

## Study Protocol

### **Correlation of IDH1-(R132H) and ATRX Expression with the Histopathological Grades of Glial Tumours: A Study Protocol**

#### **ABSTRACT:**

**Background:** Brain tumors contribute significantly to morbidity as they can lead to neurological deficits, mental alterations and have a poor survival rate. The utmost frequent varieties of primary brain tumors are gliomas. Modern data on permeated gliomas showcased persistent modification within the involved genes in the chromatin remodeling routes such as  $\alpha$ -thalassemia/mental-retardation syndrome-X-linked gene (ATRX) and IDH1. Till date, the authenticity of ATRX in predicting isocitrate dehydrogenase (IDH1) mutations in gliomas, is unclear. This study attempts to assess the correlation between IDH1 mutation and ATRX expression and histopathological grading in glial tumors.

**Methods:** This will be a retrospective analytical study conducted in Pathology Department, of JNMC, Wardha in coordination with the Department of Neurosurgery, AVBRH. This study will include histochemical analysis of 45-50 resected specimens of confirmed cases of Glial tumours. The correlation between IDH1 mutation and ATRX expression with the histopathological grades in glial tumors will be analysed in comparison with the present-day molecular advances.

**Results:** Significant correlation between IDH1 mutation and ATRX expression with the histopathological grades is expected.

**Conclusion:** Conclusion will be drawn on careful analysis of data.

**Keywords:** ATRX Marker , IDH1-(R132H), Mutation, Grading, Glioma, Glial Tumours.

#### **INTRODUCTION**

The central-nervous-system is made up of the brain & the spinal cord and their coverings. Though not frequent, brain tumors contribute significantly to morbidity as they can lead to neurological deficits, mental alterations and have a poor survival rate. Brain tumors can be benign or malignant. In contrast to other sites benign tumors may have the ability to become life threatening.

Brain tumors contribute significantly to morbidity as they can lead to neurological deficits, mental alterations and have a poor survival rate. Tumors of brain can either be benign or malignant. As stated by the Central-Brain-Tumor-Registry-of-the-United States (CBTRUS) figures, the median yearly age-adapted incidence rate of all malignant, benign and other CNS neoplasms was 23.41 per 100,000 in-between 2012 & 2016.<sup>[1]</sup> The utmost frequent intra axial CNS neoplasms with a heterogenous molecular framework are gliomas.

The utmost frequent varieties of primary brain tumors are gliomas. Gliomas arise inside the gluey supportive cells (glial cells) who embrace cells and abet them to function. CNS tumors are formed by three varieties of glial cells. Gliomas are designated as per the glial cell type occupied in the tumor, also genetic features of malignancy, which can assist in predicting which way the tumor will react gradually and the treatments most likely to be proffered with positive results. Predominantly (61.3%) gliomas occur within supra-tentorial region (frontal, temporal, parietal, and occipital lobes combined).<sup>[1]</sup> Types of glioma include:- 1) Astrocytoma's 2) Ependymomas 3) Oligodendrogliomas.

Modern data on permeated gliomas showcased persistent modification within the involved genes in the chromatin remodeling routes such as  $\alpha$ -thalassemia/mental-retardation syndrome-X-linked gene (ATRX) and IDH1. Till date, the authenticity of ATRX in predicting isocitrate dehydrogenase (IDH1) mutations in gliomas, is unclear.<sup>[2]</sup>

Reoccurrence and succession to progressive grade tumours are crucial biological evaluation & predictable actions seen in transformation progress of glioma. A slightly remaining proportion of cells habitually evades surgery and chemoradiation, developing in a specifically lethal tumor succession. IDH1-alteration and ATRX (alpha- thalassaemia/mental-retardation-X linked) loss/mutation happen in relation also prior genetic changes in the advancement of glial neoplasms may occur [28]. Nevertheless, diagnostic worth of these markers in the progression of gliomas still needs to be investigated furthermore.<sup>[3]</sup>

The histological grade of the tumour is determined by the WHO Grading System, tumours are graded from 1 to 4, with the higher numbers indicating faster growth and greater aggressiveness.<sup>[4]</sup>

- **Grade 1:** These tumours have low potential of proliferation, a constantly distinct nature, and the likelihood of cure post-operative abscission itself.
- **Grade 2:** Despite low mitotic activity, lesions present with atypical cells which are infiltrating in nature and they tend to arise more commonly in comparison with grade I malignant tumours after local therapy. Few neoplasm varieties likely to evolve to progressive class of malignancy.
- **Grade 3:** Tumours which are malignant on histology, comprehending nuclear atypia or anaplasia and accelerated mitotic activity; these tumours have anaplastic histology and ability of infiltration; these tumours are commonly managed with aggressive adjuvant radiotherapy and or chemotherapy.
- **Grade 4:** Mitotically active tumours, prone to necrosis, and are usually linked with neovascularity & infiltration of adjacent tissue, a proneness for craniospinal diffusion and a quick post-operative development and lethal results. The tumours are commonly managed with adjuvant radiotherapy and chemotherapy.

The other grading system is **The St. Anne-Mayo-grading-system**<sup>[5]</sup> which is also applied for grading astrocytomas. This system employs four morphological criterias to allocate a grade: a) Atypia of nucleus b) Mitosis c) Proliferation of endothelial cells 'piled up', NOT hypervascularity d) Necrosis.

**IDH-1** mutation is observed in around 80% of WHO Grades-II and III diffuse glial neoplasms, ODGs (80%), anaplastic ODGs (85%), and mixed oligoastrocytomas (71%), as well secondary GBMs (82%). IDH-1 mutation is rare in primary GBMs (5%), pilocytic

astrocytoma's (10%), and is absent in ependymomas. IDH-2 mutation is seen in a smaller proportion of gliomas, and that too mainly in oligodendroglial tumours. Several research have manifested that IDH-1 mutation is linked with a longer survival. IHC using IDH-1 R132H mutation-specific antibody detects IDH-1 mutation. However, this method can miss about 10% of gliomas carrying an IDH-1 mutation and all gliomas with an IDH-2 mutation. Subsequent genetic analysis is advocated in the cases associated with a negative or inconclusive IDH1 immunostaining results. This antibody also helps in differentiating gliomas from reactive gliosis where it is immune-negative.<sup>[6]</sup>

**ATDX (ATRX) syndrome, analogue** with unique hereditary disorder which involves growth retardation, intellectual deterioration, numerous bony malformations. Inborn  $\alpha$ -thalassemia-myelodysplastic-syndrome (ATMDS): unique hereditary syndrome identified by somatic point mutation in ATRX-gene with chronic myeloid disorders patients. In CNS, there is ATRX expression loss in cases of anaplastic astrocytomas i.e., 45%, in cases of anaplastic oligoastrocytomas 27% and for anaplastic oligodendrogliomas 10%. It occurs in paediatric and adult high-grade astrocytoma. Unconventional elongation of Telomeres (ALT) is a non-telomerase process of telomere elongation which happens in by and large around 10% of cancers & is specifically seen in astrocytic tumours of brain. Genetic indications, including ATRX, may enrich categorization of gliomas.<sup>[7]</sup>

There are however a-few tumors that showcase nuclear ATRX loss in the dearth of IDH1 along with a tiny portion of IDH1 mutated tumors unaccompanied by ATRX-mutation. This shows that alternate molecular ways might be engaged in patho-physiology of these neoplasm subcategory. More data are required to showcase further feasible cellular drivers in these neoplasms. In glial neoplasms, the presence of ATRX alterations can be visualized through nuclear staining loss and the presence of IDH1 mutation can be seen through strong cytoplasmic immunoreaction in immunohistochemistry.

These molecular markers aid to categorize the glial neoplasms into various molecular subcategories & in addition help to proffer diagnostic details. With the help of present study, we will assess the correlation between IDH1 mutation and ATRX expression with the histopathological grades in glial tumors in comparison with the present-day molecular advances.

## RESEARCH GAP

The present study aims to close the gap of understanding between correlation between IDH1 mutation and ATRX expression with the histopathological grades in glial tumors in comparison with the present-day molecular advances. These molecular markers aid to categorize the glial neoplasms into various molecular subcategories & enlightens the diagnostic details as well.

With the help of present study, we will be able to breach the gap of understanding the relationship between the correlation of IDH1 mutation and ATRX expression with the histopathological grades in glial tumors in comparison with the present day molecular advances on immunohistochemistry for better understanding.

## RESEARCH QUESTION:

While trying to perceive the relationship between the correlation of IDH1 mutation and ATRX expression with the histopathological grades in glial tumors the following research question is framed – Does these molecular markers assist us to categorize the glial tumors into various molecular subgroups, and do they give us any prognostic information.

## METHODOLOGY:

**STUDY DESIGN** - Observational, analytical, retrospective.

**PLACE OF STUDY** – Department of Pathology, JNMC, Sawangi (Meghe), Wardha, Maharashtra.

**DURATION** – 2020 to 2022 (2 years)

## METHODS –

- The cases confirmed as “Glial tumours” on clinical and radiological examination will be respectively operated on and the resected specimens will be received in the section of Histopathology in the Department of Pathology, J.N.M.C.
- The resected specimens will be kept in 10% Formalin for 12-24 hours for fixation.
- Gross examination of the received specimens will be done and appropriate sections will be taken.
- Specimens will be subjected to routine tissue processing after which routine Haematoxylin and Eosin (H&E) staining will be carried out.
- The histological grade of the tumour will be determined by the WHO Grading System.
- Immunostaining for IDH1 and ATRX will be carried out to evaluate IDH1 and ATRX expression in each case.
- After which, correlation of IDH1 utility and ATRX expression of each case with WHO histopathological grades of glial tumours on immunohistochemistry will be done.

## SAMPLE SIZE – 45-50 patients

The sample size was calculated by using Krejcie and Morgan formula with desired error of margin:

$$n = (Z \alpha/2)^2 \times p \times (1-p) / d^2$$

where,

$Z\alpha/2$  is the level of significance at 5% i.e. 95 % confidence interval = 1.96

p = prevalence of glial tumors = 1% = 0.01

d = desired error of margin = 3% = 0.03

$$n = (1.96)^2 \times 0.01 \times (1 - 0.01) / (0.03)^2$$
$$= 42.25$$

= Approximately 45-50 patients needed in the study.

**Inclusion Criteria:**

1. Patients suspected as gliomas on radiology.
2. Patients of all ages and gender.
3. Primary cases of gliomas.

**Exclusion Criteria:**

1. All cases of non- neoplastic lesions of the brain.
2. Non- glial tumors.
3. Recurrent cases.

**Staining Protocol: Haematoxylin and Eosin staining:<sup>[8]</sup>**

- Sections are deparaffinized in xylene: 3 changes of 10 minutes each.
- Dewaxing of sections is done. Sections are rehydrated through descending series of alcohol.
- Bring sections to water.
- Stain with Harris haematoxylin in a jar for ten minutes.
- Wash properly under tap water for 2-3 minutes.
- Separate in 1% acid alcohol (1%HCl in 70%alcohol) for few seconds.
- Wash under alkaline tap water (bluing) for 5 minutes.
- Stain in 1% aqueous Eosin for 1 minute.
- Dehydrate through 90% alcohol.
- Mount in dibutyl phthalate polystyrene xylene (DPX).

**Immunohistochemistry staining for IDH1-R132H & ATRX marker<sup>[9]</sup>**

- Immunostaining will be executed as stated by manufacturer's proprieties. Briefly, sections fixed with formalin, embedded with paraffin, cut to four micrometer will be parched for 15 min at 80°C and unwaxed in xylene, bathed in graded ethanol, and remoistened in double-distilled water.
- These sections will be processed with 3% H<sub>2</sub>O<sub>2</sub> at room temperature for 5 min. to mask endogenetic action of peroxidase. For antigen-retrieval, slides will be pre-treated by agitating in sodium citrate buffer (10 mM sodium citrate, pH 6.0) at 100°C for 15 minutes.
- Post cleansing with phosphate-buffered saline for 3 minutes, immunostaining of sections with an anti-human IDH1-R132H antibody (at 1:60 dilution, H09, Dianova, Hamburg, Germany) or an anti-human ATRX antibody (at 1:800 dilution, ab97508, Abcam) will be done, and incubation will be maintained overnight at 4°C.
- Post cleansing with 3 changes of PBS buffer, the tissues will be concealed by anti-mouse/rabbit polymer HRP-label at room temperature for about 30 min.

- Staining reaction will be performed through concealing tissue with readymade DAB chromogen solution, and incubated for about 10 min. for genuine brown colour expression.
- Standard of IDH1-R312H staining: (1) a powerful cytoplasmic immunoreaction outcome will be scored as positive; (2) a weak dispersed staining and staining of macrophages won't be scored as positive.
- Standard of ATRX staining: nuclear ATRX loss are marked as distinct if nuclei of tumour cells are not stained while non-neoplastic tumour cell nuclei such as endothelia, microglia, lymphocytes and reactive astrocytes will be definitely positive.

### Analysis of Statistics

Statistical analysis will be figured out using 't'-test and by multiple linear regression analysis to find the correlation between IDH1 mutation, ATRX expression and histopathological grades of glioma according to the WHO classification.

### EXPECTED RESULTS:

The study will be conducted for a period of 2 years and all the observations will be depicted in a well-tabulated master chart.

### DISCUSSION:

**Liu XY, Gerges N, Korshunov A, et al.** <sup>[10]</sup> conducted a study which showed frequent mutations of ATRX and adult diffuse astrocytic tumor's expression loss having IDH1/IDH2 & T53 mutations in 191 adult gliomas.

In a study conducted by **Wiestler B, Capper D, Holland-Letz T, et al**<sup>[11]</sup> they demonstrated loss of ATRX protein expression which enriches the categorization of anaplastic glial neoplasms & assimilate a subcategory of astrocytic tumors with IDH-mutation along with improved chance of recovery on 133 patients.

**Jiao Y, Killela PJ, Reitman ZJ, et al.** <sup>[12]</sup> researched upon recurrent ATRX, CIC, FUBPI and IDH1 alterations which enrich the neoplastic gliomas categorization. They perceived above mentioned allele in 363 cases of brain malignancies. ATRX is habitually altered in grade II and III astrocytomas (71%), oligoastrocytomas (68%), and secondary glioblastomas (57%), and ATRX alterations are linked with IDH1 mutations and with a substitute elongation of telomeres physical expression of DNA.

**Ikemura M, Shibahara J, Mukasa A, et al.** <sup>[13]</sup> showed ATRX usefulness in adult diffuse glial tumours identification on immunohistochemistry. 193 cases of adult diffuse gliomas went through immunohistochemical investigation. In zones where internal controls, neurones, glia and blood vessels were appropriately stained, the ATRX-immunoreactivity of tumour cells was nearly entirely negligible or thoroughly preserved in all cases. In 19 cases, there was absolute consonance among the immunohistochemical outcomes and ATRX mutation status.

Few of the related studies were reported in GBD studies<sup>[14-16]</sup>. Taksande et. al. reported a case of Glioma Presenting as an Isolated Facial Nerve Palsy <sup>[17]</sup>. Jham et. al. studied

correlation of the proliferative markers (AgNOR and Ki-67) with the histological grading of the glial tumors<sup>[18]</sup>. Similar related studies were reviewed<sup>[19-27]</sup>.

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UNDER PEER REVIEW