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Is there an association between IDH1 Mutation, MGMT-promoter Methylation and Neurotrophic Kinase receptor expression in the development and progression of CNS tumours?

Fawaz Mohamed[1], Riffat Mehboob[2], Hanadi Talal Ahmedah[3], Imad Fadl-Elmula[4] and Ahmad Taher Alsaqafi[5]

[1]Department of Pathology, Faculty of Medicine, King Abdulaziz University, Rabigh, **Saudi Arabia**
[2]Research unit, Faculty of Allied Health Sciences, Lahore Medical Research Center, LLP, Lahore, **Pakistan**
[3]Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, King Abdulaziz University, Rabigh, **Saudi Arabia**
[4]Department of Clinical Genetics, Faculty of Medicine, Al-Neelain University, Khartoum, **Sudan**
[5]Department of Anesthesia, King Abdullah medical complex, Jeddah, **Kingdom of Saudia Arabia**

***Correspondence:** fsmohammed@kau.edu.sa **Received 24-07-2022, Revised: 19-08-2022, Accepted: 21-08-2022 e-Published: 27-08-2022**

IDH mutation, MGMT methylation and Neurotrophic Tyrosine Receptor Kinase (*NTRK*) rearrangement have all been detected in different types of CNS tumours. The Pan-Trk protein plays an important role in the neuronal development and cellular growth. Our aim was to explore if Pan-Trk expression in tumour microenvironment is related to *IDH* mutation and *MGMT* methylation. This study included twenty-three patients diagnosed with different types of CNS tumours. Testing for Pan-Trk and *IDH1* was performed on all FFPE tissues. This assessment has been correlated with *MGMT* methylation and *NTRK* rearrangement. The mean age of all cases was 36 years; 19 tumours were glial, and the remaining 4 cases were non-glial. *IDH*-mutation was identified in 5 tumours (21.7%) and the remaining cases were IDH-wildtype (78.3%). *MGMT*-promoter was methylated in 4 cases (17.4%) and 19 cases (91.3%) showed unmethylated *MGMT*. Pan-Trk was expressed in 11 tumours (47.8%) and the rest (52.2%) showed no Pan-Trk expression. There was no statistically significant association between Pan-Trk expression, *IDH*-mutation and *MGMT* methylation ($p>0.05$). There was also no statistically significant relationship between these three biomarkers with recurrence-free interval (RFI). The late recurrence was only observed among tumours having expressed PanTrk, *IDH*-mutation and unmethylated *MGMT* (Median =20.32 months). About 60% of cases who received chemotherapy have showed longer RFI however, no significant association was observed ($p= 0.567$). Pan-Trk should not be used as a screener to detect *NTRK*-rearrangement in CNS tumours but can be considered as a signalling biomarker to understand the relationship between *IDH*-mutation and *MGMT*-promoter methylation in tumours microenvironment.

Keywords: CNS tumours, Pan-Trk, IDH mutation, MGMT-promoter methylation.

INTRODUCTION

Isocitrate dehydrogenase (*IDH*) is an enzyme best recognized for its function in the Krebs cycle, catalyzing the oxidative decarboxylation of isocitrate to produce alpha-ketoglutarate and carbon dioxide (Kalil et al. 2013). It involves *IDH1*, -2, and -3 that function to catalyzes a redox reaction in which isocitrate is converted to α-ketoglutarate [α- KG; also known as 2-oxoglutarate (2-OG)] while converting NADP to NADPH and releasing CO2 (Cairns and Mark, 2013). Through the cell, *IDH1* is found in the cytoplasm and peroxisomes, while *IDH2* and *IDH3* are only found in the mitochondria (Winkler et al. 1986; Kurdi et al. 2021). Al IDH genes are responsible for the production of two homodimeric proteins (*IDH1* and *IDH2*), and the remaining three are responsible for generating the heterotetrametric protein *IDH3* (Yen et al. 2010). Mutations in IDH1 and *IDH2* triggers the development of certain malignancies which occurs mostly at arginine residues. These mutations are mostly missense substitutions (Pusch et al. 2011). These active site mutations result in a gain-of-function, causing the accumulation of rare metabolite as D-2hydroxyglutarate dehydrogenase. Hence, scattered reports of point mutations in *IDH* group were documented in some tumours such as acute myeloid leukemia and diffuse glial tumours. In central nervous system (CNS) tumours, *IDH1* mutations are more frequently found in WHO grade 4 astrocytomas, with reported frequencies ranging from 50%

to 86% compared with glioblastoma, which contains the mutation only 4% to 21% of the time (Balss et al. 2008; Bleeker et al. 2009). The rates of *IDH1* mutation in low-grade gliomas (LGG) are ranging from 59% to 100% and mainly diffuse astrocytomas and oligodendrogliomas (Balss et al. 2008; Bleeker et al. 2009). *IDH* mutation has never been reported pathologically in non-glial tumours.

On the other hand, the methylation of the promoter O6-methylguanine-DNA-methyltransferase (*MGMT*) continues to be a key obstacle to the effective treatment of malignant glioma patients. It functions as a drug-selection gene and protects normal tissues from toxic effect of temozolomide chemotherapy (Kurdi et al. 2021). Numerous studies identified *MGMT*-promoter methylation to be related to *IDH1* mutation, however others have not found a significant association (Kim et al. 2010).

The association between *IDH1* mutation, *MGMT*-promoter methylation and neurotrophic tyrosine receptor kinase (*NTRK*) has never been investigated in the literature. The *NTRK* receptors (Trk-A, Trk-B, and Trk-C) are normally present in the brain, and their link to *NTRK* gene was well-explained (Solomon et al. 2019). They play significant roles in neuronal development, cellular proliferation, and cognitive memory (Amatu et al. 2016). Rearrangements in the *NTRK* gene may cause two genes fusion producing altered Trk proteins. This fusion may lead to uncontrolled growth of tumour cells (Solomon et al. 2019). The prevalence of *NTRK*-fusions has been estimated to be around 1% of all systemic tumors in both adults and children (Vaishnavi et al. 2015; Hechtman et al. 2017; Solomon et al. 2020). Few reported studies have also identified *NTRK*-fusions in CNS tumours (Amatu et al. 2016; Solomon et al. 2019; Mohamed et al. 2022). The Pan-Trk protein is considered the expression tool for *NTRK*-gene in most of CNS. Solomon et al. found that Pan-Trk was differentially expressed in cancers of lung, thyroid, and Gastrointestinal tracts (Solomon et al. 2019). However, Pan-Trk IHC must be used alone as a screening marker to detect *NTRK*-fusions in CNS tumours (Mohamed et al. 2022). Moreover, the expression level of Pan-Trk in CNS tumour microenvironment has never been correlated with *IDH* mutation and *MGMT*-promoter methylation.

In our study, we assessed the possible link between Pan-Trk protein expression, *IDH* mutation and *MGMT*-promoter methylation in the development and the progression of CNS tumours using IHC technique and methylation specific sequencing.

MATERIALS AND METHODS

Patients Sequestration

This study included twenty-three patients, with a mean age of 36 years. The tumours were segregated as glial and non-glial tumours. The glial tumours included pilocytic astrocytomas, oligodendroglioma, and WHO grade 4 astrocytomas. The non-glial tumours included astroblastoma, neurocytoma, and medulloblastoma (Table 1). The study was approved by the National Biomedical Ethics Committee at King Abdulaziz University (HA-02-J-008) under a previous ethical report with same stated purpose. Samples information were obtained from the hospital records and included patients 'age at diagnosis, gender, tumour location and type, 2021 WHO grading, *IDH1* mutational and *MGMT*-promoter methylation status, and previous genetic profiling results. The histopathological diagnosis and grading were revisited following the guidelines of 2021-WHO of CNS tumours classification (Louis et al. 2021; Weller et al. 2021).

2. Tissue Processing

4-µm formalin-fixed and paraffin-embedded (FFPE) tissues were collected from each tumour sample and each section was stained for *IDH1*^{R132H} and Pan-Trk antibodies. FFPE tissue sections were used in the process of immunohistochemistry (IHC). The IHC has been performed on two types of clones (*IDH1*^{R132H}clone, rabbit monoclonal H09) and (anti-Pan-Trk, clone EPR#17341, rabbit monoclonal antibody). The procedure was processed using the ultraView detection Kit on a BenchMark XT automated stainer from Ventana (Tuscon, AZ, USA). The whole process involved deparaffinization, heat pre-treatment cell conditioning medium and primary antibody incubation. The antibodies were optimized using a dilution of 1:50 for Pan-Trk and 1:300 for *IDH*. Each tissue section was screened at x10 then a focal area of solid tumour tissue was screened on x40 using light microscopy. The staining pattern was then classified as (i) expressed and (ii) non-expressed following the results explained by Mohamed et al (Mohamed et al. 2022). The expressed cells included tumoural and tumoural cells while the non-expressed cells were considered negative expression.

3. Genetic Testing

- MGMT-Promoter Methylation Testing using Qualitative-MSP

Qualitative detection of *MGMT*-promoter methylation was done using Methylation-specific polymerase chain reaction (MS-PCR) technique, described by Esteller et al (Esteller et al. 1999). HotStarTaq PCR kit with DNA polymerase from Qiagen was utilized in the process. Thermocycling was included as initial step at 95˚C for followed by 45 cycles of 25-30 seconds at 93˚C, 30 second at 50˚C, and 20 second at 75˚C for 15 minutes. Samples having only methylated PCR products and samples having both methylated and unmethylated PCR products were both referred as methylation positive.

-NTRK Rearrangement Detection using Next-Generation Sequencing (NGS)

Testing for *NTRK*-fusion or rearrangement was accomplished through DNA-based or RNA-based NGS

using TruSight Oncology (TSO) 500 platform from Illumina (USA). The procedure was fully explained by Mohamed et al (Mohamed et al. 2022). The run data was interpretated through the Clinical Genomics Workbench (PierianDx, France). The panel was used to correlate the Pan-Trk expression validity with *NTRK*-fusion.

Statistical methods

The data were described as frequencies and percentages. Fisher's exact test was used to test the relationship between Pan-Trk expression, *IDH* mutation, and *MGMT*-promoter methylation. P-value less than 0.05 was considered significant. All statistical analyses were performed using the IBM SPSS1 ver. 24 and R-Package statistical software programs ("Circlize" version is 0.4.13). Recurrence-free interval (RFI) is defined as the period between tumour resection and to the first date of tumour recurrence.

RESULTS

The cohort included twenty-three patients with different types of CNS tumours (Table 1). The mean age was 36 years; 19 cases were glial (astrocytic or oligodendroglial), and 4 cases were non-glial. *IDH*-mutation was identified in 5 tumours (21.7%) and the remaining cases were *IDH*-wildtype (78.3%). *MGMT*-promoter was methylated in 4 cases (17.4%) and 19 cases (91.3%) had unmethylated *MGMT*-promoter. Pan-Trk expression was detected in 11 tumours (47.8%) and the remaining tumours (n=12, 52.2%) showed no Pan-Trk expression. Among those expressed ones, two tumours (8.7%) showed *NTRK*-fusions. Radiotherapy and chemotherapy were received by 65.2% of the cases and 47.8% of the cases, respectively (Table 1).

Table 1: Demographic data of the 23 patients with CNS tumours including *IDH*-mutation, *MGMT*-promoter methylation and *NTRK* rearrangements

	Overall (n=23)
Age	
Mean (SD)	36.0 (20.7)
***IDH* Status**	
IDH-mutant	5 (21.7%)
IDH-wildtype	18 (78.3%)
PanTrk expression	
No Expression	12 (52.2%)
Expressed	11 (47.8%)
***NTRK*-fusion**	
Detected	2 (8.7%)
Not detected	21 (91.3%)
***MGMT*-methylation**	
MGMT methylated	4 (17.4%)
MGMT unmethylated	19 (82.6%)
Radiotherapy	
No	8 (34.8%)
Yes	15 (65.2%)
Chemotherapy	
Not Received	12 (52.2%)
Received	11 (47.8%)

There was no statistically significant association between Pan-Trk expression and *IDH*-mutation with RFI (p=0.057) (Figure 1).

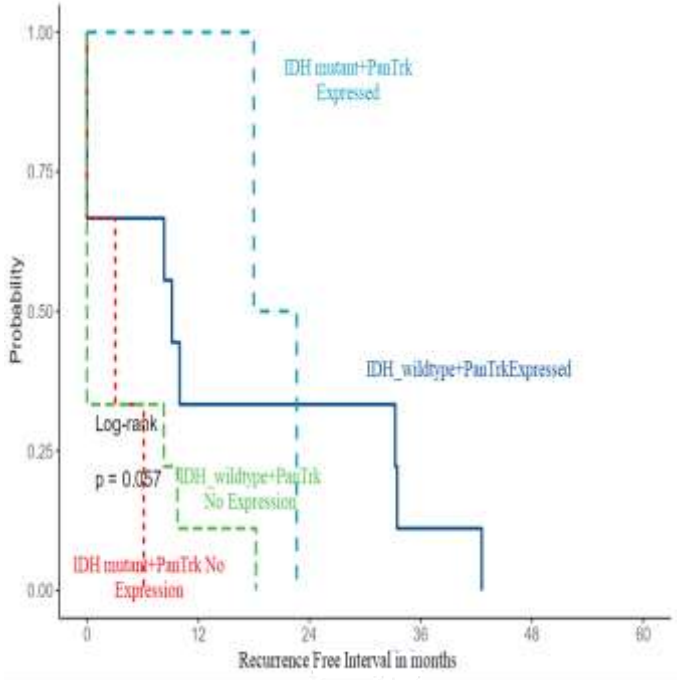

Figure 1: The association between Pan-Trk expression and *IDH*-mutation with recurrence-free interval (RFI) among all CNS tumours. Tumours with *IDH*-wildtype and Pan-Trk expression showed late recurrence compared to others.

There was also no statistically significant relationship between Pant-Trk expression and *MGMT*-promoter methylation. The late recurrence has been observed among tumours with expressed PanTrk, *IDH*-mutation and unmethylated *MGMT*-promoter (Median= 20.32 months). However, significant longer RFI has been observed among tumours with PanTrk expression, IDH-wildtype and unmethylated *MGMT* (Table 2). Among cases with expressed Pan-Trk, all cases received radiotherapy had late RFI (> 1 year), however this difference was not found significant (p=0.061). Similarly, 60% of cases who received chemotherapy have showed late RFI however, no significant association was also observed (p=0.567) (Table 3).

Table 2: The median RFI of all CNS tumours correlated with *IDH*-mutation, *MGMT*-promoter methylation and PanTrk expression. Late RFI was observed among tumours with expressed PanTrk, mutant *IDH* and unmethylated *MGMT* group (Median= 20.32 months)

			PanTrk Expression			
			Expressed		No Expression	
			n	Median RFI	n	Median RFI
***IDH*-mutant**	*MGMT* methylated		0	-	1	6.13
	MGMT unmethylated		2	20.32	2	1.53
***IDH*-wildtype**	*MGMT* methylated		1	8.33	2	14.00
	MGMT unmethylated		8	9.58	7	0.00

Table 3: The relationship between adjuvant therapies (Radiotherapy and Chemotherapy) with RFI among expressed PanTrk tumours

		Recurrence Free Interval			
		< 1 Year	**>= 1 Year**	**Total**	**p**
Radiotherapy	No	4 (66.7)	0 (0.0)	4 (36.4)	0.061[1]
	Yes	2 (33.3)	5 (100.0)	7 (63.6)	
Chemotherapy	No Received	4 (66.7)	2 (40.0)	6 (54.5)	0.567[1]
	Received	2 (33.3)	3 (60.0)	5 (45.5)	

[1] Fishers Exact Test

DISCUSSION

The association between *IDH*-mutation, *MGMT*-promoter methylation and Pan-Trk protein expression has never been investigated in the literature. Pan-Trk has been used as an effective screening method to detect *NTRK*-fusions in most body cancer types. Because Pan-Trk protein is physiologically present in the neuropil, Pan-Trk cannot be exclusively used as a tissue-efficient marker to identify *NTRK*-fusions in CNS tumours. Trk A, B, and C are encoded by the *NTR*-gene located on chromosome 1q21-q22, 9q22.1, and 15q25. Each of the Trk receptors consists of an extracellular domain, a transmembrane region and an intracellular region containing the tyrosine kinase domain. These neurotrophins were initially identified as survival molecules for sensory and sympathetic neurons but are currently known to play roles in the development and function of the nervous system. Neurotrophic binding to Trk B results in RAS-ERK pathway activation, resulting in neuronal differentiation and survival (Nakagawara, 2001; Amatu et al. 2016).

Our study revealed that 11 CNS tumours showed Pan-Trk expression while 12 tumours showed no Pan-Trk expression. Although most of the tumours that expressed Pan-Trk (n=9) were IDH-wildtype, there was no significant relationship between *IDH*-mutation and Pan-Trk expression. Among them. Moreover, the cases with Pan-Trk expression and *IDH*-wildtype showed no *MGMT*-promoter methylation. This explains that aggressive tumours with unmethylated *MGMT* may express more Pan-Trk protein in the microenvironment. Although this relationship is not widely explained in our study, the *NTRK*-signalling pathway might be activated in the tumour microenvironment.

Compared to systemic body tumours, the specificity and sensitivity of Pan-Trk expression are less in CNS neoplasms due to the normal expression of neurotrophins in CNS neuropil. The cases which showed expressed Pan-Trk, wildtype IDH, and unmethylated *MGMT* had less RFI compared to the cases with *IDH*-mutation. We can't emphasize this mechanism when Pan-Trk is not expressed in the microenvironment as the expression is significantly related to *IDH*-mutation or *MGMT*-methylation. In a meta-analysis of 5379 gliomas done by Ahrendsen et al, 8 out of 1515 IDH-mutant cases were found to have *NTRK*-fusions (Ahrendsen et al. 2021). Pan-Trk was not investigated in those cases. One limitation must be acknowledged in our study is that the total tumour cases analyzed for this association was low.

CONCLUSION

Pan-Trk should not be used as a screener to detect *NTRK*-fusions in CNS tumours but can be reflected as a signalling biomarker to understand the relationship between *IDH*-mutation and *MGMT*-promoter methylation in CNS tumours.

Ethics approval and consent to participate:

This study was approved by the National Biomedical Ethics Committee of King Abdulaziz University (HA-02-J-

008), which complies with the guidelines of the "System of ethics of research" prepared by the King Abdulaziz City for Science and Technology and approved by Royal Decree No. M/59 on 24 August 2010.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

AUTHOR CONTRIBUTIONS

FM, Conceptualization, Tissue cutting, immunohistochemistry. FM, RM, HTA and IF, Genetic interpretation, histological analysis, writing and editing**.** All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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