Pathology - Research and Practice xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Comparative assessment of three methods to analyze MGMT methylation status in a series of 350 gliomas and gangliogliomas

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ARTICLE INFO

Keywords:
Methylation
MGMT
Glioma
Pyrosequencing
Immunohistochemistry

ABSTRACT

MGMT promoter methylation is considered as a prognostic and predictive biomarker indicating response to chemotherapy and radiotherapy in glioblastoma. A number of different methods and platforms including pyrosequencing (PSQ), quantitative methylation-specific PCR (qMSP) and immunohistochemistry (IHC), methylation-sensitive high resolution melting (MS-HRM) and NGS (Next Generation Sequencing) have been used to detect MGMT promoter methylation in gliomas. However, controversy remains about the most appropriate method to use for analyzing MGMT status. The MGMT promoter methylation status of a total of 350 gliomas and gangliogliomas was examined using PSQ, qMSP and IHC in parallel. Using PSQ as a recommended standard method, the sensitivity, specificity, positive/negative predictive value and correlation with the other assays were calculated. Among 350 glioma and ganglioglioma cases, the MGMT promoter tested positive for methylation in 53.1%, 55.4%, and 70.3% of the cases by PSQ, qMSP and IHC, respectively. The sensitivity and specificity of qMSP were 97.8% and 92.7%, respectively. Twelve cases that tested positive for methylation using qMSP were negative according to PSQ, and four cases that were negative according to qMSP tested positive according to PSQ. The concordance rate between PSQ and qMSP was 90.8%. The sensitivity and specificity of IHC for the detection of MGMT at the protein level were 84.4% and 45.7%, respectively. The concordance rate between PSQ and IHC was 30.8%.

This study demonstrated that qMSP is an effective and rapid detection method for routine use in pathology laboratories for the identification of MGMT promoter methylation. A combination of IHC and qMSP assays can provide high sensitivity and specificity for the prediction of MGMT status. A few cases that tested negative with PSQ did harbor MGMT promoter methylation, as confirmed by qMSP and sequencing, and this subgroup of patients may benefit from temozolomide.

1. Introduction

The current standard of care for glioma patients involves radiotherapy and chemotherapy with temozolomide after surgery [1,2]. Studies have demonstrated that high O-6-methylguanine-DNA-methyltransferase (MGMT) expression in the tumor cells may cause tumor tolerance to chemotherapy in glioblastoma patients [2–4]. MGMT is a repair protein that protects both normal and tumor cells from DNA damage by moving alkylating groups from DNA to a cysteine residue within its own protein structure [5]. It has been found that a variety of neoplasms exhibit decreased activity of MGMT because of MGMT gene silencing through promoter CpG hypermethylation [3]. Several studies have reported that MGMT promoter methylation is frequent in diffusely infiltrating gliomas (WHO grade II–IV) and is associated with longer survival of patients with high-grade gliomas who received alkylating chemotherapy [6–8]. In addition, MGMT promoter methylation is considered as a prognostic and predictive biomarker indicating response to chemotherapy and radiotherapy in glioblastoma (GBM) [4]. Since postoperative temozolomide (TMZ) chemotherapy with concomitant radiotherapy has become the standard treatment for GBM patients [9], the application of reliable screening methods for MGMT promoter methylation is very important in order to identify the patients for which chemotherapy/radiotherapy is suitable [4,8,10].

MGMT promoter methylation can be assessed by methylation- specific polymerase chain reaction (MSP), multiplex ligation-dependent probe amplification (MLPA), pyrosequencing (PSQ) and quantitative Real-Time PCR, or by assessing protein expression using immunohistochemistry (IHC) [11,12]. PSQ has been confirmed as

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http://dx.doi.org/10.1016/j.prp.2017.10.007
Received 6 June 2017; Received in revised form 29 September 2017; Accepted 7 October 2017

statistically robust, since it allows comparative survival analyses leading to highly significant results, and was consequently recommended as the best assay for the detection of MGMT promoter methylation [13]. However, due to the cumbersome procedure and the required specialized equipment, PSQ is not widely used in clinical diagnostics when a single sample is subject to analysis. Rather, as a faster and simpler method for detecting gene mutation or methylation than PSQ, Real-Time PCR is already widely used in clinical practice. The IHC method is rapid and easy to perform as well as relatively low-cost, and was therefore also recommended as another option to detect MGMT methylation status [14]. In this study, we compared the specificity and sensitivity of 3 methods (pyrosequencing, quantitative methylation-specific PCR and immunohistochemistry) in the detection of MGMT status in a series of 350 gliomas to determine which method offers the optimal balance of resource expenditure and benefits for the patients.

2. Materials and methods

The study has been approved by the ethics committee of Xuanwu Hospital, Beijing, China. Three hundred and fifty formalin-fixed paraffin-embedded (FFPE) tumor samples from patients diagnosed with glioma or ganglioglioma were collected from Xuanwu Hospital, Capital Medical University, Beijing, China, between January 2013 and March 2016. Patient and tumor characteristics, such as age, gender, location, histology and grade were summarized. Histological characteristics of the tumors were as follows: 25 (7.1%) ganglioglioma (GG, WHO grade I), 4 (1.1%) pilocytic astrocytoma (PA, WHO grade I), 1 (0.3%) subependymal giant cell astrocytoma (SEGA, WHO grade I), 6 (1.7%) pleomorphic xantho-astrocytoma (PXA, WHO grade II), 84 (24%) astrocytoma or oligodendroglioma (WHO grade II), 76 (21.7%) anaplastic astrocytoma or oligodendroglioma (WHO grade III), and 154 (44%) glioblastoma (GBM, WHO grade IV). Before DNA extraction, representative sections were stained with hematoxylin and eosin (H & E), and the tumors were reviewed by two pathologists and histologically classified according to the 2016 WHO criteria [15]. This study is retrospective and the data were analyzed anonymously.

2.1. Immunohistochemistry (IHC)

4 μm-thick sections were obtained from well-preserved paraffin blocks for each case. The immunostaining for MGMT was performed using a monoclonal antibody (clone MT3.1, Chemicon International, Temecula, California, USA; working dilution 1:200) using a BOND-MAX immunostainer (Leica Microsystems). Normal brain tissue was used as external positive control. A negative control with omission of the primary antibody was also included. Antigen retrieval was performed in citrate buffer (pH 9.0) for 20 min at 100 ˚C. The primary antibodies were incubated overnight at 4 ˚C. All sections were counterstained with hematoxylin. The immunohistochemical results were evaluated according to a previous report [14]. Endothelial cells and histiocytes were used as internal positive controls. Areas with negative staining of endothelium were excluded from reading and interpretation. Gliomas with loss of MGMT protein (IHC-) were considered as being MGMT promoter-methylated.

2.2. DNA extraction and bisulfite treatment

An appropriate area comprising tumor tissue was selected for Real-Time PCR and PSQ analysis. Three 4 μm-thick sections of each specimen were cut from paraffin-embedded tissue, treated twice with xylene, and washed twice with ethanol. Genomic DNA was extracted using the Dneasy Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Quantification was performed on a Nanodrop 2000 spectrophotometer (Thermo-Fisher Scientific, USA). 300–3000 ng each of the extracted DNA and controls comprising CpGenome Universal Methylated DNA and CpGenome Universal Unmethylated DNA, respectively, were subjected to bisulfite treatment using the Bisulfite Kit (Gene Tech Co., Ltd, Shanghai, China), according to the manufacturer's protocol. The products were purified and the efficiency of the bisulfite conversion was checked by analyzing the control DNA by pyrosequencing.

2.3. Quantitative methylation-specific (real-time) PCR (qMSP)

The methylation status of 5 CpG sites within the MGMT promoter region (genomic sequence on chromosome 10 from the 129,467,243 position to the 129,467,263 position) was analyzed by Real-Time PCR using the MGMT Gene Methylation Detection Kit (Gene Tech Co., Ltd, Shanghai, China) according to manufacturer's instructions. Briefly, bisulfite-treated DNA was used as template for PCR amplification using the following temperature program: an initial denaturation at 95 ˚C for 3 min, followed by 45 cycles of 95 ˚C for 15 s, and 60 ˚C for 60 s. The quantities were determined based on the fluorescence signals. Positive and negative controls were also performed. Samples with ΔCt values ≤ 7 and DNA methylation levels $\geq 1\%$ were regarded as positive (methylated), according to the recommendation by the manufacturer.

2.4. Pyrosequencing

The pyrosequencing methylation assay was performed using the PyroMark Q96 CpG MGMT Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. MGMT primers were provided in the detection kit and the following PCR procedure was used: an initial denaturation step at 95 ˚C for 3 min followed by 45 cycles comprising 95 ˚C for 20 s, 53 ˚C for 20 s and 72 ˚C for 20 s, followed by a final elongation step at 72 ˚C for 5 min. The samples were processed in the PyroMark Q96 ID instrument (Qiagen, Hilden, Germany), and data were analyzed using the instrument's PyroMark CpG Software. Samples were dichotomized upon a mean methylation level threshold of 8% of the 5 CpGs tested, according to the manufacturer's instructions and previous reports [11,16].

2.5. Statistical analysis

Comparisons of MGMT promoter methylation status as determined by PSQ, qMSP and IHC were performed using Pearson's chi-squared test and Fisher's exact probability test. Given that PSQ is the recommend standard for MGMT promoter methylation detection, the sensitivity, specificity and concordance of IHC and qMSP were calculated based on it. Statistical analyses were performed using SPSS software version 21.0 (SPSS, Chicago, IL, USA). Differences were considered statistically significant when the p value was less than 0.05.

3. Results

3.1. MGMT IHC results

When the MGMT promoter is methylated, the MGMT protein expression should be low [3]. Therefore, the MGMT promoter methylation status as determined by PSQ was compared with the loss of MGMT expression determined by IHC staining (IHC-). The MGMT IHC staining results were recorded as either methylated (IHC-) or unmethylated (IHC+) by two pathologists. Contaminating cells, such as histiocytes/microglia, endothelial cells, and lymphocytes, were excluded. Of the 350 cases studied, 246 (70.3%) were IHC-. Weaker nuclear staining of the tumor cells than normal glial cells in white matter was identified in 107 cases, an no staining in 139 cases (Fig. 1). Conversely, 104 cases (29.7%) were IHC+, with stronger nuclear staining of the tumor cells in 23 cases, and moderate staining in 81 cases (Fig. 1).