A new method for testing MGMT gene promoter methylation status of glioblastoma tissue using a direct real-time fluorescence-based methylation-specific PCR

Stéphane Califice[1], Annie-Claire Diserens[2], Ilse Vlassenbroeck[1], Eugenia Migliavacca[3,4], Josef Straub[1], Ivano Di Stefano[1], Fabrice Moreau[1], Marie-France Hamou[2], Isabelle Renard[1], Mauro Delorenzi[3,4], Katja Bierau[1], Monika E. Hegi[2,4] [1] OncoMethylome Sciences SA, Liège, Belgium, [2]Laboratory of Tumor Biology and Genetics, Department of Neurosurgery, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland, [3]Swiss Institute of Bioinformatics, [4]National Center of Competence in Research Molecular Oncology at the Swiss Institute of Experimental Cancer Research, Epalinges, Switzerland.

Abstract

The treatment options for primary malignant brain cancers are limited and available therapies produced modest benefit highlighting the need for more targeted, patient specific treatments. Epigenetic silencing of the DNA repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT) by promoter methylation in the tumor tissue is associated with longer survival in patients with glioblastoma who receive alkylating agents, such as carmustine (1) and temozolomide (2, 3). OncoMethylome Sciences (ONCO) has developed a direct real-time fluorescence based methylation-specific PCR assay (real-time MSP assay) to define the methylation status of the MGMT promoter. After DNA isolation of formalin fixed paraffin embedded tissue, the real-time MSP protocol was followed. This comprises a sequence specific PCR reaction after DNA modification using sodium bisulphite. The chemical modification is the essential step for distinguishing methylated and unmethylated DNA sequences. The assay specificity is obtained by using methylation specific primers during the PCR reaction. The results of the real-time MSP assay were compared to the already validated nested gel-based MSP assay developed at the Centre Hospitalier Universitaire Vaudois (CHUV), which yields a binary outcome ("presence or absence" of a band for methylated MGMT (m_MGMT)). In conclusion, the real-time MSP assay has good evaluability, is reproducible, and correlates well with the already validated gel-based MSP assay. Moreover the use of a real-time PCR platform provides the possibility for high throughput analysis and it also opens the possibility of determining in the future a test threshold optimized for clinical applications.

Materials and Methods

The results of the real-time MSP assay (performed at ONCO) were compared to the already validated nested gel-based MSP assay (performed at the CHUV). The laboratories were blinded to the results obtained in the other assay. Figure 1 shows the workflow of the real-time MSP assay.

Sample preparation: 134 tissue samples (formalin fixed paraffin embedded (FFPE) sections) were tested in this study. When processing FFPE samples a deparaffination step (standard reagents) was performed prior to DNA isolation using the classical phenol/chloroform extraction procedure (standard reagents). DNA was quantified using a PicoGreen® assay.

Real-time MSP: Up to 1.5µg of purified DNA was bisulphite modified using the commercially available EZ DNA Methylation kit from Zymo Research. In addition to MGMT, the independent reference gene β-actin (ACTB) was also measured. The analyte (m_MGMT and ACTB) quantitations were done in real-time PCR assays. These consisted of parallel amplification / quantitation processes. The amplicons created during the amplification process were quantified by real-time measurement of emitted fluorescence. The ratio between m_MGMT and ACTB was calculated. This ratio was defined as the test result. The samples were classified as methylated, non-methylated, grey zone or invalid based on the decision tree shown in Figure 2.

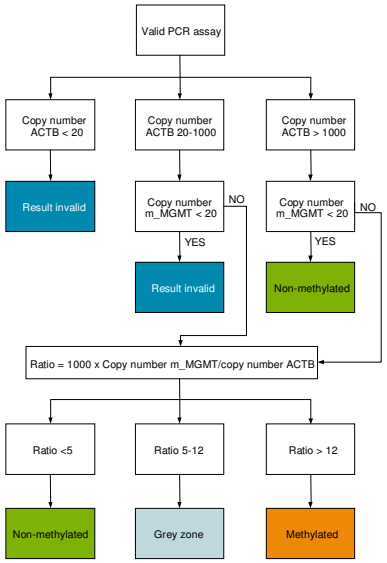

Figure 2: Decision tree for determination of the MGMT methylation status in clinical samples (real-time MSP).

Gel-based MSP: The same samples were analyzed with the already validated gel-based MSP assay using a nested PCR approach (2, 3).

Figure 1: Workflow of the real-time MSP assay.

Results

Validity rate: The assays were compared by evaluating 134 FFPE glioma samples by real-time MSP (29 single and 105 duplicate tests) and 125 FFPE glioma samples by gel-based MSP (all single tests as defined in references 2 and 3). The real-time MSP assay produced valid results in 94.2% versus 75.2% of valid tests for the gel-based assay (Table 1). Among the duplicate real-time MSP tests 5 of 6 invalid results were duplicated. The validity rates are based on the respective validation criteria for each assay (for gel-based MSP see references 2 and 3, for real-time MSP see Figure 2).

Table 1: Summary of the samples evaluated by both assays

Site	Samples	Duplicate test	Single test	Total tests	Valid tests [%]
ONCO	134	105 (210 tests)	29	239	225/239 [94.2%]
CHUV	125	0	125	125	94/125 [75.2%]

Table 2: Concordance between real-time and gel-based MSP results (m_MGMT and non-m_MGMT measurements)

		Real-time MSP		
		m_MGMT	non-m_MGMT	Total
Gel-based MSP	m_MGMT	34	4	38
	non-m_MGMT	1	52	53
	Total	35	56	91

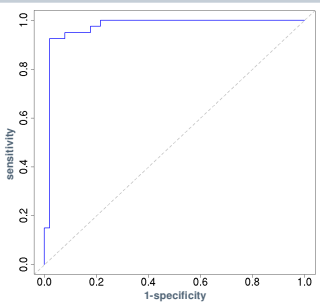

Figure 3: ROC curve for real-time MSP assay to predict a methylated MGMT status as classified based on 91 samples. AUC = 0.96.

Concordance between real-time and gel-based MSP assays: 91 FFPE glioma samples yielded a valid result for both real-time and gel-based MSP assays (Table 2). Previous work (4) suggested the use of a grey zone between non-methylated and methylated ratio cut-offs of 5 and 12 respectively, due to the limited availability of samples between those cut-offs. For the present study ratio values of 5 or greater were classified as methylated (Table 2).

Comparison between real-time and gel-based MSP tests for the 91 cases showed high concordance between the results (Cohen's Kappa of 0.89). Considering the gel-based MSP assay as the "truth", the real-time MSP assay had an AUC (area under the Receiver Operating Characteristic (ROC) curve) of 0.96, with sensitivity and specificity of 0.90 and 0.98 respectively, at the chosen ratio cut-off of 5 (Figure 3).

Reproducibility of the real-time MSP assay: 99 of the 105 duplicate samples yielded a valid result for both real-time MSP replicates. The reproducibility of the replicates can be seen in Figure 4. An application of a ratio cut-off of 5 yielded the concordance of the results shown in Table 3 (Cohen's Kappa of 0.98). The single discrepant sample, upon retesting, was seen to be non-methylated in a third replicate which would have yielded complete concordance.

Table 3: Concordance between real-time MSP duplicates (ONCO 1 and ONCO 2) for m_MGMT and non-m_MGMT measurements

		ONCO 1		
		m_MGMT	non-m_MGMT	Total
ONCO 2	m_MGMT	37	0	37
	non-m_MGMT	1	61	62
	Total	38	61	99

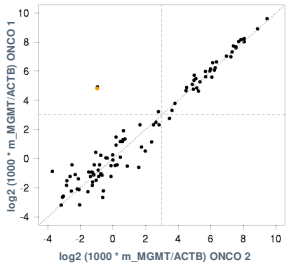

Figure 4: Reproducibility of duplicate measurements. Dotted line represents identity line (x = y). The one sample (shown in orange) that gives very poor reproducibility is most probably due to a technical error in the first testing. A third replicate closely matched the low ratio value seen in the second testing.

Conclusions: **The real-time MSP assay has good evaluability, yielded similar results as compared to the established method and is technically more robust. Comparison of processing duplicates demonstrated an excellent reproducibility. The use of real-time MSP allows standardization of the assay, facilitating quality control as required for diagnostic purposes, and provides the possibility for high throughput analysis. Also, the real-time PCR platform opens the possibility of determining in the future a test threshold optimized for clinical applications, such as individualized management of patients considered for treatment with temozolomide or other alkylating agent chemotherapy. The real-time MSP assay is currently being used for an ongoing prospective trial (RTOG 0525 / EORTC 26052-22053) which will yield data to establish a clinically relevant cut-off for m_MGMT.**

References: 1) Esteller M *et al.* N Engl J Med 2000; 343(19):1350-4., 2) Hegi ME *et al.* Clin Cancer Res 2004; 10(6):1871-4., 3) Hegi ME *et al.* N Engl J Med 2005; 352(10):1036-8, 4) Califice S et al. Poster 2005 "O6-Alkylguanine-DNA Alkyltransferase Meeting", Keele, UK, 06 - 09/08/2005

More information: Stephane.Califice@oncomethylome.com or www.OncoMethylome.com