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Research Article

ROLE OF IDH1 MUTATION BY R 132 H ANTIBODY CLONE H09 IN GRADE II & III GLIOMAS AND SECONDARY GLIOBLASTOMA (As per current classification of CNS Tumors 2016)

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ABSTRACT

As per WHO CNS classification 2016, grade II diffuse astrocytomas and grade III anaplastic astrocytomas are now divided into Isocitrate Dehydrogenase (IDH)-mutant, IDH-wildtype and not otherwise specified(NOS) categories. About 10 % of cases of low grade glioma that arises in young and having IDH1 mutation progress to secondary glioblastoma(GBM). So IDH sequencing is highly recommended. The study aims to show the presence of IDH1 mutation in low grade gliomas (grade II & III) and GBM and classify them as per new WHO CNS Classification 2016.Two out of five cases (40%) of anaplastic astrocytoma, nine out of fifteen cases(60%) were diffuse astrocytoma, three out of three(100%) of secondary GBM(sGBM) and none out of twenty-four of twenty-fourof primary GBM(pGBM) were IDH1 positive. Classifying grade II & III gliomas into IDH mutant and wild type categories reduces NOS Catagories. Immunohistochemistry (IHC) is a simple and valuable diagnostic tool in assessing the IDH1 mutational status.

INTRODUCTION

Gliomas, the most common type of primary brain tumors, are classified as Grade- I to Grade- IV basing onhistopathological and clinical criteria established by the World Health Organization (WHO) (Louis DN *et al*, 2007). WHO Grade-I gliomas, considered to be benign,ascured with complete surgical resectionand rarely, if ever, evolve into higher-grade lesions (Burger PC *et al*, 2000). By contrast, Grade II or III (diffuse gliomas) are invasive, progress to higher-grade lesions, and have a poor outcome.Grade IV tumors (glioblastomas), the most invasive form, have a dismal prognosis (Stupp R *et al*, 2005; Wen PY and Kesari S, 2008)

Glioblastomas (glioblastomamultiforme [GBM]) are the most commonand aggressive malignant brain tumor, with a median survival from diagnosis of approximately 12 to 14 months (Louis DN *et al*, 2007). The majority of glioblastomas (90%) occur without evidence of a less malignant precursor lesion (primaryglioblastomas) in older patients, whereas secondary glioblastomas progressfrom low-grade diffuse astrocytoma or anaplastic astrocytoma, and occur inyounger patients (Burger PC *et al*, 2000).Secondary glioblastomas have a significantly betterprognosis than primary glioblastoma (Burger PC *et al*, 2000).

2016 update of the 2007 WHO classification incorporates well-established molecular parameters into the classification of diffuse astrocytoma. Presently Grade-II and Grade-III astrocytomas are grouped together as diffuse astrocytoma, based on growth pattern, behaviour and mutation of Isocitrate Dehydrogenase1 (IDH1) gene. WHO 2016 update of 2007 classification of Grade II-IV astrocytoma are groped as IDH1-wildtype, IDH1-mutant and IDH1-NOS(not otherwise specified). The IDH1 gene is located on chromosome 2p33. The IDH1 protein is localized in the cytoplasm and in peroxisomes and catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate, resulting in the production of reduced nicotinamide adenine dinucleotide phosphate (NADPH). NADPH is an important intracellular antioxidant (Watanabe T *et al*, 2009).

Anti IDH1-R132H immunostaining is a reliable method for evaluation of IDH1 gene mutation status. Advantages of this method are the easy implementation in most standard neuropathology laboratories, time and cost efficiency, and the possibility of evaluating the morphologic expression pattern of the aberrant protein (Matthias P *et al*, 2011). Anti-IDH1-R132H immunostaining has been suggested to be useful for identification of single tumor cells in small biopsy in contrast

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to time taking and expensive gene sequencing technology. IDH1 mutation is linked to the genomic profile of the astrocytoma Grade II-IV and is a good prognostic marker for targeted therapeutic purpose(Parsons DW *et al*, 2008;Sjoblom T *et al*, 2006; Wang TL *et al*, 2004; Batinic-Haberle I *et al*, 2008). This study illustrates IDH1 mutation in low grade gliomas (grade II & III) &glioblastoma cases and classify them as per the recent WHO classification OF CNS TUMOUR 2016 maintaining the multilayering reporting format (David NL *et al*, 2016).

MATERIALS AND METHODS

This prospective study was carried out in the Department of Pathology and Neurosurgery in a Medical College, between January-2016to December-2017 and comprised of 47 cases of astrocytoma occurring in frontal, parietal, temporal and occipital regions of the brain. Cases included- Diffuse astrocytoma, Anaplastic astrocytomaand GBM cases(both primary & secondary) .The study was undertaken after obtaining Institutional Ethical Clearance and patient consent.

Formaldehyde fixed biopsy specimens were received and subjected to routine histopathological examination. Paraffin embedded sections were subjected to routine histopathology using Haematoxylin & Eosin (H&E). All the cases were classified and graded morphologically according to WHO diagnostic criteria for CNS neoplasms. Imminohistochemistry (IHC) was done by using, Glial Fibrillary Acidic Protein(GFAP) and Ki67 for typing and grading of the tumour. Molecular study was done using Anti IDH1 R 132 H / DIA-HO9 kit; BIOINFINITY, GERMANY. The physical state of the antibody: Lyophilized powder, Isotype: Mouse IgG2a, Clone: H09, Concentration: 0.2mg/ml using distil water. Protocol: immunostaining performed manually using HRP/DAB MR Polymer Detection kit dianova as the following stepsg: 1) Dewaxe and rehydrate sections: Xylol: 3x10min / EtOH: 2x100%, 2x 95%, 1x70%, 1xH2O; 3min each, 2) Perform heat induced antigen retrieval (HIER) using citrate buffer at pH6 (CC2 solution, Ventana) by cooking for 60min in a steamer, 3) Cool slides for 5min. Wash with 3 changes of PBS buffer, 3min incubation per step, 4) Blocking endogenous peroxidases: Place slides in PeroxidasePlus-Block (dianova) for 10min. at RT, 5) Wash with 3 changes of PBS buffer, 3min incubation per step, 6) Blocking: Place slides in PBS buffer with 5% FCS and incubate for 30min at RT. 20. Cover tissue with primary antibody anti-IDH1 R132H/clone H09: Dilute 1:20-1:40 in PBS with 5% FCS and incubate at 4°C over night, 7) Wash with 3 changes of PBS buffer, 3min incubation per step, 8) Secondary antibody: Cover tissue with Anti-mouse/ rabbit polymer HRP-label (dianova, ready or use) for 30min at RT,9) Wash with 3 changes of PBS buffer, 3min incubation per step, 10) Prepare DAB by adding 2 drops of DAB-chromogen per 1ml DAB-substrate buffer (dianova) and mix, 11) Staining reaction: Cover tissue with prepared DAB chromogen solution, incubate approximately for 10min. to allow for proper brown colour development, 12) Wash slides thoroughly in H2O, 13) Counterstain with hemalun for 2min, 14. Wash slides in H2O and 15) Coverslip with mounting medium (Immunoselect, dianova). Positive control was 1) neuroglial cell from normal brain tissue and 2) Colour card provided by the manufacturer.The interpretation of IDH1 cytoplasmic staining: single cell with dark brown cytoplasm is considered positive.Few GBM patients (04) and one anaplastic astrocytoma patient died during this study period due to some complications. 3 months follow up continuing for the all cases and GBM patients during follw-up.

RESULTS

0ut of 47 cases 38% belonged to age group of 50-60 yrs, youngest being child was 2yr and oldest 65 years and majority 59% cases were male indicating male predominance (Table-1). AntiIDH1-R132H antibodies can be demonstrated in the cytoplasm of astrocytes. A total of 47 cases were studied; 15 cases of Diffuse Astrocytoma, 05 cases of Anaplastic Astrocytoma , 24 cases of primary Glioblastoma and 03 cases of Secondary Glioblastoma(Table-2). Regarding distribution of these tumours according to site, 37 out of 47 cases (79%) were supratentorialand rest 10 cases (21%) were infratentorial (Figure-1)

Table I showing age and sex distribution of patients

Age in distribution	Male	Female	Total	Percentage
0-10 YEARS	01	01	02	04
10-20 YEARS	00	02	02	04
20-30YEARS	03	01	04	09
30-40YEARS	02	01	03	06
40-50YEARS	09	04	13	29
50-60YEARS	13	05	18	38
60-70YEARS	04	01	05	10
TOTAL	32 (68%)	15 (32%)	47	100

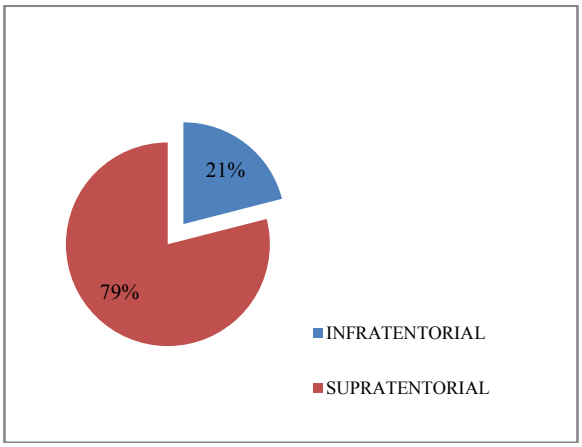

Figure 1 Sitewise Distribution of Tumors

Anti IDH1-R132H immunostaining of tumor cells was found in 15 (31.9%) out of the 47 cases using both antibodies (DIA-H09) (Table-II). Diffuse staining of all tumour cell seen in 12 cases, whereas 03 cases showed patchy and single cell pattern of cell positivity. 09 out of 15(60%) cases of diffuse astrocytoma were IDH1 positive, 02 out of 05(40%) cases of anaplastic astrocytoma were IDH1 positive, none of the 24 cases of primary GBM (pGBM) were IDH1 positive and all 3 secondary GBM (sGBM) cases were IDH1 positive. Diffuse astrocytoma CT scan and IDH1 positivity is shown in Figure-2 and Figure-3 respectively. IDH1 positivity in IHC and histomophology of anaplastic astrocytoma are shown in Figure-4 and Figure-5 respectively.

Table II Idh1 Mutation in Different Tumors

Type of tumor	Male	Idh1 +ve	Female	Idh1 +ve	Total No. of cases	Total idh1 +ve	% of idh1 +ve
Diffuse Astrocytoma	10	07	05	2	15	09	60 %
Anaplastic Astrocytoma	04	02	01	0	05	02	40%
Primary GBM	16	0	08	0	24	0	0%
Secondary GBM	02	02	01	1	03	03	100%
TOTAL	32	12	15	3	47	15	32%

Male to female ratio were 2:1, 4:1 and 2:1 in diffuse astrocytoma, anaplastic astrocytoma and GBM cases respectively. Out of 47 number of astrocytic tumour 12 cases showed a strong cytoplasmic and often a weak nuclear staining of tumour cells with diffuse staining of the fibrillary tumour matrix. The diffuse staining was limited to the tumour containing area and was consider a specific antibody staining.In positive cases, all tumour cell are marked positive by R132H antibody where as endothelial cells, perivascular lymphocytes and residual brain glial cells are negative.

Reactive gliosis are not recognized by R132H antibody. All 03 negative controls (reactive gliosis, meningioma, ependymoma) donot show binding to R132H antibody with a weak diffuse background staining. A three tier reporting system is there for interpretation of IDH1 positivity but in this study a single cell positive case is also consider positive.

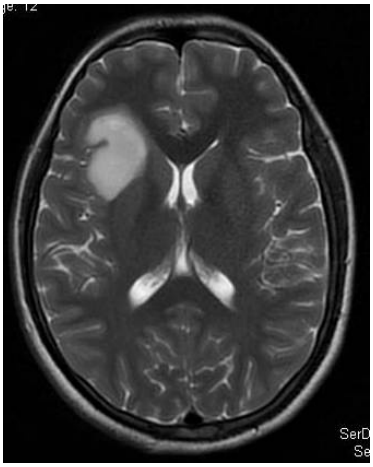

Figure 2 CT Scan showing NonEnhancing Lesion in Right Frontal Lobe

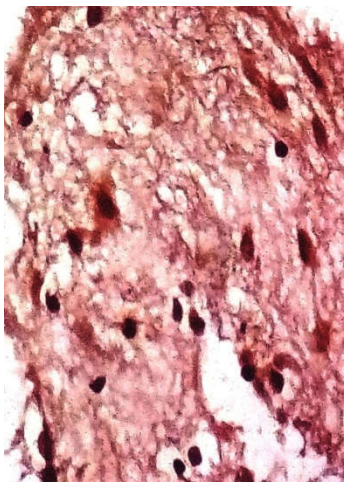

Figure 3 shows Anti IDH 132 H positivity,(single cell with dark brown cytoplasm) in Diffuse astrocytoma (grade II)

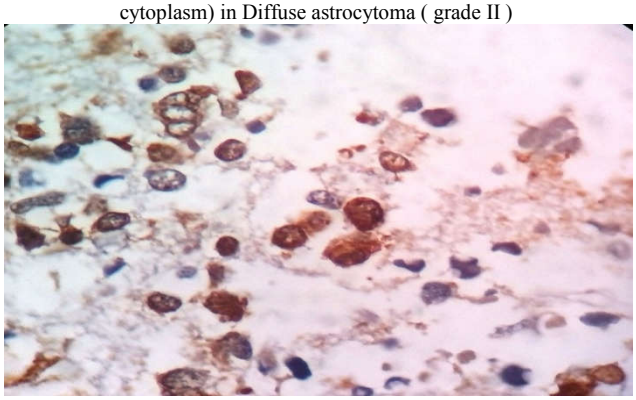

Figure 4 Anti IDH 123 H positivity in tumor cells in Anaplastic astrocytoma (grade III)

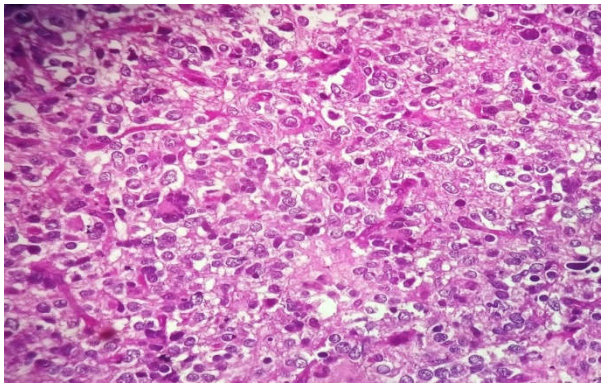

Figure 5 increased cellularity as compared to the grade II , distinct nuclear atypia and mitotic activity suggestive of Anaplastic astrocytoma (III)

DISCUSSION

GLIOMAS, the most common type of primary brain tumors, are classified as grade I to grade IV on the basis of histopathological and clinical criteria established by the WHO (Pressure M *et al*, 2011). This group of tumors includes specific histologic subtypes, the most common of which are astrocytomas, oligodendrogliomas, and ependymomas. WHO grade I gliomas, often considered to be benign, are generally curable with complete surgical resection and rarely, if ever, evolve into higher-grade lesions (Ohgaki H *et al*, 2007). By contrast, gliomas of WHO grade II or III are invasive, progress to higher-grade lesions, and have a poor outcome (Burger PC *et al*, 2000). WHO grade IV tumors (glioblastomas), the most invasive form, have a dismal prognosis (Stupp R *et al*, 2005). On the basis of histopathological criteria, it is impossible to distinguish a secondary glioblastoma, defined as a tumor that was previously diagnosed as a lower-grade glioma, from a primary tumor (Wen PY *et al*, 2008).

Several genes, including TP53, PTEN, CDKN2A, and EGFR, are altered in gliomas (Lynda C and MeyersenM , 2008; Li J *et al*, 1997; Nigro JM *et al*, 1989; Ueki K *et al*, 1996). These alterations tend to occur in a defined order during the progression to a high-grade tumor. The TP53 mutation appears to be a relatively early event during the development of an astrocytoma, whereas the loss or mutation of PTEN and amplification of EGFR are characteristic of higher-grade tumors (Ueki K *et al*, 1996; Wong AJ *et al*, 1987). In a recent genomewide analysis, somatic mutations at codon 132 of the isocitrate dehydrogenase 1 gene (IDH1) is identified in

glioblastomas. The multilayer reporting formatas per WHO 2016 CNS TUMOR classification includes four basic layers namely Layer 1: Integrated diagnosis (incorporating all tissue based information); Layer 2: Histological Classification; Layer 3: WHO grade (reflecting natural history) and Layer 4: Molecular information. IHC may be may be more sensitive method for detection in IDH1 in astrocytoma in comparison to traditional Sanger sequencing and polymerase chain reaction (PCR) because of their ability to detect even single IDH-mutant-containing tumor cells and distinguish them from non-neoplastic brain tissue or tissue contaminants. This method is simple, fast and reliable.

Immunostains GFAP and MIB index using KI67 antibody help in histological typing and grading. The molecular information in astrocytoma can be done by demonstrating IDH mutation. Cytosolic IDH1 mutation has emerged as a major diagnostic and prognostic biomarker for gliomas. Important earlier findings in a fraction of GBM tumours led to the identification of IDH1 mutations in a vast majority of diffuse astrocytomas, oligodendrogliomas, and mixed oligoastrocytomas of WHO grades II and III. The current study is to validate the IHC expression of IDH1 mutant protein across various grades of glioma. This study detected IDH1 mutations and its mutant protein expression in diffusely infiltrating astrocytoma samples of different grades.

In the study, diffuse astrocytoma (grade II) were 15 cases; out of which 09 cases (60%) were IDH1 mutant as it showed R132H IDH1 protein expression and. rest 06 cases are IDH1 wild type. In studies by Pressure M *et al* (2011)[and David Capper *et al* (2010), positivity of grade II diffuse astrocytic tumour were73 % and 83% respectively.

In our study, anaplastic astrocytoma (grade III) were 05 cases; outof which two cases (40%) were IDH1 mutant and rest 03 cases were IDH1 wild type. Similar studies by David Capper *et al*(2010)and Hartmann C *et al* (2009) showed positive results in 81% and 61% of cases.

In pre IDH era , the median survival of anaplastic astrocytoma (grade III) patient was reported to be in the range of 3-5 years. But once the tumour becomes IDH1 positive, median survival time becomes 9.3 years (Hai Yan *et al*, 2009).It is recommended that WHO grading is retained for both IDH1-mutant and IDH1-wild type astrocytomas, although the prognosis of the IDH1-mutant cases appears more favourable in both grades.

Out of 27 cases of glioblastoma (GBM) in the study; 04 cases were IDH1 positive(15 % of GBM cases and 12% of total study population) . Of the 24 cases of pGBM none were IDH1 positive whereas all three cases(100%) of sGBMwere IDH1 positive. However in literature pGBM with IDH1 mutation is absent. This study is in concordance with David Capper *et al* (2010) study where the IDH1 positivity in secondary GBM was 96%. IDH1 immunostain differentiate a grade III astrocytoma from pGBM andpGBM from sGBM (David Capper *et al*,2010)

Parsons DW*et al* (2008) first reported on on improved survival in patientswith GBM with IDH1 mutations (45.6 vears 13.2 months in IDH1-mutations versus IDH1-wildtype respectively).In addition to improved Overall Survival, Houillier C *et al* (2010), were able to demonstrate improved progression free survival (PFS) as well in their set of patients with GBM, with 55 months PFS in patients with IDH1 mutation versus 8.8 months PFSin those without it. The analysis was extended to anaplastic (WHO gradeIII) tumors because many groups were readily able to show an improved OSin grade III tumors that harboured the IDH mutation compared with those thatdid not in both univariate and multivariate analyses. In a prospective analysis,

It is still unclear if IDH1 mutational status is a prognostic indicator ora predictive measure of response to treatment. Houillier*et al* (2010) stratified a cohort of LGG into 3 groups based on prognostic factorsbased on the presence of 1p 19q deletion, IDH1 mutation, or bothtogether. They found that each of these factors was an independentpredictor of improved clinical outcome in response to treatment with thechemotherapeutic agent temozolomide and that the group of patientswith both mutations had the best treatment response (objective responsein 80% with both mutations, 61% of IDH1-mutants without 1p19qdeletion, 17% without either mutation). These findings support the notion that IDH1mutations may be an important predictor to treatment response. However,Gravendeel LA *et al* (2009) reported that improved prognosis was foundregardless of adjuvant therapy when investigating IDH1-mutant gliomain response to procarbazine (Matulane), lomustin (CCNU), andvincristine (Oncovin) chemotherapy. Future studies are necessary to betterdetermine the prognostic versus predictive role of IDH1 status in humanglioma.

The limitations of the study are: i) the study is of one and half year duration and has a small sample size ii) Four GBM patients (four) and one anaplastic astrocytoma patient died during this study period due to some complications and iii) Grade II & III astrocytic tumour takes a period of 08 to 10 years to develop into GBM and due to this longer duration of survival time, follow up results were not available.

CONCLUSION

Cytosolic IDH1 mutation has emerged as a major diagnostic and prognostic biomarker for gliomas. Important earlier findings in a fraction of GBM tumors led to the identification of IDH1 mutations in a vast majority of diffuse astrocytomas, oligodendrogliomas, and mixed oligoastrocytomas of WHO grades II and III.

The current study validates the frequency of IDH1 mutations across different grades of diffusely infiltrating astrocytomas. In my study we used IDH1 R132 H 09 stain immunohistochemistry by manual method and classified grade II & III gliomas into IDH mutant and wild type categories there by reducing NOS Catagories. Also we classified GBM cases into primary and secondary types.

The practical aspects of routine IDH1 R 132 H IHC are clear-Faster turnaround time, Lower costs and ability to detect just a few single positive cells for that antibody based detection of IDH1 R132H mutation appears to be superior in sensitivity compared with direct sequencing, especially in low grade diffuse astrocytoma. Study reveals the usefulness of a simple laboratory technique, IHC, as a valuable diagnostic tool in

assessing the IDH1 mutational status and suggests the need for future studies on larger prospective cohorts to elicit the prognostic significance of this molecular marker in patients with newly diagnosed GBM.

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