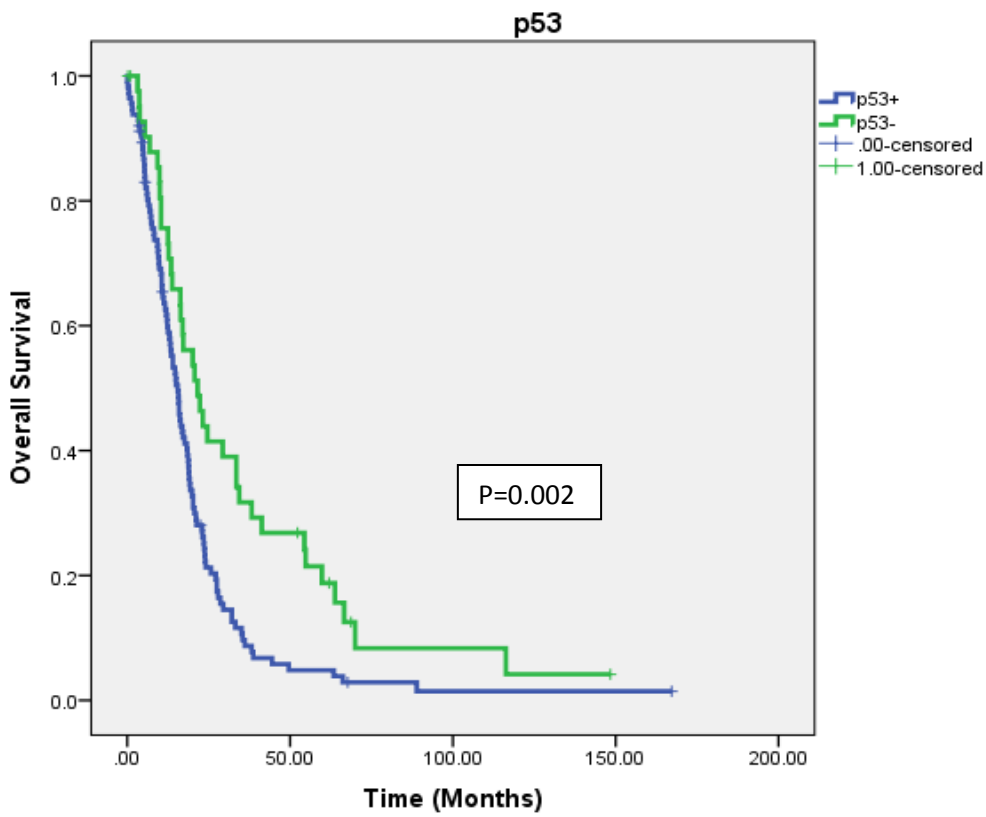


Figure 3 GBM patient survival curve (Kaplan-Meier curve) by P53 gene mutation status

Survival of GBM patients with p53 mutation (n=113) and without p53 mutation (n=43). Patient survival with normal p53 expression is better than patients with p53 Mutation, with difference being statistically significant (p=0.002).

4.3.3 ASSOCIATION OF IDH MUTATION WITH BETTER SURVIVAL IN GBM

IDH mutation positivity was seen in 16 of 156 (10.2%) patients with GBM. In these GBM patients, longer median survival was observed with IDH immunopositivity as compared to absent IDH protein expression. The difference was statistically significant (p=0.008). Kaplan- Meier survival curve for IDH mutation positive versus mutation negative GBM patients is shown in figure 4. Patients with IDH mutant GBM had a median survival of the 28.6 months, compared to 15.5 months for patients with wild type IDH GBM (p=0.008).

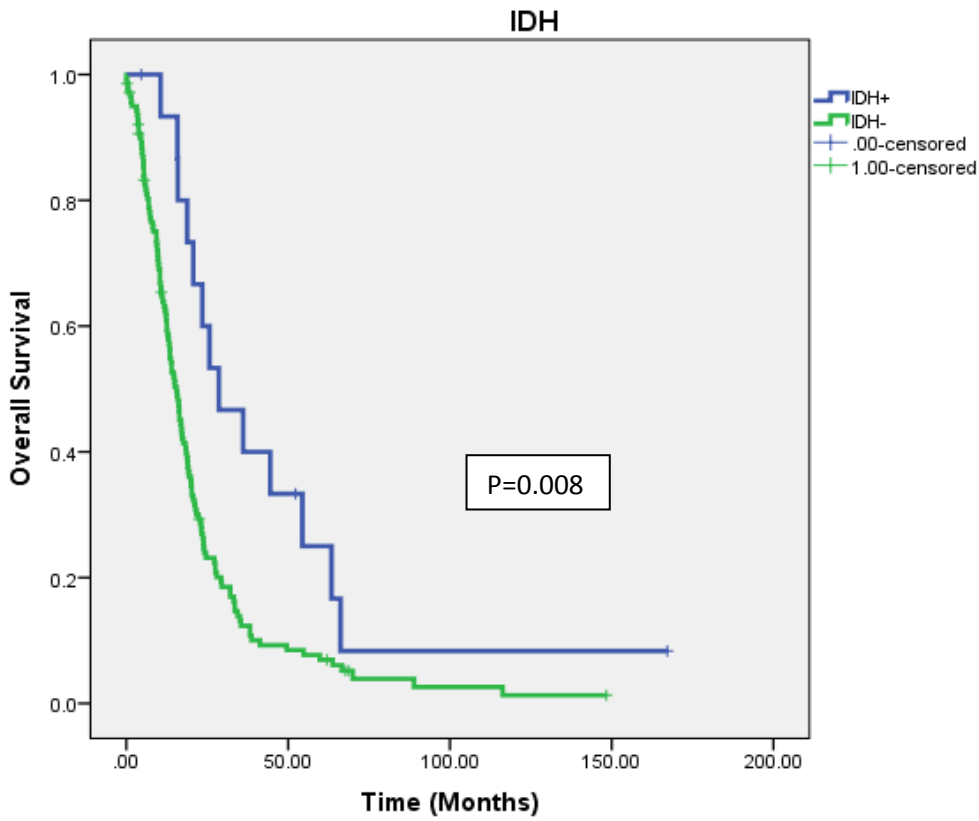


Figure 4 GBM patient survival curve (Kaplan-Meier curve) by IDH gene mutation status

Survival of GBM patients with IDH mutation (n=16) and without IDH mutation (n=140). Patient survival with IDH mutation is better than patients with no IDH Mutation, with p value reaching statistical significance (p=0.008)

4.4 SURVIVAL ANALYSIS FOR GBM IMMUNOHISTOCHEMICAL VARIANTS (COMBINED GENE ANALYSIS)

SURVIVAL ANALYSIS OF GBM IMMUNOHISTOCHEMICAL VARIANTS DEPENDING ON ATRX - p53, ATRX- IDH, p53- IDH (DOUBLE GENE) GENE EXPRESSION SIGNATURES.

Combined immunohistochemical analysis for, ATRX- p53, ATRX-IDH and IDH-p53 gene pairs expressed four GBM tumor variants in each pair of genes (total 12 variants), based on the presence or absence of mutation of ATRX -p53, ATRX-IDH, and p53-IDH. Table 4, 5, 6 summarizes the distribution of the four GBM variants of each pair and their corresponding fraction among the total tumor number of 156. Figure 5, 6, and 7 shows the Kaplan-Meier survival plots for different two gene combinations, and corresponding GBM variants.

4.4.1 SURVIVAL ANALYSIS OF GBM IMMUNOHISTOCHEMICAL VARIANTS DEPENDING ON ATRX - p53GENES

Analysis of the gene combination of ATRX and p53 discovered four different combinations, ATRX-/ p53- , ATRX+/p53 +, ATRX-/p53+ and ATRX+/p53-. The median survivals for patients with ATRX-/ p53- and ATRX+/p53 + was 33.5 and 13.2 months, respectively. The median survival for double mutation positive ATRX-/p53+ and double mutation negative ATRX+/p53- patients was 18.5 and 13.5 months, respectively. This pair showed the highest median survival of 33.5 months for ATRX-/p53- gene pair, and lowest of 13.2 months for ATRX+/p53+ gene pair. The difference in survival was statistically significant (p value= 0.001). Figures 5 show the Kaplan-Meier survival plots for ATRX and p53 gene pair, and corresponding GBM variants.

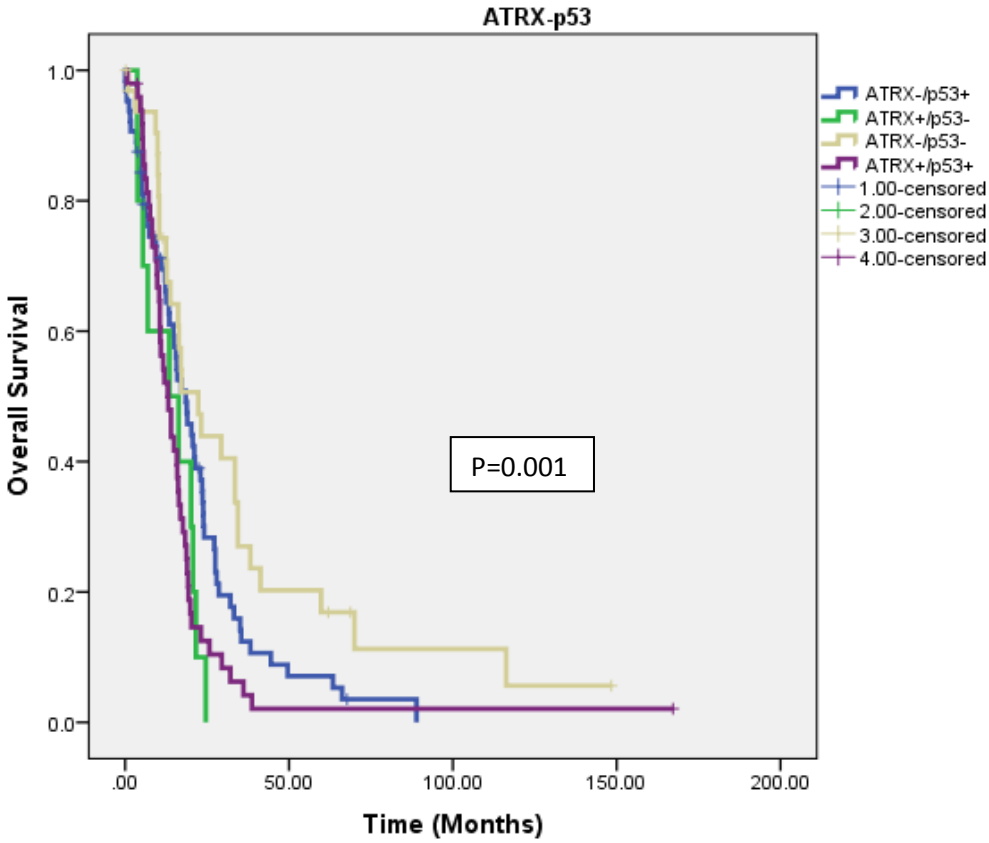


Figure 5 GBM patient survival curve (Kaplan-Meier curve) by combined ATRX and p53 gene mutation status

Survival of patients with ATRX-/p53+ (n=64), ATRX+/ p53- (n=10), ATRX-/p53- (n=33) and ATRX+/ p53+ (n=49). Highest median OS of 33.5 months was found in patients with ATRX-/ p53-mutation status. The difference in survival was statistically significant (p value= 0.001).

4.4.2 SURVIVAL ANALYSIS OF GBM IMMUNOHISTOCHEMICAL VARIANTS DEPENDING ON ATRX – IDH GENES

Analysis of the gene combination of ATRX and IDH discovered four different combinations; ATRX-/ IDH-, ATRX+/ IDH +, ATRX-/ IDH+ and ATRX+/ IDH-. The median survival for patients with ATRX-/ IDH- and ATRX+/ IDH+ was 17.2 and 20.7 months, respectively while the median survival for double positive ATRX-/ IDH+ and double negative ATRX+/IDH- patients was 44.5 and 12.0 months, respectively. This pair showed the highest median survival of 44.5 months for ATRX-/ IDH+ gene pair, and lowest of 12.0 months for ATRX+/ IDH- gene pair. The difference in survival was statistically significant (p value= 0.001). Figures 6 show the Kaplan-Meier survival plots for ATRX and IDH gene pair, and corresponding GBM variants.

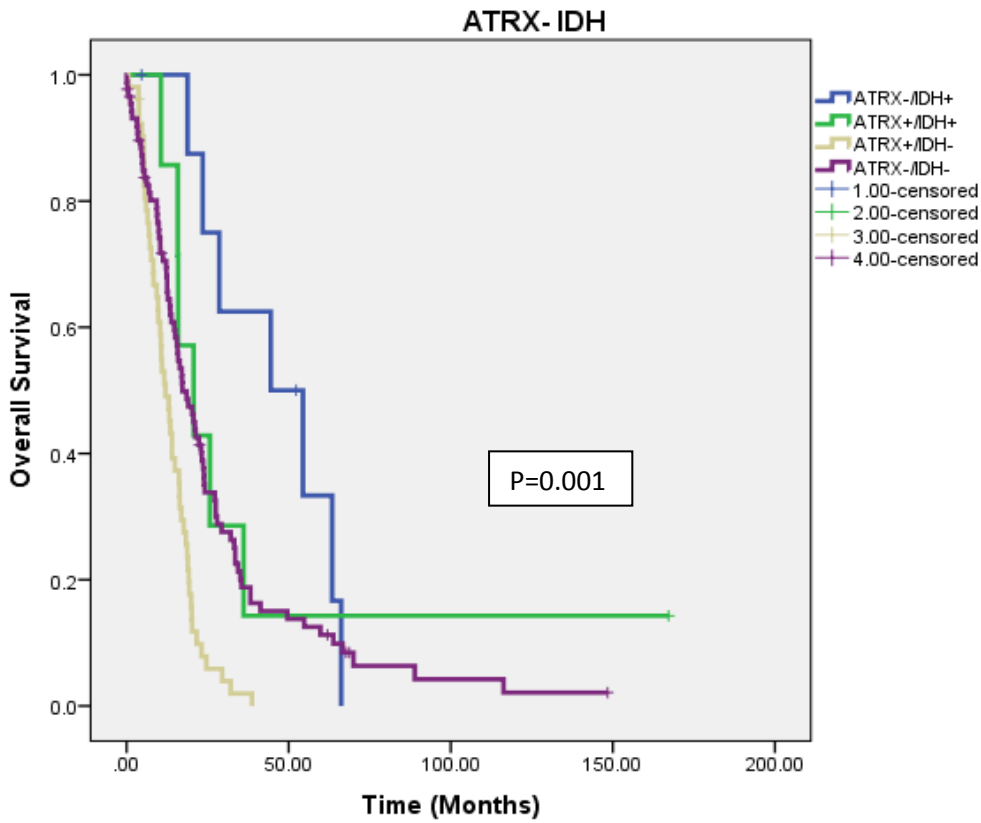


Figure 6 GBM patient survival curve (Kaplan-Meier curve) by combined ATRX and IDH gene mutation status

Survival of patients with ATRX-/IDH+ (n=9), ATRX+/IDH+ (n=7), ATRX+/IDH- (n=52) and ATRX-/IDH- (n=88). Highest median OS of 44.5 months was found in patients with ATRX-/IDH+ mutation status. The difference in survival was statistically

4.4.3 SURVIVAL ANALYSIS OF GBM IMMUNOHISTOCHEMICAL VARIANTS DEPENDING ON IDH - p53 GENES

In the gene combination of p53 and IDH gene, four different combinations were IDH+/ p53-, IDH-/p53 +, IDH+/p53+ and IDH-/p53-. The median survival for patients with IDH+/ p53- and IDH-/p53 + was 54.4 and 14.0 months respectively and the median survival for double mutation positive IDH+/p53+ and double mutation negative IDH-/p53- patients was 25.7 and 20.1 months, respectively. This pair showed the highest median survival of 54.4 months for IDH+/ p53- gene pair, and lowest of 14.0 months for IDH-/p53 + gene pair. The difference in survival was statistically significant (p value= 0.001). Figures 7 show the Kaplan-Meier survival plots for p53 and IDH gene pair, and corresponding GBM variants.

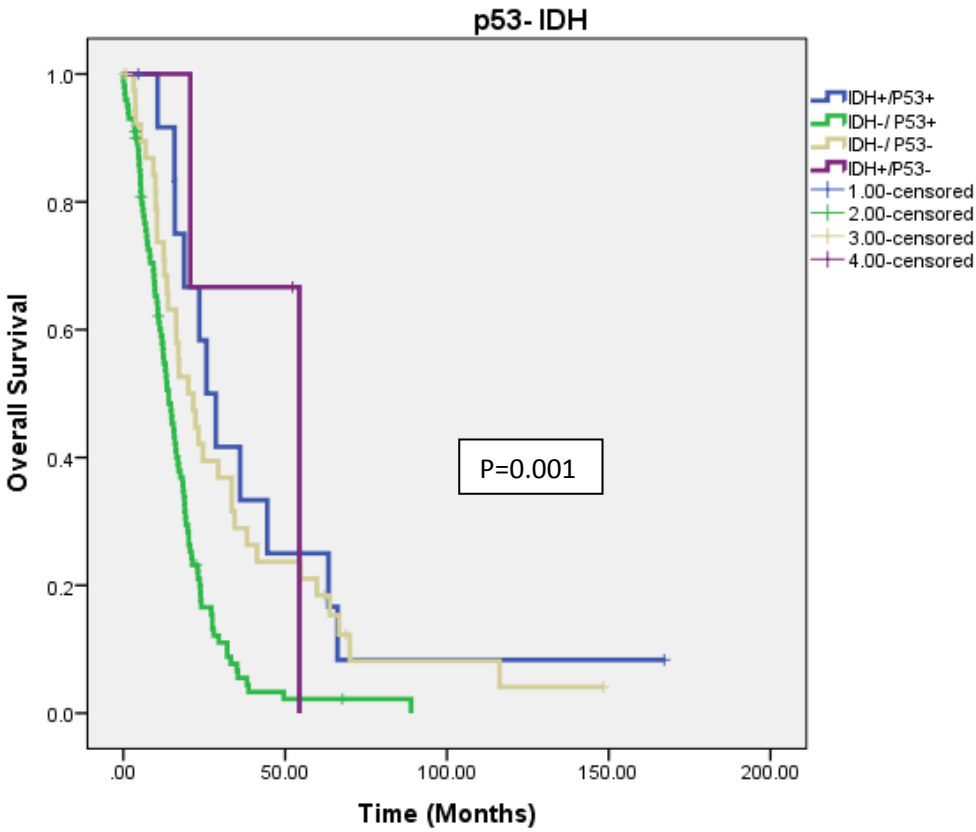


Figure 7 GBM patient survival curve (Kaplan-Meier curve) by combined p53 and IDH gene mutation status

Survival of patients with IDH+/p53+ (n=13), IDH-/ p53+ (n=100), IDH-/ p53- (n=40) and IDH+/p53- (n=3). Highest median OS of 54.4 months was found in patients with IDH+/p53-mutation status. The difference in survival was statistically significant (p value= 0.001).

4.4.4 SURVIVAL ANALYSIS OF GBM IMMUNOHISTOCHEMICAL VARIANTS, DEPENDING ON ATRX, p53, IDH (TRIPLE GENE) IMMUNOPROFILING

Eight distinct subgroups were established depending on combined immunohistochemical results of all three genes. Table 7 summarizes the distribution of the eight GBM variants and their corresponding fraction among the total tumor number of 156.

In these subgroups highest median survival of 54.4 months was for ATRX-/p53-/IDH+ gene combination, and lowest of 12.0 months for ATRX+/p53+/IDH- gene combination. The difference in survival was statistically significant (p value= 0.001).

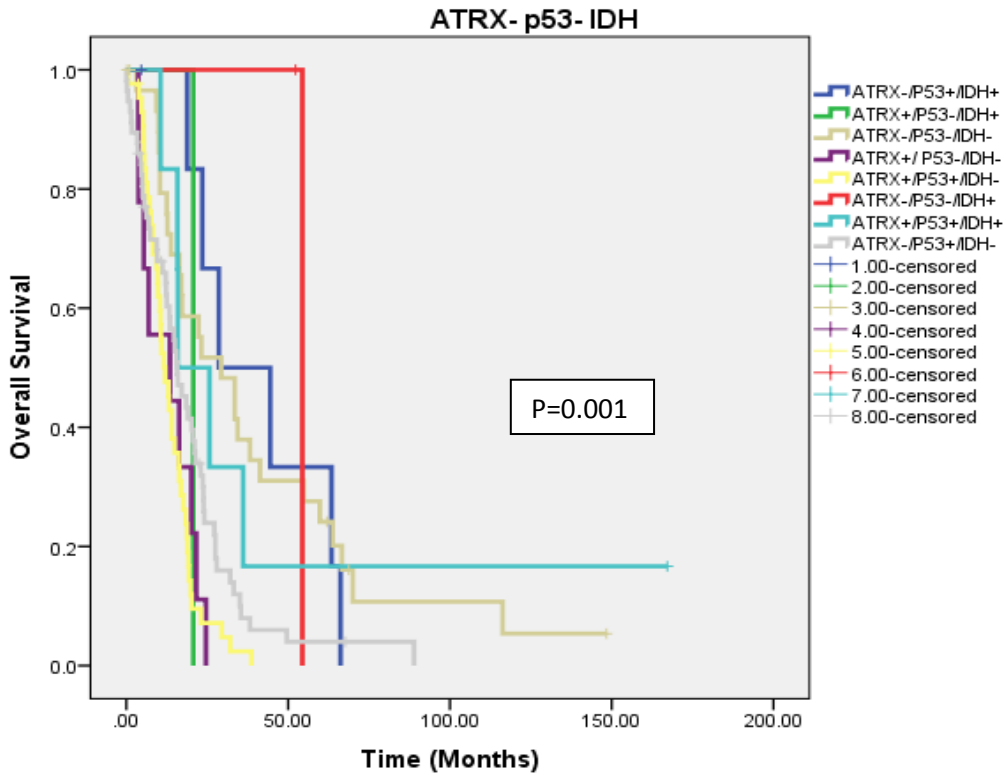


Figure 8 GBM patient survival curve (Kaplan-Meier curve) by combined ATRX, p53 and IDH gene mutation status

Survival of patients with ATRX-/p53+/IDH+ (n=7), ATRX+/p53-/IDH+ (n=1), ATRX-/p53-/IDH- (n=31) ATRX+/ p53-/IDH- (n=9), ATRX+/p53+/IDH-(n=43), ATRX-/p53-/IDH+(n=2),ATRX+p53+/IDH+(n=6), ATRX-/p53+/IDH-(n=57). Highest median OS of 54.4 months was found in patients with ATRX-/p53-/IDH+ mutation status. The difference in survival was statistically significant (p value= 0.001).

4.5 IMMUNOHISTOCHEMICAL AND SURVIVAL ANALYSIS IN PEDIATRIC GBM CASES.

To explore the gene expression signatures of ATRX, IDH and p53 in our pediatric patients we separately analyzed pediatric GBM for mutation status of these three genes and compared them with adult GBM to check out if gene expression signatures for these genes in pediatric and adult GBM are similar or different.

Out of 156 GBM 10 cases were pediatric GBM. Patient characteristics of pediatric GBM cohort are shown in table 8. Age range for pediatric GBM in our cohort was from 4 to 18 years. 7 cases out of 10 showed loss of follow up, one patient was still alive and two patients have died. Median age at diagnosis was 9.5 years for this cohort. No sex predominance was seen.

Table 8 Demographic summary of pediatric patient cohort with GBM

Parameter	
TOTAL NUMBER	10
ALIVE	1
DIED	2
LOSS OF F/U	8
MEDIAN AGE	9.5 YEARS
GIRLS:BOYS	1:1

4.5.1 IMMUNOHISTOCHEMICAL ANALYSIS OF ATRX, p53 AND IDH GENE IN PEDIATRIC GBM CASES

The immunohistochemical characters of the GBM population are summarized in Table 9. Figure 9 and table 9 shows comparison of gene expression signatures in pediatric and adult GBM cases. In this pediatric GBM population of 10, 4 cases (40%) showed defective ATRX expression, 5 (50 %) showed defective p53 expression. These results are in agreement with the results by previous studies (28, 32, 36, 39-41). Here it is to be noted that though IDH mutations are found exclusively in adult GBM (42), in our pediatric cohort exceptionally one case also showed defective IDH expression.

Table 9 ATRX, p53, IDH immunohistochemical staining characters in pediatric and adult GBM

Gene	Number of mutation +ve	Number of mutation -ve	Mutation +ve adult GBM
ATRX	4/10 (40%)	6/10 (60%)	93/146 (63.7%)
p53	5/10 (50%)	5/10 (50%)	108/146 (73.9)
IDH	1/10 (10%)	9/10 (90%)	15/146 (10.3)

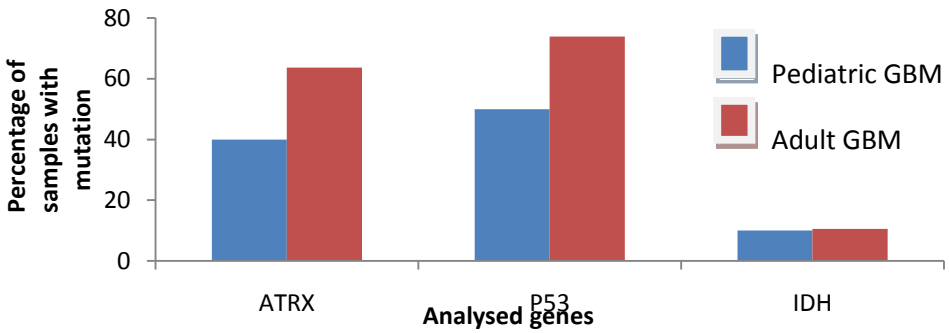


Figure 9 comparison of gene expression signatures in pediatric and adult GBM

Survival analysis by gene expression profiling in pediatric GBM showed inconclusive results because of censoring of 8 cases out of 10 and hence showed no significant association of any of the three gene mutation signature with survival in our pediatric cohort with p values being $p > 0.05$ in all three gene analysis. (p values-ATRX =0.64, P53 =0.93, IDH =0.72).

4.6 IMMUNOHISTOCHEMICAL AND SURVIVAL ANALYSIS IN ADULT GBM CASES.

Figure 9 and table 9 shows comparison of gene expression signatures in pediatric and adult GBM cases and conclude that molecular aberrations/ gene expression signatures are different in adult and pediatric GBM implying that pediatric GBM and adult GBM are different genetically. Survival analysis of adult GBM cases depending on ATRX, p53 and IDH genes individually and in different combinations revealed similar survival results as of the unselected GBM survival

results. Median overall survival for adult group was 16.2 months as compared to 16.3 months of unselected GBM cases.

4.6.1 SURVIVAL ANALYSIS OF ATRX, p53 AND IDH GENE IN ADULT GBM CASES

Analyzing individual genes immunohistochemically; ATRX, IDH gene mutations and WT p53 showed statistically significant better survival as compared to WT ATRX, IDH and overexpression of p53 protein respectively ($p<0.05$).

Table 10 ATRX, p53, IDH gene specific mutation and survival outcome in adult GBM (Months)

Gene	Median OS mutation +ve	Median OS mutation -ve	P value
ATRX	19.0	13.5	0.001
p53	15.0	20.7	0.006
IDH	28.6	15.0	0.005

Immunohistochemical analysis of two gene and three gene combinations in adult GBM cases also revealed similar results as of unselected GBM cases. Here also survival differences reached statistical significance in two gene combinations of ATRX- p53, ATRX- IDH, IDH- p53, and three gene combinations with p values less than 0.001 in all categories. Gene combination specific survival results were also similar to the unselected GBM results. Table11 shows the survival analysis of

different gene combinations. Like overall survival, Kaplan-Meier curve were also similar to the unselected GBM cases.

Table 11 Survival analysis of different gene combinations in adult GBM

Gene combination	Gene pair with Longest **:** Shortest survival	Longest / shortest survival	p value
ATRX,p53	ATRX-/P53- : ATRX+/ P53+	29.3/13.2 months	0.001
ATRX,IDH	ATRX-/IDH+ : ATRX+/IDH-	44.5/12.0 months	0.001
IDH,p53	IDH+/P53- : IDH-/ P53+	54.4/13.5 months	0.001
ATRX,p53,IDH	ATRX-/P53-/IDH+: ATRX+/P53+/IDH-	54.5/12.0 months	0.001

4.7 ASSESSMENT OF TREATMENT MODALITIES AS PROGNOSTIC FACTORS IN OUR COHORT

Extent of surgical resection and concomitant use of chemotherapy and radiotherapy are prognostic factors independent of the grade, histology and molecular subclasses of the GBM tumors. However, the effect of extensive resection on prolonging survival in patients with GBM is less clear because extensive resection of malignant astrocytomas is made difficult by the frequent invasive and widely infiltrative nature of these tumors which often involve eloquent areas (43). Previous studies have shown that the addition of concomitant and adjuvant chemotherapy (temozolomide) to standard postoperative radiotherapy improved median survival relative to postoperative radiotherapy alone (44).

To investigate status of treatment modalities (surgery ± adjuvant chemoradiotherapy) as prognostic factors we analyzed the treatments that our cohort received and subsequently we could classify these treatments in to 7 major treatment categories. These 7 treatment categories cover the treatments received by our 142 (out of total 156) patients. Categories which had minor number of patients (1 or 2) were ignored and not included in survival analysis. Treatment details for 2 patients were unavailable. Surgical resections were defined as biopsy (<10% resected), gross-total resection (GTR) - no residual enhancement, near-total resection (NTR) as having thin rim enhancement of the resection cavity only, and subtotal resection (STR) as having residual nodular enhancement on post operative imaging (43).

Table 12 Survival analysis of different Treatment modalities in GBM

Treatment modalities	Number of cases	Survival time (months)	P value
GTR + CT+RT	42	18.5	
NTR+ CT+RT	16	21.3	
STR+ CT+RT	50	20.2	
Biopsy+ CT+RT	11	10.9	0.006
STR+ CT	6	6.9	
GTR +RT	5	12.7	
STR	12	9.8	

Table 12 shows different treatment categories, number of patients in different treatment categories, survival outcome of different categories and the p values of statistical significance. Figure 10 shows Kaplan-Meier survival curves for different treatment categories in our GBM cohort.

It is evident from the survival curves and survival table that near total resection (NTR) combined with CT+RT has the highest (21.3months) survival outcome as compared to other treatment categories and the difference is statistically significant (p value 0.006). Next in the treatment category for higher survival outcome is subtotal resection (STR) combined with CT+RT (20.2 months) followed by GTR combined with CT+RT (18.5 months).

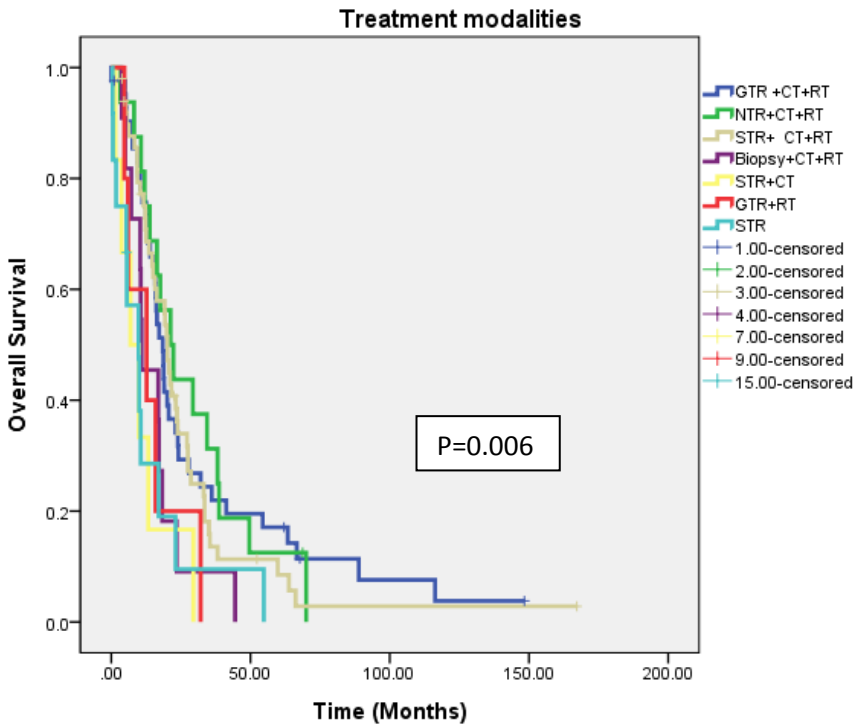


Figure 10 GBM patient survival curve (Kaplan-Meier curve) by Treatment modalities

Survival analysis of patients with different treatment categories. NTR+ CT+RT (n=16) category shows highest median survival of 21.3 months followed by STR+ CT+RT (n=50) with 20.2 months and GTR + CT+RT (n=42) with 18.5 months. The difference in survival was statistically significant (p value= 0.006).

It is clear from the above that it is NTR with CT+RT which has the highest survival outcome and not the GTR with CT+RT. In conclusion, the use of aggressive surgical resection such as NTR, GTR and STR along with adjuvant chemo-radiotherapy (CT+RT) makes long-term survival more likely in GBM patients as compared to surgery or biopsy alone and surgery with either CT or RT.

4.8 ASSOCIATION OF IDH MUTATIONS WITH CLINICAL CHARACTERISTICS IN THE COHORT

In our unselected cohort we found 16 GBM cases with IDH mutations and rest 140 GBM had wild type IDH (WT-IDH). None of the 16 IDH+ cases have history of previous diagnosis of a lower grade glioma; all these cases were diagnosed as GBM at first incidence. Because there was no evidence or history of previous diagnosis of a lower grade glioma in any of the 16 IDH+ cases; these all cases were diagnosed as primary GBM in accordance to the conventional definition of primary (de novo) glioblastoma. These patients were relatively younger with a median age 42 years. These patients have a median overall survival of 28.6 months.

Table 13 Characteristics of IDH mutations in our cohort

Parameter	IDH +ve GBM
Number of cases	16
Median age at diagnosis (Years)	42
Median OS (Months)	32.3
M:F	2.2:1
alive	3
died	13

4.9 ASSOCIATION OF CO-EXPRESSION OF GENE MUTATIONS IN GLIOBLASTOMA.

Each of the three gene mutations was tested in pair wise manner for any synergistic (positive) or mutually exclusive (negative) relationship. Significance of association of co-expression of two mutations together was assessed by the Pearson Chi square test or Spearman correlation coefficient analysis. Association of co expression between ATRX-p53, ATRX-IDH and p53-IDH were assessed.

Table 14 Association of co-expression of gene mutations in glioblastoma

	ATRX loss (n=97)	ATRX no loss (n=59)	p value
IDH+	9	7	0.9
IDH -	88	52	
p53+	64	49	
p53-	33	10	
	IDH+ (n=16)	IDH- (n=140)	
ATRX loss	9	88	
ATRX no loss	7	52	
p53+	13	100	
p53-	3	40	0.5
	p53+ (n=113)	P53-(n=43)	
ATRX loss	64	33	
ATRX no loss	49	10	0.02
IDH +	13	3	
IDH -	100	40	

There were no statistically significant positive or negative associations between co-expression of ATRX and IDH or p53 and IDH, the p values being 0.9 and 0.5 respectively. But we found a statistically significant association of co-expression between ATRX and p53 mutations, with p value= 0.02. Implying that GBM tumors having ATRX mutations tended to show stronger presence of associated p53 mutations. 66% (64/97) tumors that were mutant for ATRX had associated p53 mutations, while 56.6% (64/113) tumors showing p53 mutations had associated ATRX mutations. Only 9.3% tumors with ATRX loss and 11.5% tumors with p53 mutations had associated IDH mutation. Table 14 and figure11 shows the associations of different genetic variables to one another.

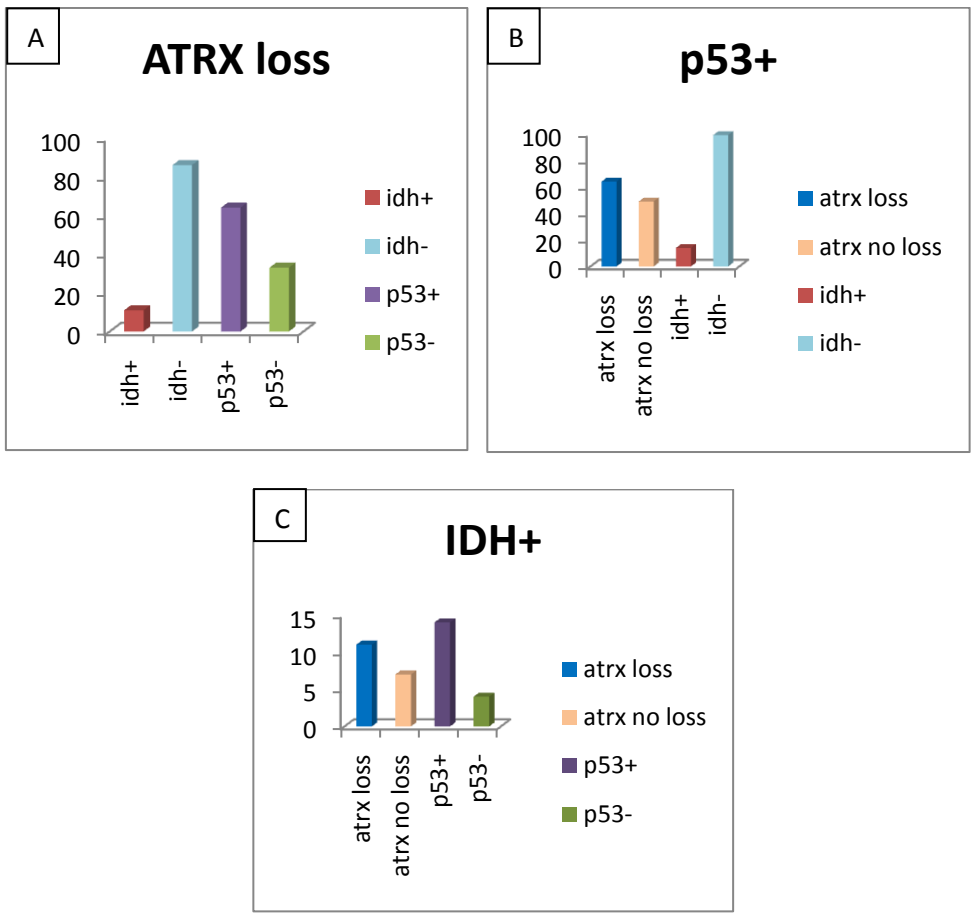


Figure 11 A. B. C. immunohistochemical correlations of ATRX loss, p53 over expression and IDH positivity (n=156)

A. Distribution of IDH+, IDH negative, p53+, and p53- in ATRX mutated GBM.
B. Distribution of ATRX loss, ATRX no loss, IDH+, and IDH- in p53 mutated GBM.
C. Distribution of ATRX loss, ATRX no loss, p53+, and p53- in IDH mutated GBM

5. DISCUSSION

The major goal of immunohistochemical and molecular studies in the neuro-oncology is to establish a steady, reproducible relationship between the presence of particular molecular alterations and the clinical or survival outcome of the patient. In our cohort all patients had GBM, which is astrocytic tumor of WHO grade IV. GBM as the name suggests is very heterogeneous neoplasm with a broad range of molecular and histological diversity at presentation, and efforts to definitely correlate any particular molecular factor with patient survival and clinical outcome has mostly been ineffective. At present the prognostic criterions which are constantly associated with longer patient survival are Karnofsky performance score (KPS) and younger age at the time of diagnosis.

It is seen in various previous studies that there is some relationship between mutations of ATRX, p53, and IDH in GBM and the overall patient survival. Though with less confidence; these studies suggests a better overall survival outcomes in GBM patients with presence of mutations of ATRX, IDH and in some studies associated mutations of p53 gene. Aim of this immunohistochemical study was to establish a relationship between median overall survival outcomes of GBM patients and mutation status of ATRX, IDH and p53 genes individually, as well as in different combinations of these gene expressions. Immunohistochemically we evaluated 156 GBM for mutations of ATRX, p53, and IDH genes to determine their significance as prognostic factors.

Unselectively, immunohistochemical analysis of individual genes found ATRX mutations in 62.2%, cases (32, 34, 35), p53 mutations in 72 % cases and IDH mutations in 10.2% cases. These results were in accordance with the previous studies (4, 13, 26). Mutations of p53 were present in about 72% GBM (113/156) and were the most prevalent mutation present, while IDH mutation were the least common mutations, found only in 10.2% (16/156) GBM patients.

While analyzing individual genes immunohistochemically; ATRX and IDH gene mutations showed statistically significant differences in survival and the Kaplan-Meier survival curves of the same showed a statistically significant better median overall survival in GBM with ATRX and IDH mutations as compared to the GBM with wild type of ATRX and IDH respectively. These results were in agreement with the results of previous studies and hence confirming our hypothesis of better patient survival in GBM with ATRX loss and IDH mutation.

Immunostaining analysis for p53 mutation/ p53 protein accumulation had been done in previous studies. Even though a linear association was never observed between p53 protein accumulation and the presence of p53 gene mutations in any GBM study; approximately 90% of the cases which shows immunohistochemical evidence of p53 protein accumulation have been found to have detectable p53 aberrations on sequence analysis (24, 45, 46). Until now some studies have demonstrated a relationship between p53 gene mutation and poor median survival (47-49), while others haven't found any such relation (50-52). In our cohort

patients with p53 mutant GBM showed shorter median survival (15.5 months) as compared to patients with normal p53 expressing GBM (21.6 months) (p=0.002). These results are in line with the results obtained by Elizabeth W. Newcomb et al. (24). In their study Elizabeth W. Newcomb et al. found short median survival for p53 mutant GBM patients as compared to normal expressing p53 GBM patients in 40 -60 years age group. Similar results in our cohort may be because the median age for our cohort is 46 years which is well in the range of 40-60 years.

Among the category of individual gene mutation analysis the highest median OS was for GBM with IDH mutations (28.6 months) while the lowest median OS was for the GBM with ATRX no loss (13.5 months). Survival differences were statistically significant in mutation analysis for all three ATRX, IDH and p53 genes. For p53, survival difference showed a trend for better overall survival in GBM without mutation while for ATRX and IDH mutations the survival difference showed a trend for better overall survival in GBM with respective gene mutations.

Immunohistochemically analyzing two gene and three gene combinations we asked whether certain gene combination alterations were more likely to be associated with better overall survival. We found that the different gene combinations were associated with distinct survival differences. The survival differences reached statistical significance in two gene combinations of ATRX- p53, ATRX-IDH, IDH-p53, and three gene combinations with p values less than 0.001 in all categories. While analyzing gene combination of ATRX and p53 the ATRX-/p53- mutation

status showed the highest median OS (33.5 months) while the lowest median OS was for ATRX+/ p53+ (13.2 months). Analysis of ATRX and IDH gene combination revealed ATRX-/IDH+ mutation status with the highest OS (44.5 months) while ATRX+/IDH- status showed the lowest median OS (12.0 months). Analysis of p53 and IDH gene combination revealed IDH+/p53- mutation status with highest median OS of 54.4 months while IDH-/ p53+ mutation status showed the lowest median OS (14.0 months). Analysis of three gene combinations, revealed median survivals ranging from highest 54.4 months for ATRX-/P53-/IDH+ gene combination to lowest 12.0 months for ATRX+/P53+/IDH- mutation status.

Glioblastoma is grade IV brain tumor in adults and children but molecular aberrations/ gene expression signatures are different in adult and pediatric GBM implying that adult and pediatric GBM are different genetically rather than being same. In previous studies mutations of ATRX and p53 are found in approximately 35% and 55% pediatric GBMs respectively. In our pediatric cohort we found ATRX and p53 mutations in 40% and 50% GBM respectively which are similar to results of previous studies. These results support the concept that adult and pediatric GBM are different in respect of gene expression signatures. Here it's worth noting that though IDH mutations are found exclusively in adult GBM, exceptionally in our pediatric cohort one GBM showed defective IDH expression. This exceptional case was spinal GBM in a 16 years old male child.

While analyzing the prognostic significance of different treatments, ironically, NTR with CT+RT showed the highest survival outcome followed by STR+ CT+RT and GTR + CT+RT; putting forward that the use of aggressive surgical resection such as NTR, GTR and STR along with adjuvant chemo-radiotherapy (CT+RT) makes long-term survival more likely in GBM. This suggests that increasing extent of tumor resection is not necessarily associated with better patients' survival; in other words operation type does not essentially affect patients' survival.

During the past few years, evidence has accumulated that primary GBM and secondary GBM constitute distinctive disease entities that develop through different genetic pathways, affect patients of different age, show different genetic alteration profiles, and may differ in their response to therapy (30). Data from a group of studies show that only approximately 5.6% of primary GBM are IDH mutant while more than 80% of secondary GBMs carry the IDH mutation. Along with higher incidence in secondary GBM, lower-grade gliomas (LGG) also carry a high incidence of IDH mutations. IDH is mutated in more than 75% of Grade II and 62% of Grade III astrocytomas, approximately 80% of Grade II and nearly 70% of Grade III oligoastrocytomas, and approximately 80% of Grade II and 70% of Grade III oligodendrogliomas (53). These finding suggests that IDH mutations occur early in the development of a glioma from a stem cell that can give rise to both astrocytes and oligodendrocytes. These findings also imply that IDH

mutations might drive the progression of the lower grade glioma to GBM (27). Together, given the high incidence of IDH mutation in lower-grade gliomas and secondary GBM and the comparatively low incidence in primary glioblastoma, it seems probable that IDH-mutant primary GBMs may in fact represent misdiagnosed secondary GBM progressed very rapidly from previously undiagnosed lower-grade gliomas that escaped clinical diagnosis—therefore suggesting that primary GBM should be considered separately as a purely IDH-WT disease (53). In our cohort we had 16 IDH mutated GBM. Among these none had a history of previous cranial surgery for lower grade glioma; and so were diagnosed as primary GBM without previous evidence of lower grade glioma. Here it's worth saying that on histopathological examinations of the tumor it is not possible to distinguish between primary GBM and secondary GBM. As previous studies indicated IDH mutations are specific for secondary GBM and rarity of their presence in primary GBM is well accepted, these 16 IDH mutant GBM in our cohort without previous diagnosis of lower grade glioma which seems to be primary GBM with IDH mutations may actually be secondary GBMs that rapidly progressed from less malignant precursor lesions that escaped clinical diagnosis. Analysis pertaining to association of co-expression of two gene mutations revealed a positive association between ATRX and p53mutations with a statistically significance (p value 0.02), while there were no significant associations of co-expression between ATRX and IDH or p53 and IDH.

6. CONCLUSION

In this GBM study immunohistochemical analysis for gene mutation of ATRX, p53, and IDH genes was done from 156 GBM cases who attended Seoul national university hospital from 1999 to December 2014. First we analyzed the occurrence of different gene mutations in our GBM samples, individually and in two and three gene combinations to investigate the relationship of association of different mutations with GBM. Then gene mutation status was analyzed individually and in different two and three gene combinations with patient survival outcome to co-relate their mutation status with prognosis.

Summarizing the observations, we found that the presence of ATRX, p53 and IDH gene mutations were common in our GBM cohort and the results were in line with outcomes of previous studies. Mutation analysis of these three genes was also found to be linked with patient survival outcomes while analyzing them individually and in two and three gene combinations as well.

In conclusion we have shown that aberrant expressions of different tumor regulatory genes ATRX, p53, and IDH frequently occur in GBM. Our immunohistochemical analysis of ATRX, p53 and IDH gene individually and in different combination revealed that mutant ATRX, mutant IDH and WT p53 individually and mutation combinations of ATRX-/p53-, ATRX-/IDH+, IDH+/p53-, and ATRX-/p53-/IDH+ are associated with a distinct and statistically significant better survival outcomes and as such these individual gene mutations as

well as these gene mutation combinations can be considered GBM subgroups with prognostically better survival outcome and may be used as prognostic factors of better patient survival.

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