Clinical Neuropathology practice news 1-2014: Pyrosequencing meets clinical and analytical performance criteria for routine testing of MGMT promoter methylation status in glioblastoma

Matthias Preusser¹,³, Anna S. Berghoff¹,³, Claudia Manzl², Martin Filipits¹,³, Andreas Weinhäuser⁴, Walter Pulverer⁴, Karin Dieckmann⁵,³, Georg Widhalm⁶,³, Adelheid Wöhrer³,⁷, Engelbert Knosp⁶,³, Christine Marosi¹,³, and Johannes A. Hainfellner³,⁷

¹Department of Medicine I, Medical University of Vienna, Vienna, ²Division of Pathology, Medical University of Innsbruck, Innsbruck, ³Comprehensive Cancer Center – CNS Tumors Unit (CCC-CNS), Medical University of Vienna, Vienna, ⁴Molecular Diagnostics, Austrian Institute of Technology, ⁵Department of Radiotherapy, ⁶Department of Neurosurgery, and ⁷Institute of Neurology, Medical University of Vienna, Vienna, Austria

Abstract. Testing of the MGMT promoter methylation status in glioblastoma is relevant for clinical decision making and research applications. Two recent and independent phase III therapy trials confirmed a prognostic and predictive value of the MGMT promoter methylation status in elderly glioblastoma patients. Several methods for MGMT promoter methylation testing have been proposed, but seem to be of limited test reliability. Therefore, and also due to feasibility reasons, translation of MGMT methylation testing into routine use has been protracted so far. Pyrosequencing after prior DNA bisulfite modification has emerged as a reliable, accurate, fast and easy-to-use method for MGMT promoter methylation testing in tumor tissues (including formalin-fixed and paraffin-embedded samples). We performed an intra- and inter-laboratory ring trial which demonstrates a high analytical performance of this technique. Thus, pyrosequencing-based assessment of MGMT promoter methylation status in glioblastoma meets the criteria of high analytical test performance and can be recommended for clinical application, provided that strict quality control is performed. Our article summarizes clinical indications, practical instructions and open issues for MGMT promoter methylation testing in glioblastoma using pyrosequencing.

Background

Glioblastoma is the most frequent primary brain tumor of adults and is highly malignant with a median overall survival time of only 12 – 14 months [1]. The standard therapy options include maximal safe neurosurgical resection, radiotherapy and chemotherapy preferentially with the alkylating drug temozolomide, antiangiogenic treatment with the antibody bevacizumab, symptom control through corticosteroids for increased intracranial pressure and anticonvulsants and best supportive care [1, 2, 3, 4]. Important clinical prognostic factors comprise patient age at diagnosis, patient performance status, and the extent of resection [5]. At the molecular level, the O6-methylguanine-methyltransferase (MGMT) promoter methylation status carries relevant prognostic and, at least in the subset of elderly patients, also predictive information for therapy planning [6, 7, 8].

MGMT is a DNA-repair protein that counteracts the effect of alkylating chemotherapy by removing methyl groups from the O6-position of guanine. In line with this assumption, the MGMT promoter methylation status influences the outcome of patients with glioblastoma treated with temozolomide. In 2005, Hegi et al. [6] demonstrated in a post hoc analysis of a study cohort from a phase III study that glioblastoma patients aged 18 – 70 years with intratumoral MGMT gene silencing by promoter hypermethylation had significantly better outcomes when treated with combined radiochemotherapy with temozolomo-
mide as compared to patients treated with radiotherapy alone. However, these prognostic data did not clearly support withholding temozolomide from patients with unmethylated MGMT promoter in the absence of effective treatment alternatives. More recently, two independent phase III studies showed that the MGMT promoter methylation status is of predictive value in the subpopulation of elderly glioblastoma patients [7, 8]. It was shown that patients with methylated MGMT promoter specifically benefited from temozolomide therapy, while patients with unmethylated MGMT promoter fared better when treated with radiotherapy alone. These results strongly argue for implementation of MGMT testing into everyday clinical patient care for informed patient allocation to radiotherapy or chemotherapy in order to improve patient outcomes, avoid unnecessary treatment toxicities and save costs.

A number of different methods for analysis of MGMT testing have been reported, but many have been shown to be difficult to standardize. One of the most frequently used methods, methylation specific polymerase-chain reaction (MSP), has proven unreliable and is regarded by many laboratories as inadequately accurate for clinical decision making [9]. A fairly large number of modifications of this technique or alternative methods for MGMT testing have been proposed, but a consensus on a specific protocol reliably yielding high quality test results has not been reached so far [10].

In 2007, Mikeska et al. [11] optimized pyrosequencing (see Box A for methodological background) for testing of MGMT promoter methylation status in glioblastoma specimens and demonstrated feasibility and reliability in snap-frozen and formalin fixed paraffin-embedded specimens. Since then, several research groups have reported similarly favorable experience with this technique and demonstrated high reliability of test results and a better analytical performance as compared to other methods [11, 12, 13, 14, 15]. Furthermore, the MGMT status determined by pyrosequencing was shown to correlate with progression-free and overall survival of glioblastoma patients in several independent patient cohorts [12, 14, 16, 17]. Also the predictive value of the MGMT promoter methylation status seen in elderly glioblastoma patients in the NOA-08 and the Nordic glioma trials could be reproduced using pyrosequencing [7, 8, 18].

Herein, we assessed repeatability and reproducibility of MGMT testing by pyrosequencing among independent laboratories.

### Study design

#### Tumor specimens

For this study we used 18 glioblastoma specimens of 9 patients (1 formalin-fixed/paraffin-embedded (FFPE) and 1 RCL2-fixed/paraffin-embedded (RCLPE) sample per patient) [19]. The MGMT promoter methylation status has been determined for every included sample in a previous study using a methylation-sensitive restriction enzyme (MSRE)-based quantitative PCR (qPCR) assay in an independent laboratory (reference laboratory: Molecular Diagnostics, Austrian Institute of Technology). MGMT results obtained in the reference laboratory were validated by a MALDI-Epityper assay in another external laboratory as contract service (Sequenom, Hamburg, Germany) as described previously [20]. In brief, 5 patients were shown to harbor a methylated and 4 patients to harbor an unmethylated MGMT promoter in their tumor tissue. In each sample, the tumor cell content was more than 60% as determined by hematoxylin and eosin stained sections. Of each of the 18 tumor tissue blocks, we cut at least 30 µm material into an Eppendorf tube at the coordinating center (Institute of Neurology, Medical University of Vienna, Vienna, Austria) [20].
The set-up of this interlaboratory study is summarized in Figure 1.

The coordinating center sent the material to test laboratory 1 (Division of Pathology, Medical University of Innsbruck) and test laboratory 2 (Institute of Cancer Research, Medical University of Vienna). The investigators at test laboratories 1 and 2 were blinded to the MGMT status of the samples determined in the reference laboratory.

In test laboratory 1, MGMT pyrosequencing of each FFPE tissue sample was performed at least twice and up to 5 times in independent runs and by two independent technicians (Table 1) (Figure 1). Of each RCLPE tissue sample, MGMT pyrosequencing was performed once by each of the two technicians.

In test laboratory 2, MGMT pyrosequencing of each FFPE and each RCLPE tissue block was performed once by one technician (Table 1) (Figure 1).

MGMT pyrosequencing results were reported back to the coordinating center by test laboratories 1 and 2 only after completion of all MGMT pyrosequencing analyses for un-blinding and comparison of test results.

**DNA isolation, bisulfite modification, and pyrosequencing**

DNA isolation was performed using the EZ1 DNA investigator Kit (Qiagen, Germany, test laboratory 1) and the EpiTect FFPE Lysis Kit (Qiagen) (test laboratory 2) according to manufacturer’s recommendations.

In both laboratories, bisulfite modification was performed using the Epi Tect Fast FFPE Bisulfite kit (Qiagen) according to the manufacturer’s recommendations.

In both laboratories, analysis of the MGMT promoter methylation status was performed on a PyroMark Q24 (Qiagen, Germany) system and using the Therascreen MGMT Pyro Kit (Qiagen).

MGMT-pyrosequencing yields a quantitative result giving the percentage of methylated alleles for each of the four investigated CpG sites (Figure 3). For definition of cases with methylated vs. unmethylated MGMT promoter, the percentage mean value of the methylation percentage obtained at each of the four investigated CpG dinucleotides was calculated. According to the definitions elaborated in a previous publication, cases with a mean methylation percentage of < 8% were regarded as MGMT promoter unmethylated and cases with a mean methylation percentage of ≥ 8% were regarded as MGMT promoter methylated [18].

**Results**

All 9 cases fulfilled the histopathological criteria of glioblastoma according to the current edition of the WHO Classification of Tumors of the Central Nervous System [21]. In each sample, the tumor cell content was more than 60% as determined on he-
matoxylin and eosin stained sections. Five patients were shown to harbor a methylated and four patients to harbor an unmethylated MGMT promoter in their tumor tissue. MGMT-pyrosequencing results of the individual runs are detailed in Table 1 and Table 2. We found a perfect correlation (100% concordance, Kappa value of 1, p < 0.001) of binary MGMT status (methylated vs. unmethylated) in all samples irrespective of tissue fixation conditions, testing laboratory, testing technician and time point of testing. Cut-offs for defining MGMT promoter methylated vs. unmethylated cases: for MSRE: 0.32%, for PSQ: 8%. PSQ-% represent the mean-methylation values from four CpG analyzed and calculated from pyrograms.

| ID   | Test lab    | Test run | Fixation | Method  | Technician | Mean value | Result      |
|------|-------------|----------|----------|---------|------------|------------|-------------|
| Case 1 | Reference lab | 1        | RCLPE    | MSRE    | 1          | 0          | unmethylated |
| Case 1 | Sequenom    | 2        | RCLPE    | Epityper| 1          | 5.33       | unmethylated |
| Case 1 | Test lab 1  | 3        | FFPE     | PSQ     | 1          | 2.25       | unmethylated |
| Case 1 | Test lab 1  | 4        | FFPE     | PSQ     | 1          | 1.75       | unmethylated |
| Case 1 | Test lab 1  | 5        | RCLPE    | PSQ     | 1          | 2.25       | unmethylated |
| Case 1 | Test lab 1  | 6        | RCLPE    | PSQ     | 2          | 2.75       | unmethylated |
| Case 1 | Test lab 2  | 7        | RCLPE    | PSQ     | 1          | 2.27       | unmethylated |
| Case 1 | Test lab 2  | 8        | FFPE     | PSQ     | 1          | 2.55       | unmethylated |
| Case 5 | Reference lab | 1        | RCLPE    | MSRE    | 1          | 0          | unmethylated |
| Case 5 | Sequenom    | 2        | RCLPE    | Epityper| 1          | 3.9        | unmethylated |
| Case 5 | Test lab 1  | 3        | FFPE     | PSQ     | 1          | 1.75       | unmethylated |
| Case 5 | Test lab 1  | 4        | FFPE     | PSQ     | 1          | 2          | unmethylated |
| Case 5 | Test lab 1  | 5        | FFPE     | PSQ     | 2          | 1.75       | unmethylated |
| Case 5 | Test lab 1  | 6        | RCLPE    | PSQ     | 1          | 1.25       | unmethylated |
| Case 5 | Test lab 1  | 7        | RCLPE    | PSQ     | 2          | 1.5        | unmethylated |
| Case 5 | Test lab 2  | 8        | FFPE     | PSQ     | 1          | 2.29       | unmethylated |
| Case 5 | Test lab 2  | 9        | RCLPE    | PSQ     | 1          | 1.27       | unmethylated |
| Case 7 | Reference lab | 1        | RCLPE    | MSRE    | 1          | 0          | unmethylated |
| Case 7 | Sequenom    | 2        | RCLPE    | Epityper| 1          | 2.5        | unmethylated |
| Case 7 | Test lab 1  | 3        | FFPE     | PSQ     | 1          | 3.25       | unmethylated |
| Case 7 | Test lab 1  | 4        | FFPE     | PSQ     | 1          | 3          | unmethylated |
| Case 7 | Test lab 1  | 5        | FFPE     | PSQ     | 2          | 3.25       | unmethylated |
| Case 7 | Test lab 1  | 6        | FFPE     | PSQ     | 2          | 4          | unmethylated |
| Case 7 | Test lab 1  | 7        | RCLPE    | PSQ     | 1          | 4.25       | unmethylated |
| Case 7 | Test lab 1  | 8        | RCLPE    | PSQ     | 2          | 3.5        | unmethylated |
| Case 7 | Test lab 2  | 9        | RCLPE    | PSQ     | 1          | 2.48       | unmethylated |
| Case 7 | Test lab 2  | 10       | FFPE     | PSQ     | 1          | 2.81       | unmethylated |
| Case 9 | Reference lab | 1        | RCLPE    | MSRE    | 1          | 0.02       | unmethylated |
| Case 9 | Sequenom    | 2        | RCLPE    | Epityper| 1          | 1.9        | unmethylated |
| Case 9 | Test lab 1  | 3        | FFPE     | PSQ     | 1          | 5          | unmethylated |
| Case 9 | Test lab 1  | 4        | FFPE     | PSQ     | 1          | 5.25       | unmethylated |
| Case 9 | Test lab 1  | 5        | FFPE     | PSQ     | 2          | 5.25       | unmethylated |
| Case 9 | Test lab 1  | 6        | FFPE     | PSQ     | 2          | 5.75       | unmethylated |
| Case 9 | Test lab 1  | 7        | RCLPE    | PSQ     | 1          | 1.5        | unmethylated |
| Case 9 | Test lab 1  | 8        | RCLPE    | PSQ     | 2          | 1.75       | unmethylated |
| Case 9 | Test lab 2  | 9        | RCLPE    | PSQ     | 1          | 1.06       | unmethylated |
| Case 9 | Test lab 2  | 10       | FFPE     | PSQ     | 1          | 3.84       | unmethylated |

FFPE = formalin-fixed and paraffin-embedded; MSRE = methylation-specific restriction enzyme based quantitative polymerase chain reaction; PSQ = pyrosequencing; RCLPE = RCL2-fixed and paraffin-embedded.
In this study, we show high reproducibility and repeatability of MGMT promoter methylation testing by means of pyrosequencing using a commercial assay. Our findings corroborate findings previously reported by other laboratories and confirm that pyrosequencing fulfills the prerequisites for MGMT testing in the routine clinical setting. Based on the results of two recent phase III clinical trials and the confirmed assay reliability, MGMT pyrosequencing fulfills the criteria for high clinical utility and is recommended for therapy planning in elderly glioblastoma patients (Box B) (Table 3) [7, 8]. In other glioblastoma sub-cohorts, the clinical utility of MGMT testing is lower and at present does not have direct implications for clinical management [24], although knowledge of the MGMT promoter methylation status may support prognostic considerations that influence indirectly patient management.

Although the evidence level for clinical MGMT testing is high, some issues remain to be addressed in further studies in order to refine its application. An important and not completely resolved issue is the definition of cut-offs for defining MGMT methylated vs. unmethylated cases based on the quantitative test results (the percentage of methylated alleles for each of the investigated CpG sites). For the assay used in our laboratories, an average methylation rate of the four analyzed CpG sites of 8% has been elaborated as clinically relevant cut-off in previous studies using MGMT pyrosequencing [12, 18]. Based on our data, this threshold seems reasonable, but it needs to be validated in prospective and adequately powered studies. Importantly, MGMT pyrosequencing provides quantitative results and there seems to be prognostic/predictive value of the continuous assay read-out. Dunn et al. [17] described that glioblastomas with the highest mean methylation levels (> 35%) showed the longest survival times. Similarly, Reifenberger et al. [18] reported that patients with strongly methylated tumors (> 25% MGMT methylated alleles) showed a significantly better outcome than patients with tumors with < 25% methylated alleles, if treated with alkylating chemotherapy. For the clarification and definition of universally applicable prognostic and predictive cut-off
values, correlations of MGMT pyrosequencing results with patient outcomes in independent and large patient cohorts from prospective clinical trials are needed.

Another important issue is the selection of appropriate CpG sites for methylation analysis. The MGMT gene contains 98 CpG sites in the first of five exons and the promoter re-

| ID  | Test lab     | Test run | Fixation | Method | Technician | Mean value | Result     |
|-----|--------------|----------|----------|--------|------------|------------|------------|
| Case 2 | Reference lab 1 | RCLPE    | MSRE 1   | 0.56   | methylated |
| Case 2 | Sequenom 2    | RCLPE    | Epityper 1 | 16.9 | methylated |
| Case 2 | Test lab 1 3 | FFPE PSQ | 1  | 21.25 | methylated |
| Case 2 | Test lab 1 4 | FFPE PSQ | 1  | 21.75 | methylated |
| Case 2 | Test lab 1 5 | FFPE PSQ | 2  | 20.50 | methylated |
| Case 2 | Test lab 1 6 | RCLPE PSQ | 1  | 26.25 | methylated |
| Case 2 | Test lab 1 7 | RCLPE PSQ | 2  | 20  | methylated |
| Case 2 | Test lab 2 8 | FFPE PSQ | 1  | 31.35 | methylated |
| Case 2 | Test lab 2 9 | RCLPE PSQ | 1  | 25.64 | methylated |
| Case 3 | Reference lab 1 | RCLPE MSRE | 1  | 0.69  | methylated |
| Case 3 | Sequenom 2    | RCLPE    | Epityper 1 | 20.9 | methylated |
| Case 3 | Test lab 1 3 | FFPE PSQ | 1  | 28.5  | methylated |
| Case 3 | Test lab 1 4 | FFPE PSQ | 1  | 30.25 | methylated |
| Case 3 | Test lab 1 5 | FFPE PSQ | 2  | 34.75 | methylated |
| Case 3 | Test lab 1 6 | RCLPE PSQ | 1  | 58  | methylated |
| Case 3 | Test lab 1 7 | RCLPE PSQ | 2  | 50.75 | methylated |
| Case 3 | Test lab 2 8 | FFPE PSQ | 1  | 36.21 | methylated |
| Case 3 | Test lab 2 9 | RCLPE PSQ | 1  | 48.94 | methylated |
| Case 4 | Reference lab 1 | RCLPE MSRE | 1  | 7.35  | methylated |
| Case 4 | Sequenom 2    | RCLPE    | Epityper 1 | 15  | methylated |
| Case 4 | Test lab 1 3 | FFPE PSQ | 1  | 17.75 | methylated |
| Case 4 | Test lab 1 4 | FFPE PSQ | 1  | 18.25 | methylated |
| Case 4 | Test lab 1 5 | FFPE PSQ | 2  | 18.75 | methylated |
| Case 4 | Test lab 1 6 | FFPE PSQ | 2  | 16.5  | methylated |
| Case 4 | Test lab 1 7 | FFPE PSQ | 2  | 18.3  | methylated |
| Case 4 | Test lab 1 8 | RCLPE PSQ | 1  | 55.25 | methylated |
| Case 4 | Test lab 1 9 | RCLPE PSQ | 2  | 35  | methylated |
| Case 4 | Test lab 2 10 | FFPE PSQ | 1  | 18.03 | methylated |
| Case 4 | Test lab 2 11 | RCLPE PSQ | 1  | 34.94 | methylated |
| Case 6 | Reference lab 1 | RCLPE MSRE | 1  | 11.85 | methylated |
| Case 6 | Sequenom 2    | RCLPE    | Epityper 1 | 23.4 | methylated |
| Case 6 | Test lab 1 3 | FFPE PSQ | 1  | 20.25 | methylated |
| Case 6 | Test lab 1 4 | FFPE PSQ | 1  | 20.5  | methylated |
| Case 6 | Test lab 1 5 | FFPE PSQ | 2  | 15.25 | methylated |
| Case 6 | Test lab 1 6 | FFPE PSQ | 2  | 14.5  | methylated |
| Case 6 | Test lab 1 7 | RCLPE PSQ | 1  | 48  | methylated |
| Case 6 | Test lab 1 8 | RCLPE PSQ | 2  | 38.25 | methylated |
| Case 6 | Test lab 2 9 | RCLPE PSQ | 1  | 24.42 | methylated |
| Case 6 | Test lab 2 10 | RCLPE PSQ | 1  | 36.23 | methylated |
| Case 8 | Reference lab 1 | RCLPE MSRE | 1  | 69.38 | methylated |
| Case 8 | Sequenom 2    | RCLPE    | Epityper 1 | 31.3 | methylated |
| Case 8 | Test lab 1 3 | FFPE PSQ | 1  | 30.25 | methylated |
| Case 8 | Test lab 1 4 | FFPE PSQ | 1  | 31.25 | methylated |
| Case 8 | Test lab 1 5 | FFPE PSQ | 2  | 40.25 | methylated |
| Case 8 | Test lab 1 6 | RCLPE PSQ | 1  | 48.75 | methylated |
| Case 8 | Test lab 1 7 | RCLPE PSQ | 2  | 41.75 | methylated |
| Case 8 | Test lab 2 8 | RCLPE PSQ | 1  | 37.75 | methylated |
| Case 8 | Test lab 2 9 | FFPE PSQ | 1  | 33.98 | methylated |
It is not entirely clear so far which of the CpG sites in the promoter region determine MGMT expression and have the greatest influence on the clinical course. The commercial kit we used in this study analyses four distinct CpG sites and a correlation with patient outcome has been shown for this particular assay [18]. It must be noted that implementation of alternative assays targeting other CpG sites will require to verify not only the analytical but also the clinical performance i.e. the prognostic/predictive impact.

From a practical point of view, MGMT pyrosequencing is fraught with the disadvantage that it requires particular equipment and is relatively expensive. On the other hand, pyrosequencing performs very well not only on frozen but also on FFPE and RCLPE tissue samples. This facilitates centralization of MGMT testing in geographical regions, as paraffin-embedded samples can easily be transferred between institutions and pathology laboratories. In our opinion, however, regular accreditation of test laboratories through inter-laboratory ring trials seems vital to ensure high and sustained quality of MGMT testing. Quality assurance should also include histopathological verification of sufficient tumor content in the sample submitted for MGMT promoter methylation testing.

In conclusion, we confirm in this study a high analytical performance of MGMT promoter methylation analysis by means of pyrosequencing (Table 3). Based on our results we can recommend this technique for clinical application in glioblastoma patients, given that strict quality controls including inter-laboratory ring trials are performed.

Acknowledgment

We thank Anita Brandstetter for excellent technical assistance.
Conflict of interests

The authors declare no conflict of interest.

References

[1] Preusser M, de Ribaupierre S, Währer A, Erridge SC, Hegi M, Weller M, Stupp R. Current concepts and management of glioblastoma. Ann Neurol. 2011; 70: 9-21. CrossRef PubMed

[2] Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphorn MJ, Janzer RC, Ludwin SK, Allgeier A, Fisher B, Belanger K, Hau P, Brandes AA, Gijtenbeek J, Marosi C, Vecht CJ, Mokhtar K, Wesseling P, Villa S, Eisenbauer E, Gorlia T, et al. European Organisation for Research and Treatment of Cancer Brain Tumour and Radiation Oncology Groups; National Cancer Institute of Canada Clinical Trials Group. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol. 2009; 10: 459-466. CrossRef PubMed

[3] Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenbauer E, Mirimanoff RO. European Organisation for Research and Treatment of Cancer Brain Tumour and Radiation Oncology Groups; National Cancer Institute of Canada Clinical Trials Group. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med. 2005; 352: 987-996. CrossRef PubMed

[4] Weller M, Cloughesy T, Perry JR, Wick W. Standards of care for treatment of recurrent glioblastoma – are we there yet? Neuro-oncol. 2013; 15: 4-27. CrossRef PubMed

[5] Sanai N, Berger MS. Glioma extent of resection and its impact on patient outcome. Neurosurgery. 2008; 62: 753-764., discussion 264-266. CrossRef PubMed

[6] Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, Kрос JM, Hainfellner JA, Mason W, Mariani L, Bromberg JE, Hau P, Mirimanoff RO, Cairncross JG, Janzer RC, Stupp R. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med. 2005; 352: 997-1003. CrossRef PubMed

[7] Wick W, Platten M, Meissner C, Felsberg J, Tabatabai G, Simon M, Nikkhah G, Papsdorf K, Steinbach JP, Sabel M, Combi SE, Vesper J, Braun C, Meinsenberger J, Ketter R, Mayer-Steinacker R, Reifenberger G, Weller M, NOA-08 Study Group of Neuro-oncology Working Group (NOA) of German Cancer Society. Temozolomide chemotherapy alone versus radiotherapy alone for malignant astrocytoma in the elderly: the NOA-08 randomised, phase 3 trial. Lancet Oncol. 2012; 13: 707-715. CrossRef PubMed

[8] Malmström A, Grunberg BH, Marosi C, Stupp R, Frappaz D, Schultz H, Abacioglu U, Tavelin B, Lhermitte B, Hegi ME, Rosell J, Henriksson R; Nordic Clinical Brain Tumour Study Group (NCBTSG). Temozolomide versus standard 6-week radiotherapy versus hypofractionated radiotherapy in patients older than 60 years with glioblastoma: the Nordic randomised, phase 3 trial. Lancet Oncol. 2012; 13: 916-926. CrossRef PubMed

[9] Preusser M, Eble I, Hainfellner JA. Reliability and reproducibility of PCR-based testing of O6-methylguanine-DNA methyltransferase gene (MGMT) promoter methylation status in formalin-fixed and paraffin-embedded neurosurgical biopsy specimens. Clin Neuropathol. 2008; 27: 388-390. CrossRef PubMed

[10] Berghoff AS, Preusser M. Clinical neuropathology practice guide 06-2012: MGMT testing in elderly glioblastoma patients – yes, but how? Clin Neuropathol. 2012; 31: 405-408. CrossRef PubMed

[11] Mikeska T, Bock C, El-Maarri O, Hübner A, Ehrentraut D, Schramm J, Felsberg J, Kahl P, Bütner R, Pietsch T, Waha A. Optimization of quantitative MGMT promoter methylation analysis using pyrosequencing and combined bisulfite restriction analysis. J Mol Diagn. 2007; 9: 368-381. CrossRef PubMed

[12] Felsberg J, Thon N, Eigenbrod S, Hentschel B, Sabel MC, Westphal M, Schackert G, Kreth FW, Pietsch T, Löffler M, Weller M, Reifenberger G, Tonn JC; German Glioma Network. Promoter methylation and expression of MGMT and the DNA mismatch repair genes MLH1, MSH2, MSH6 and PMS2 in paired primary and recurrent glioblastomas. Int J Cancer. 2011; 129: 659-670. CrossRef PubMed

[13] Quillien V, Lavenu A, Karayan-Tapon L, Carpentier C, Labussière M, Lesimple T, Chinot O, Wager M, Honnorat J, Saikali S, Fina F, Sanson M, Figarella-Branger D. Comparative assessment of 5 methods (methylation-specific polymerase chain reaction, MethyLight, pyrosequencing, methylation-sensitive high-resolution melting, and immunohistochemistry) to analyze O6-methylguanine-DNA-methyltransferase in a series of 100 glioblastoma patients. Cancer. 2012; 118: 4201-4211. CrossRef PubMed

[14] Karayan-Tapon L, Quillien V, Guilhot J, Wager M, Fromont G, Saikali S, Echeverry A, Hamlat A, Loussouarn D, Campion L, Campone M, Vallette FM, Gratas-Rabahi-Re C. Prognostic value of O6-methylguanine-DNA-methyltransferase status in glioblastoma patients, assessed by five different methods. J Neurooncol. 2010; 97: 311-322. CrossRef PubMed

[15] Christians A, Hartmann C, Benner A, Meyer J, von Deimling A, Weller M, Wick W, Weiler M. Prognostic value of three different methods of MGMT promoter methylation analysis in a prospective trial on newly diagnosed glioblastoma. PLoS ONE. 2012; 7: e33449. CrossRef PubMed

[16] Håvik AB, Brandal P, Home H, Dahlbäck HS, Scheie D, Hektoen M, Meling TR, Helseth E, Heim S, Lothe RA, Lind GE. MGMT promoter methylation in gliomas-assessment by pyrosequencing and quantitative methylation-specific PCR. J Transl Med. 2012; 10: 36. CrossRef PubMed
Preusser, Berghoff, Manzl, et al. 14

[17] Dunn J, Baborie A, Alam F, Joyce K, Moxham M, Sithon R, Crooks D, Husband D, Shenoy A, Brodbelt A, Wong H, Liloglou T, Haylock B, Walker C. Extent of MGMT promoter methylation correlates with outcome in glioblastomas given temozolomide and radiotherapy. Br J Cancer. 2009; 101: 124-131. CrossRef PubMed

[18] Reifenberger G, Hentschel R, Felsberg J, Schackert G, Simon M, Schnell O, Westphal M, Wick W, Pietsch T, Loeffler M, Weller M; German Glioma Network. Predictive impact of MGMT promoter methylation in glioblastoma of the elderly. Int J Cancer. 2012; 131: 1342-1350. CrossRef PubMed

[19] Preusser M, Plumer S, Dirnberger E, Hainfellner JA, Mannhalter C. Fixation of brain tumor biopsy specimens with RCL2 results in well-preserved histomorphology, immunohistochemistry and nucleic acids. Brain Pathol. 2010; 20: 1010-1020. CrossRef PubMed

[20] Pulverer W, Hofner M, Preusser M, Dirnberger E, Hainfellner JA, Weinhaeusel A. A simple quantitative diagnostic alternative for MGMT DNA-methylation testing on RCL2 fixed paraffin embedded tumors using restriction coupled qPCR. Clin Neuropathol. 2013; 33: 50-60. CrossRef PubMed

[21] Louis D, Ohgaki H, Wiestler O, et al. WHO Classification of Tumours of the Central Nervous System. 4th ed. 2007, Lyons, France: IARC Press.

[22] Löf-Ohlin ZM, Nilsson TK. Pyrosequencing assays to study promoter CpG site methylation of the O6-MGMT, hMLH1, p14ARF, p16INK4a, RASSF1A, and APC1A genes. Oncol Rep. 2009; 21: 721-729. PubMed

[23] Tuononen K, Tyninnin O, Sarhadi VK, Tyybakimoja A, Lindlöf M, Antikainen M, Nääpäkangas J, Hirvonen A, Mäenpää H, Paetau A, Knuutila S. The hypermethylation of the O6-methylguanine-DNA methyltransferase gene promoter in gliomas—correlation with array comparative genome hybridization results and IDH1 mutation. Genes Chromosomes Cancer. 2012; 51: 20-29. CrossRef PubMed

[24] Berghoff AS, Stefanits H, Woehrer A, Heinzl H, Preusser M, Hainfellner JA; Vienna Comprehensive Cancer Center Central Nervous System Unit. Clinical neuropathology practice guide 3-2013: levels of evidence and clinical utility of prognostic and predictive candidate brain tumor biomarkers. Clin Neuropathol. 2013; 32: 148-158. CrossRef PubMed

[25] Ronaghi M, Uhlen M, and Nyren P. A sequencing method based on real-time pyrophosphate. Science. 1998; 281(5375): 363, 365.

[26] Nyren P. The history of pyrosequencing. Methods Mol Biol. 2007; 373: 1-14. PubMed

[27] Ronaghi M, Karamohamed S, Pettersson B, Uhlen M, Nyren P. Real-time DNA sequencing using detection of pyrophosphate release. Anal Biochem. 1996; 242: 84-89. CrossRef PubMed

[28] Ahmadian A, Ehn M, Hober S. Pyrosequencing: history, biochemistry and future. Clin Chim Acta. 2006; 363: 83-94. CrossRef PubMed