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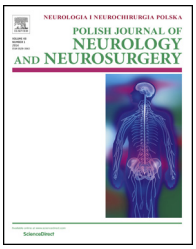

Letter to Editor

O^6-methylguanine-DNA-methyltransferase immunostaining intensity in glioblastoma

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ABSTRACT

Immunohistochemistry (IHC) for O^6-methylguanine-DNA-methyltransferase (MGMT) has shown heterogeneous results. Cell staining intensity has not been included as a quantifiable variable in IHC analyses. We performed MGMT IHC in 29 patients diagnosed as glioblastoma classifying cells into three categories based on nuclear staining intensity compared with adjacent endothelium. The median proportions of strong-moderate, weak and no staining cells were 10%, 16% and 71%, respectively. The proportion of positive cases for MGMT expression varies from 38% to 52% depending on the classification of weakly stained cells. This letter challenges previous studies that have not included intensity as a variable for IHC analysis.

Dear Editor,

Even though tremendous advances in the molecular and clinical characterization of glioblastoma (GBM) reached during the last decade, 5-year survival remains limited to 5% [1] and median survival with the current standard treatment is 14.6 months [2]. Nevertheless, median survival can vary from 12.7 to 21.7 months depending on the O^6-methylguanine-DNA-methyltransferase (MGMT) promoter methylation status [3], one of the more powerful genetic prognosticator of response to alkylating agent therapy in malignant gliomas [4]. To date, genetic testing has not become a routine investigation and the discordances between different techniques to analyze MGMT and clinical outcomes have promoted an interest on methodological sources of variability.

MGMT gene (10q26) codifies a reparative enzyme that removes methyl groups from DNA. The MGMT cytosine-guanine (CpG) island promoter methylation leads to the partial or complete gene silencing [5] that has been associated with better response to alkylating chemotherapy [3]. Despite many testing methods have been proposed, MGMT promoter testing still present many obstacles and has not become a routine investigation in GBM [6]. Although Methylation-specific polymerase chain reaction (MSP) and sequencing methods are well established techniques, they are expensive and not always available in general hospitals.

Immunohistochemistry (IHC) is a fast and less expensive method than promoter analyses. However, the association between MGMT protein expression and clinical outcome remains controversial [6,7]. Observer variability, non-standardized cut-off point to define MGMT positivity and the lack of methods to exclude normal cells expressing MGMT have been pointed as sources of variability in IHC results [7]. However, the evaluation of staining intensity has not been included as parameter for IHC analyses in most of previous studies. Although a global score of intensity has been proposed [8], the proportion and impact of different patterns of cell staining intensities in the same IHC field of analyses is not known. We present our preliminary observations using a method for MGMT IHC analyses that includes cell staining intensity as a quantitative variable.

This study was performed with the Institutional Review Board approval and ethical approval. A convenience sample of 30 patients diagnosed as glioblastoma during 2013 and 2014 in the Institute of Neurosurgery Dr. Alfonso Asenjo in Santiago of Chile was selected. Two cases were considered secondary glioblastoma with the previous histological diagnosis of diffuse fibrillary astrocytoma (grade II) and anaplastic

astrocytoma (grade III) respectively. Average age of the patients was 54.8 years (ranging from 34 to 72) and 12 (40%) were women. Presence of suitable tumor tissue in paraffin-embedded blocks was confirmed by hematoxylin eosin staining before immunohistochemistry procedures. MGMT IHC was performed following the avidin/biotin-based peroxydase system (VECTASTAIN® Elite ABC HRP kit, Peroxidase Universal) using a monoclonal mouse anti-MGMT antibody (MT3.1, ab39253; Abcam) in a dilution defined by titration test (1:25). All tissues were counterstained with hematoxylin to visualize non MGMT stained cells. Four high magnification fields (400×) from each case were selected for analyses excluding areas close to edges and necrosis to avoid false positives. Visual assessment was performed using a multistep algorithm and non-tumor cells expressing MGMT, including endothelium and lymphocytes were visually excluded based on histological features. Cells were classified in three categories based on nuclear staining intensity compared with adjacent endothelium (Fig. 1A): strong-moderate (similar or stronger than endothelium), weak (heterogeneous or weaker than endothelium) and no staining. We chose 10% as cut-off because it has been used in most previous studies [7]. However, the cut-off point to allocate a case as positive ranges from 5 to 35% and remain largely arbitrary [7].

We obtained results of 29 cases because the quality of one sample impeded an adequate staining. The hematoxylin eosin staining confirmed the presence of tumor tissue fulfilling the World Health Organization (WHO) criteria for glioblastoma in all cases. In the MGMT immunohistochemistry analysis, the median number of cells counted per case was 692 (interquartile range [IQR] 494–928). The median proportions of strong-moderate, weak and no staining cells were 10% (IQR 4–24%), 16% (IQR 10–26%) and 71% (IQR 52–85%), respectively (Fig. 1B). Excluding weak staining cells from positive cells group and using 10% as the cut-off we classified 15 (52%) as positive for MGMT expression. However, when we include all stained cells as positives regardless intensity, 11 (38%) negative cases according to the first criteria are classified as positive (Fig. 1C).

This is the first study reporting cell counting by predefined categories based on nuclear staining intensity criteria. Our preliminary observation of a variety of intensity patterns in a same field of observation emphasizes the importance of include a quantifiable measure of cell staining intensity in MGMT IHC analyses. Although an intensity score has been previously proposed [8], this method is based on a global case scoring rather than a cell by cell classification system as we did. The method that we propose challenges the results from previous studies that have not reported a criterion to deal with different staining intensities in IHC for MGMT analyses.

We assessed the effect of the weakly stained cells – that represented 16% (IQR 10–26%) of all counted cells – on the case diagnosis of MGMT expression. Using 10% as cut-off to define a positive MGMT expression status, the proportion of positive MGMT expression cases varied from 52% to 90% depending on the classification of weak staining cells and the cut-off chosen. In this study, we have not tested MGMT promoter status and most of the patients in our sample did not receive alkylating chemotherapy. Despite these limitations to establish the prognostic value of our results, this change, the variation on the expression diagnosis highlights the unrecognized role of intensity as a source of heterogeneity in IHC results. Moreover, this potential improvement on the reproducibility and reliability of the ICH analysis can contribute to determine an ideal cut-off point for future studies. Since the clinical significance of every improvement in IHC procedure must be carefully tested, our next step will be to correlate this IHC analysis with the MGMT promoter status and clinical outcomes.

Although genetic testing for MGMT promoter status has been used in most of studies linking MGMT and response to chemotherapy, it has not become a routine investigation in GBM. Moreover, comparative studies have shown that promoter status is not a gold standard, with the combined study of MGMT including the assessment of protein expression levels resulting in the best predictive value [9]. For that reason, quantifying of tumor cells by staining intensity could contribute to a better understanding of the underlying mechanisms of discordances between promoter methylation, protein expression and clinical outcome shown in previous studies [7,9]. Our strategy is in keeping with the necessity to elucidate the role of methylation independent pathways for MGMT expression that could explain the discordances between promoter methylation and mRNA expression [10]. Finally, our method may help to

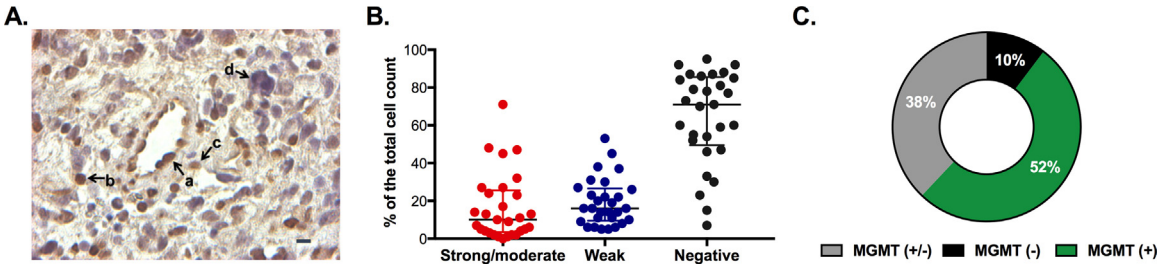

Fig. 1 – (A) Examples of the staining intensity categories used for immunohistochemistry analyses of O^6-methylguanine-DNA-methyltransferase (MGMT) and hematoxilyn in glioblastoma (400×): (a) MGMT positive endothelium used as positive control and reference of moderate MGMT staining intensity; (b) tumor cell with a strong-moderate nuclear staining intensity; (c) tumor cell with weak nuclear staining intensity; (d) negative MGMT staining cell. Scale bar 10 μm (B) Scatter plot showing the percentage of cells corresponding to each staining intensity category for each case. The median and interquartile range of percentage are presented for each category. (C) Diagnosis of MGMT expression by immunohistochemistry in 29 cases. In 11 cases (MGMT+/−) diagnosis depends on the inclusion or exclusion of the weak staining intensity cells group.

understand the interaction between MGMT methylation status and IDH1 gene mutations on the overall survival in chemoradiotherapy-treated patients [11]. However, further studies should be testing these hypotheses.

In conclusion, our preliminary results show that different staining intensity cells can be identified in the same tissue sample and the weak staining intensity cells group can be quantified to estimate its impact on the diagnosis of MGMT expression. Since the intensity of MGMT staining is a missed variable in most previous studies, this report is an alert for neuropathology groups working on MGMT immunohistochemistry regarding the relevance of reporting the proportion of weakly stained cells in future research.

Statement of authorship

All the authors have made a significant contribution to the manuscript. Daniel Jiménez, Jose Manuel Matamala and Alessandra Chiti planned the experiments, interpreted the data and prepared the manuscript. Daniel Jiménez and Carmen Vergara performed every immunohistochemistry procedure. Claudia Tissera, Romulo Melo and Luis Cartier supervised the project and gave conceptual input. All authors read and approved the final manuscript.

Conflict of interest

None declared.

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Daniel Jiménez[a,b,*]
[a]Laboratory of Biomedical Sciences, Department of Neurological Sciences, Faculty of Medicine, University of Chile, Santiago, Chile
[b]Neurology Service, Hospital del Salvador, Santiago, Chile

José Manuel Matamala[a,c]
[a]Laboratory of Biomedical Sciences, Department of Neurological Sciences, Faculty of Medicine, University of Chile, Santiago, Chile
[c]Biomedical Neuroscience Institute (BNI), Faculty of Medicine, University of Chile, Santiago, Chile

Alessandra Chiti
Laboratory of Biomedical Sciences, Department of Neurological Sciences, Faculty of Medicine, University of Chile, Santiago, Chile

Carmen Vergara
Laboratory of Biomedical Sciences, Department of Neurological Sciences, Faculty of Medicine, University of Chile, Santiago, Chile

Claudia Tissera
Instituto de Neurocirugía Dr. Asenjo, Santiago, Chile

Romulo Melo[d,e]
[d]Instituto de Neurocirugía Dr. Asenjo, Santiago, Chile
[e]Department of Neurological Science, Faculty of Medicine, University of Chile, Santiago, Chile

Luis Cartier[a,b]
[a]Laboratory of Biomedical Sciences, Department of Neurological Sciences, Faculty of Medicine, University of Chile, Santiago, Chile
[b]Neurology Service, Hospital del Salvador, Santiago, Chile

*Corresponding author at: Department of Neurological Science, Faculty of Medicine, University of Chile, José Miguel Infante

553, 7500691 Santiago, Chile
E-mail address: danieljimenez@med.uchile.cl (D. Jiménez)

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