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BRAF^{V600E} Mutation in Gliomas in Iraqi Patients Immunohistochemical Study

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Abstract: Gliomas considered the most common primary malignant brain tumors in adults. *BRAF*^{V600E} mutation occurs more in pediatric gliomas but less frequent in adult gliomas. This study aims to assess the frequency of *BRAF* mutation in Iraqi patients with gliomas by immunohistochemical study and to correlate its immunoreactivity with some clinicopathological parameters This work did on formalin fixed, paraffin embedded tumor tissue took from 66 patients with different grades of intracranial gliomas of both gender and all age groups in the Baghdad city. Ten normal brain tissue samples in form of paraffin blocks took from forensic medicine unit. New technique used, that was manual tissue microarray, in which twenty-four small cores (each measure 2mm) of represented tissue make, and then cut by microtome. Immunohistochemical detection of *BRAF*^{V600E} antibody did by Dako autostainer link 48. *BRAF*^{V600E} expressed as cytoplasmic staining in seven (10.60%) cases of glioma. It detected in pleomorphic xanthastrocytoma, oligodendroglioma, anaplastic astrocytoma, anaplastic oligodendroglioma, anaplastic oligoastrocytoma and primary glioblastoma (100%, 9.1% 8.6%, 20.0%, 25% and 6.7%). It had a strong association with pediatric gliomas. From the work concluded that *BRAF*^{V600E} mutation occurred in low percentage in gliomas especially adult types and significantly express in pediatric gliomas and some rare glial tumors.

Keywords: *BRAF*, Adults, Pediatric, Manual Tissue Microarray, Glioma, Immunohistochemical Study

1. Introduction

"Glioma" is a universal term used to label any tumor that arises from the supportive "gluey" tissue of the brain [1]. The most common type of central nervous system tumors in adults is malignant gliomas (about 86%), which are divided into low grade and high grade [2], [3]. According to histopathological and clinical criteria established by the WHO gliomas are categorized as grade I to grade IV [4], [5], [6]. WHO grade I gliomas, have an idle growth, often considered benign, and hardly ever, evolve into higher-grade lesions [7]. By contrast, gliomas of WHO grade II or III are aggressive tumors, usually invasive, diffuse, advance to higher grade (grade III or IV) lesions, and have a poor outcome [7], [8]. Knowledge about the molecular aberrations driving the development and progression of gliomas is steadily growing [8]. Counting recent discoveries of novel genetic alterations in both pediatric and adult brain tumors, such as the frequent mutations in the *IDH1* gene in diffuse gliomas, *BRAF* gene mutations in pediatric gliomas, the combined complete loss of chromosomal arms 1p and 19q in oligodendroglial tumors and others molecular abnormalities [8]. Those molecular markers considered as attractive targets for molecular diagnostic testing aiming to improve treatment stratification and prognostic assessment of the glioma's patients [8]. One of the molecules has been of greater recent apprehension to molecular neurooncology is *BRAF*. Because it is, a key gene altered in most pediatric low-grade gliomas [9]. *BRAF*, a member of the RAF family including *ARAF*, *BRAF* and *RAF1*, which is a serine/threonine protein kinase encoded by *BRAF* gene on chromosome 7q34 that serves as an immediate downstream effector of RAS in the RAS/RAF/MEK/ERK signaling cascade [10], [11]. That transmits mitogenic signals from activated growth factor

receptors on the cell surface and modulates various processes, including cell proliferation and survival [10], [11]. In contrast to other serine/threonine kinase family members, *BRAF* is expressed most highly in neuronal tissues, melanocytes and hematopoietic cells [10], [11], [12]. Oncogenic activation usually results from point mutations rather than gene rearrangements. The most common *BRAF* mutation is the T1796A point mutation, resulting in a valine (V) to glutamic acid (E) substitution at position 600, so named as $BRAF^{V600E}$ [12], [13]. The $BRAF^{V600E}$ mutation interrupts the hydrophobic interaction, enabling the BRAF kinase to fold into a catalytically active formation, resulting in an almost 500-fold increase in kinase activity [13], [14].

The finding that $BRAF^{V600E}$ displays increased kinase activity relative to the wild-type protein and has transforming capacity further reinforces this proof [11].

Regarding *BRAF* mutations and glioma; activation of the MAP kinase/ERK-signaling pathway appears to play an important role in the pathogenesis of a subset of glial/glioneuronal tumors [10], [15], [16], [17].

$BRAF^{V600E}$ mutations have been found in approximately 10–15% of pilocytic astrocytoma [15], [16], [17], [18], [19], [20], and in approximately 5–10% of pediatric diffusely infiltrating gliomas, including diffuse astrocytomas (WHO grade II), anaplastic astrocytomas (WHO grade III) and glioblastomas (WHO grade IV) [11], [15], [16], [17] but in less than 2% of comparable adult gliomas [11].

Mutations have been also identified in gangliogliomas (GGs), as well as in pleomorphic xanthoastrocytomas (PXA) [10], [15], [16], [17], [19], [21]. Among ganglioglioma, the incidence of $BRAF^{V600E}$ mutations varied from 30 to 60% and the mutated protein seems to be predominantly localized to the neuronal compartment [22]. In PXA, the incidence of *BRAF* mutations is app The $BRAF^{V600E}$ mutation in brain tumors did not have prognostic value but is certainly a diagnostic marker and therapeutic target, not only for pediatric low-grade gliomas but also for malignant gliomas, because they are rarely but also found in high-grade gliomas [15]. In PXA, the incidence of *BRAF* mutations is approximately 60% and appears to be age related [10], [21], [23], [24].

The $BRAF^{V600E}$ mutation in brain tumors did not have prognostic value but is certainly a diagnostic marker and therapeutic target, not only for pediatric low-grade gliomas but also for malignant gliomas, because they are rarely found in high-grade gliomas [15].

The aims of this study were to validate the frequency of *BRAF* mutation in Iraqi patients in Baghdad city with gliomas by immunohistochemical study and to correlate its positivity with certain clinicopathological variables.

2. Patients, Materials and Methods

2.1. Patients and Materials

This is a retrospective and prospective randomized study. In a period extended from October 2013- June 2016, 66 cases of intracranial gliomas of both gender and all age groups in the Baghdad city were included in this study. Formalin fixed, paraffin embedded brain excisional biopsies of the cases retrieved from the archival materials of a pathology laboratories of Neurosurgical hospitals in Baghdad and some private clinical laboratories.

In addition, ten normal of different age groups of brain tissue samples in form of paraffin blocks took from forensic medicine unit.

The clinical data of the patients including age and gender, radiological findings of site and side of affection and the provisional clinical diagnosis obtained from archival histological reports.

Hematoxylin and Eosin stained section from each case revised, concerning the pathological type and grade to prove the diagnosis of gliomas. The cases graded and classified according to WHO classification of the central nervous system tumors [5].

2.2. Methods

a. Tissue Microarray Technique (TMA)

This achieved by using manual TMA kit. Manual TMA kit contains two components (Moulder and Puncher extractor tool) [25]. In the tissue microarray procedure, a hollow needle used to cutting tissue cores as small as 2 mm in diameter from regions of concern, (areas of glial tumors that detected previously in (H & E) staining slides) in paraffin-embedded tissues [25]. A microarray recipient‘s paraffin block contains 24 small cores of demonstrative tissue samples, each measure 2 mm in diameter. Sections from microarray block cut using a microtome, equestrian on a single microscope slide and then evaluated by staining with H and E stain, then another sections made on 3-micron thickness for the immunohistochemical stain for $BRAF^{V600E}$ antibodies.

b. Immunohistochemical technique

The immunohistochemical work in this study was done by Dako automated Autostainer Link 48 with Dako EnVision™ FLEX detection system used in immunohistochemistry work [26].

Three-micron sections obtained from formalin fixed, paraffin embedded tissue blocks and mounted on Dako FLEX IHC slides, then allowed to fix overnight in oven at temperature 65ºC. Then performed Pre-treatment procedure, which recommended three in one specimen preparation procedure using PT Link: Deparaffinization, rehydration and heat-induced epitope retrieval (HIER) on the tissue sections. Prepared a working solution by diluting the EnVision™ FLEX Target Retrieval Solution concentrate 1:50 in distilled or deionized water then PT Link tanks filled with sufficient quantity (1.5 L) of working solution to cover the tissue sections. PT Link established, to pre-heat the solution to 65°C. Immersed the mounted, formalin-fixed, paraffin-embedded tissue sections into the pre-heated EnVision™ FLEX Target Retrieval Solution in PT Link tanks and incubate for 20 minutes at 97°C then the sections put to cool in PT Link to 65°C for 20 minutes. Each autostainer slide

rack removed with the slides from the PT Link tank and immediately dip slides into a tank (PT Link Rinse Station, Code PT109) with diluted, room temperature EnVision™ FLEX Wash Buffer (20x). The slides placed in the diluted, room temperature EnVision™ FLEX Wash Buffer (20x) for five minutes and sited them on a Dako Autostainer Link 48 and proceeded with staining. Staining procedure, anti $BRAF^{V600E}$ antibody (Rabbit Monoclonal Antibody, Clone RM8; Catalog No. 31-1042-00, manufactured by Rev MAb Biosciences, USA). added in dilution 1:100 in autostainer system, the staining steps and incubation times are pre-programmed into the software of Dako Autostainer/Autostainer Plus instruments, using the protocols, Template protocol: FLEX_200 (200 µL dispense volume)

Autoprograms for staining runs, FLEX (FLEX protocol) in *BRAF*. The Auxiliary step should be set to "rinse buffer" in staining runs with ≤10 slides. For staining runs with ≥10 slides, the Auxiliary step should be set to "none". This ascertains comparable wash times. All incubation steps performed at room temperature. Regarding incubation times, for *BRAF* it was 60 minutes.

Finally, the sections lightly counterstained with hematoxylin, dehydrated and mounted. Negative control sections treated in the same way, but by the substitution of primary antibody with PBS. Positive control sections took from positive cases and performed in each batch of staining.

Immunohistochemical staining assessment: For $BRAF^{V600E}$ antibody, cytoplasmic staining intensity scored as:

- 0 no staining
- 1 faint, weak, and/or focal cytoplasmic staining was present.
- 2 moderate diffuse and granular cytoplasmic staining observed
- 3 strong mainly granular cytoplasmic staining detected.

A tumor considered positive for $BRAF^{V600E}$ if it displayed a staining intensity of number two or three irrespective of the percentage of tumor cells stained [10], [27].

Statistical analyses performed using SPSS statistical package for Social Sciences (version 17.0 for windows, SPSS, Chicago, IL, USA). The data were qualitative. So relations analyzed by Chi square test. P value of less than or equal to 0.05 was considered statistically significant.

3. Results

3.1. Clinical Analysis of the Samples

During the period of one year, a total of (66) tissue samples in the form of paraffin blocks of brain gliomas specimens was included in this study. The patients' age range from (1-75) years, distribution among age groups revealed that the mean age of cases in this study was 38.41, standard deviation= 18.15 years and median of 37 years. Most of cases were in the third and fourth decades. There were 9 (13.44%) pediatric patients and 57 (86.36%) adults.

Thirty-four (51.51%) were males and 32 (48.49%) were females and the male to female ratio was 1.06:1.

3.2. Histopathological Findings

Histological examination of Hematoxylin and Eosin stain (H&E) sections confirmed and grading was done to the cases according to the criteria established by WHO 2007. There were 2 cases WHO grade I (pilocytic astrocytomas and subependymal giant cell astrocytoma), 25 cases were WHO grade II (11 cases were diffuse astrocytoma, 1 case was pleomorphic xanthoastrocytoma, 2 cases were ependymoma and 11 cases were oligodendroglioma), 16 cases were WHO grade III (7 cases were anaplastic astrocytoma, 5 cases were anaplastic oligodendroglioma and 4 cases were mixed anaplastic oligoastrocytoma) and 23 cases were WHO grade IV glioblastoma (15 cases were primary glioblastoma and 8 cases were secondary glioblastoma).

3.3. BRAF ^{V600E} Immunohistochemical Expression in Gliomas

Positive $BRAF^{V600E}$ strong cytoplasmic staining observed in seven (10.60%) cases of glioma. This positive staining was appear only in tumor cells and not shown in normal brain tissue cases.

Correlation between BRAF Mutation and Some Clinicopathological Parameters

a. Age: Regarding adult and pediatric glioma cases;

Four cases of pediatric gliomas which were *BRAF* positive, and three cases of adult gliomas were *BRAF* positive (pathological types of these positive cases in pediatric were; anaplastic astrocytoma, primary glioblastoma, anaplastic oligodendroglioma and oligodendroglioma). Although 2 of adult gliomas were young adult (25, 32 years old) only one case was 39 years old. Difference in age distribution of *BRAF* positivity between adult and pediatric is statistically significance. P< 0.017. Table 1

Table 1. *Correlation of BRAF positivity with age.*

			BRAF (IHC)		Total
			Positive	Negative	
Age group	<18 year	Count	4	5	9
		%	44.44%	55.56%	100.0%
	>=18 year	Count	3	54	57
		%	5.3%	94.7%	100.0%
Total		Count	7	59	66
		%	10.6%	89.4%	100.0%

X^2 test, P< 0.017 S.

b. *BRAF* status and the pathological types of glioma

Positive *BRAF* expression saw in pleomorphic xanthastrocytoma 1 (100%) case, 1(9.1%) case oligodendroglioma, 2 (28.6%) cases anaplastic astrocytoma, 1 (20.0%) case anaplastic oligodendroglioma, 1 (25%) anaplastic oligoastrocytoma and 1 (6.7%) case primary glioblastoma. NO satirical association between BRAF positivity and pathological type of glioma (p =0.233). Table 2

Table 2. *BRAF status and types of gliomas.*

Type of Glioma		*BRAF* (IHC) Positive	*BRAF* (IHC) Negative	Total
Subependymal giant cell astrocytoma	Count	0	1	1
	%	0.0%	100.0%	100.0%
Pilocytic astrocytoma	Count	0	1	1
	%	0.0%	100.0%	100.0%
Diffuse astrocytoma	Count	0	11	11
	%	0.0%	100.0%	100.0%
Pleomorphic xanthastrocytoma	Count	1	0	1
	%	100%	0%	100.0%
Ependymoma	Count	0	2	2
	%	0.0%	100.0%	100.0%
Oligodendroglioma	Count	1	10	11
	%	9.1%	90.9%	100.0%
Anaplastic astrocytoma	Count	2	5	7
	%	28.6%	71.4%	100.0%
Anaplastic oligodendroglioma	Count	1	4	5
	%	20.0%	80.0%	100.0%
Anaplastic oligoastrocytoma	Count	1	3	4
	%	25.0%	75.0%	100.0%
Primary glioblastoma	Count	1	14	15
	%	6.7%	93.3%	100.0%
Secondary glioblastoma	Count	0	8	8
	%	0.0%	100.0%	100.0%
Total	Count	7	59	66
	%	10.6%	89.4%	100.0%

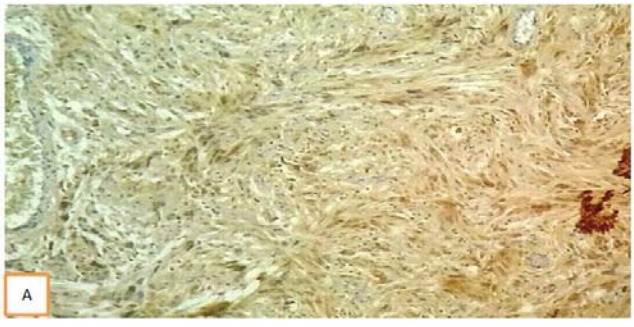
X^2 test, P=0.233 NS.

c. Concerning the grades of gliomas, *BRAF* expression was high in grades III, IV in 5 (71.42%) cases in compares to grades (I, II) 2 (28.58%), no significant relationship was identified between the grades of tumors and positivity of *BRAF* (P = 0.182). Table 3

Table 3. *Correlation of BRAF positivity and the grades of gliomas.*

			BRAF (IHC) Positive	*BRAF* (IHC) Negative	Total
Grade	I	Count	0	2	2
		%	.0%	100.0%	100.0%
	II	Count	2	23	25
		%	8.0%	92.0%	100.0%
	III	Count	4	12	16
		%	25.0%	75.0%	100.0%
	IV	Count	1	22	23
		%	4.3%	95.7%	100.0%
Total		Count	7	59	66
		%	10.6%	89.4%	100.0%

X^2 test, P=0.182 NS.



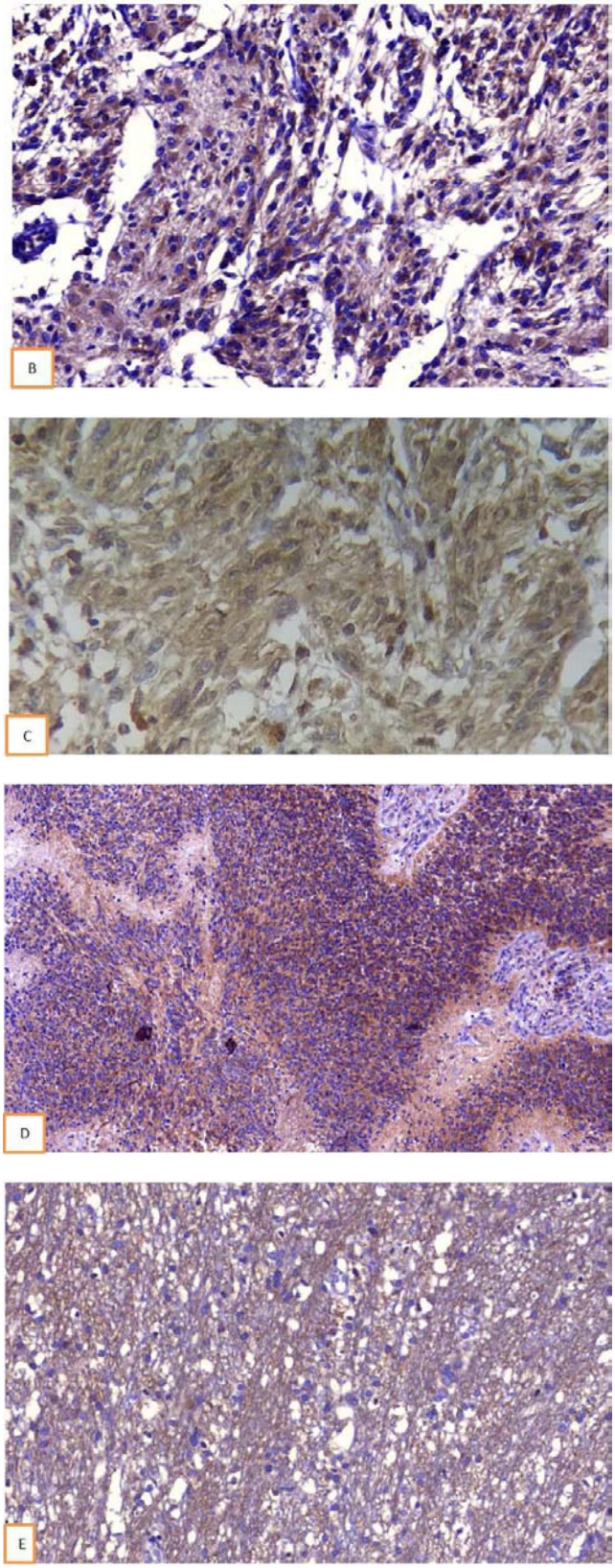


Figure 1. *Positive BRAF immunoreactivity in glioma cases: A- Pleomorphic xanthoastrocytoma (BRAF x100). B- Primary glioblastoma (BRAF x200). C- Anaplastic astrocytoma (BRAF x400). D- Anaplastic oligodendroglioma (BRAF x100). E- Anaplastic oligoastrocytoma (BRAF x200).*

4. Discussion

BRAF^{V600E} expression was detected in (10.6%) of whole gliomas cases, this agree with many studies that reported; *BRAF*^{V600E} was mutated in some brain tumors, including astrocytic tumors, oligodendroglial tumors, and ependymal tumors, but at relatively low rates [15], [17], [23], [24], [28], [29], [30], [31].

According to pathological types, one case of adult Pleomorphic xanthoastrocytoma (PXA) was present in the existing study, and was positive for *BRAF*^{V600E}, this agrees with many studies [10], [15], [17], [21], [32] that demonstrated the highest frequencies of *BRAF*^{V600E} mutation in primary central nervous system neoplasms have been reported in this type of tumors.

About age difference in this tumor type, Schindler et al found that 38% of adult PXAs showed this mutation, compared to 100% of pediatric PXAs [17].

This association of *BRAF*^{V600E} with PXAs suggested that the presence of this mutation might be helpful in distinguishing PXAs from histological mimics.

Regarding other types of gliomas in the current study, *BRAF*^{V600E} was positive in oligodendroglioma (9.1%), anaplastic astrocytoma (28.6%), anaplastic oligodendroglioma (20%), anaplastic oligoastrocytoma (25%) and primary glioblastoma (6.7%).

These results in spite of little higher percentages, went with line of Myung et al, Schindler et al and Dias-Santagata et al, in those studies *BRAF*^{V600E} mutation frequency in PXAs (60-66%), moderate frequency in GGs (18%) and PAs (9%), and a low frequency (0–5.9%) in other gliomas [15], [17], [21]. The possible explanations for this difference are the selection of cases in those studies, the large number of PAs, GGs, PXAs tumors in compare with the current study and the molecular testing method that used in those studies.

Myung et al analyzed *BRAF* mutation in 223 brain tumors by PCR and FISH techniques [15]. Schindler et al used 1,320 samples in the study and investigated *BRAF* mutation by PCR [17]. Dias-Santagata et al used SNaPshot method that consists of multiplexed PCR and multiplexed single-base extension, followed by capillary electrophoresis on 26 PXAs and 71 glioblastomas samples [21].

No statistical significant association was seen between pathological types and grading of glioma cases used in the current study and *BRAF*^{V600E} expression, this agree with Myung et al, Schindler et al and Dias-Santagata et al [15], [17], [21].

BRAF^{V600E} was highly expressed in high grades III and IV gliomas in the presented work (71.42%) in compare with low grades (28.58%), the presence of this mutations exclusively in high-grade lesions suggests that they may represent a late event in the gliomagenesis, and this variance with melanoma, where *BRAF* mutations are an early event [24].

Regarding pediatric and adult gliomas; 4 cases from 9(44.4%) of pediatric cases was positive for *BRAF*^{V600E} in compares' with adult cases, three cases from 57(5.7%) was positive for this mutation and there was a significant correlation regarding age groups. This clue went with the fact that *BRAF*^{V600E} mutation occurs more in pediatric and adolescent patients rather than adults [15], [16], [24], [29], [30], [33].

However, in the pediatric population, a *BRAF*^{V600E} result is not quite as helpful as *BRAF* fusion in differentiating non-infiltrative from infiltrative gliomas, particularly if the differential is between a ganglioglioma and a diffuse glioma overtaking gray matter [23].

BRAF gene mutations are not equally occurring in gliomas, this evidence reinforced by recent study. Nicolaides et al showed that targeted *BRAF* inhibition with vemurafenib only worked on *V600E* glioma cells in vivo and actually made *BRAF* wild xenografts a little worse [34].

Depending on the success of recent clinical trials, ''low-grade glioma with BRAF fusion'' or ''low-grade glioma with *BRAF*^{V600E}'' could become mandatory for the pediatric neuro-oncology world [23].

5. Conclusions

BRAF^{V600E} mutation occurs in low percentage in gliomas especially adult types and significantly express in pediatric gliomas and some rare glial tumors. *BRAF*^{V600E} mutation alone is not reliable to give definite diagnosis in gliomas. However, the presence of this mutation could be considered as good prognostic factor.

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