Digital PCR quantification of *MGMT* methylation refines prediction of clinical benefit from alkylating agents in glioblastoma and metastatic colorectal cancer

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Abstract

Background: O6-Methyl-Guanine-Methyl-Transferase (*MGMT*) silencing by promoter methylation may identify cancer patients responding to the alkylating agents dacarbazine or temozolomide.

Patients and methods: We evaluated the prognostic and predictive value of *MGMT* methylation testing both in tumor and cell-free circulating DNA (cfDNA) from plasma samples using an ultra-sensitive two-step digital PCR technique (Methyl-BEAMing). Results were compared to two established techniques, Methylation specific PCR (MSP) and Bs-pyrosequencing.

Results: Thresholds for *MGMT* methylated status for each technique were established in a training-set of 98 glioblastoma patients. The prognostic and the predictive value of *MGMT* methylated status was validated in a second cohort of 66 glioblastoma patients treated with temozolomide in which Methyl-BEAMing displayed a better specificity than the other techniques. Cut-off values of *MGMT* methylation specific for metastatic colorectal cancer (mCRC) tissue samples were established in a cohort of 60 patients treated with dacarbazine. In mCRC, both quantitative assays Methyl-BEAMing and Bs-pyrosequencing outperformed MSP, providing better prediction of treatment response and improvement in progression-free survival (PFS)($p<0.001$). Ability of Methyl-BEAMing to identify responding patients was validated in a cohort of 23 mCRC patients treated with temozolomide and pre-selected for *MGMT* methylated status according to MSP. In mCRC patients treated with dacarbazine, exploratory analysis of cfDNA by Methyl-BEAMing showed that *MGMT* methylation was associated with better response and improved median PFS ($p=0.008$).

Conclusions: Methyl-BEAMing showed high reproducibility, specificity and sensitivity and was applicable to formalin fixed paraffin embedded tissues and cfDNA. This study

supports the quantitative assessment of *MGMT* methylation for clinical purposes since it could refine prediction of response to alkylating agents.

Key Words: *MGMT*; DNA methylation; digital PCR; Metastatic colorectal cancer; alkylating agent; cell free circulating DNA.

Key Message:

Improved assessment of MGMT methylation by a digital PCR method in glioblastoma as well as in colorectal cancer samples could be used to identify patients most likely to derive clinical benefit from treatment with alkylating agents such as dacarbazine or temozolomide.

Introduction

Alkylating agents such as dacarbazine and temozolomide (TMZ) are currently used in the clinical management of lymphomas, melanomas and as first-line treatment for glioblastoma (GBM) in addition to surgical resection and radiotherapy. Action of these drugs is enhanced in tumors with inactive O6-Methyl-Guanine-Methyl-Transferase (*MGMT*), which is the DNA repair enzyme in charge of removing DNA alkylated adducts [1]. Defective MGMT function mainly results from its transcriptional silencing by gene promoter methylation. Therefore, *MGMT* methylation has been proposed as a predictive marker of response to alkylating agents [2-5]. Nevertheless, not all patients with *MGMT* hypermethylated tumors respond to treatment with alkylating agents [6, 7].

MGMT silencing has also been found to occur in several other malignancies [8, 9], including colorectal cancer (CRC) [8, 9]. The reported high prevalence of this marker in CRC (30-40%) has led to several trials which have recently evaluated the clinical activity of alkylating agents in the metastatic setting [10-13]. Collectively, these studies showed that clinical benefit could be achieved in up to 40% of heavily pre-treated patients [11-13]. Despite minor differences in response rates and progression-free survival (PFS), all the above studies reported that only a fraction of *MGMT* methylated cases derived clinical benefit from treatment with dacarbazine or TMZ. We hypothesize that the relatively poor specificity of *MGMT* status as a predictive marker of response to alkylating agents could be explained by an inaccurate assessment of methylation due to sampling issues, tumor heterogeneity or suboptimal detection methods.

Here we implemented the detection of *MGMT* methylation through the methyl beads, emulsion, amplification, and magnetics protocol also known as Methyl-BEAMing assay

[14]. We validated the predictive prognostic value of MGMT methylation testing in two GBM cohorts. We tested whether this technique could improve the assessment of *MGMT* methylation and the selection of CRC patients with higher probability of response to alkylating agents. We then compared it to commonly used methods, including Methylation Specific PCR [MSP] [15] and Bisulfite Pyrosequencing [Bs-Pyrosequencing] [16]. Finally, we evaluated the ability of the Methyl-BEAMing assay to detect tumor methylation status directly from plasma samples of CRC patients to evaluate the feasibility of patient selection for treatment via a blood test.

Material and Methods

Patients and sample preparation

A first GBM training-set included tissue samples from 98 patients who had undergone brain surgery at the Academic Medical Center in Amsterdam, between 1988 and 2006 [17]. A second GBM validation-set consisted of 66 tissue samples from patients with newly-diagnosed GBM, who had surgery and chemoradiation (radiotherapy and concomitant TMZ, followed by six monthly cycles of adjuvant TMZ) with a follow-up of at least two years at the VU University Medical Center in Amsterdam. The DETECT-01 trial composed the CRC training-set, in which 68 patients with chemorefractory metastatic CRC (mCRC) were treated with dacarbazine [11]. The validation-set consisted of 23 samples from a phase II trial, in which 32 patients with chemorefractory metastatic CRC (mCRC) were treated with TMZ [13]. Further details about the cohorts and the sample preparation can be found in Data S1. The studies followed the Declaration of Helsinki and were approved by local ethics committees.

MGMT **methylation assays**

MGMT methylation was retrospectively assessed in tissue sample DNA using MSP, Bs-pyrosequencing and Methyl-BEAMing. Analyses were performed in a blinded fashion without prior knowledge of *MGMT* methylation status. Cell-free circulating DNA (cfDNA) (cfDNA) was assessed by Methyl-BEAMing. All the assays targeted CpGs within the differentially methylated region number 2 previously associated with TMZ response [18]. Detailed protocols are provided in Data S1. Sensitivity, reproducibility and specificity of MGMT Methyl-BEAMing assays can be found in Data S2.

Quality control of cfDNA

Three different assays were used to evaluate the presence of cfDNA from tumor origin (circulating tumor DNA, ctDNA): Methyl-BEAMing assays specific for *SEPT9* and *VIM* methylation (markers highly prevalent in mCRC) and Droplet Digital™ PCR assays for the *KRAS* mutational status for samples known to be mutated in the tumor tissue (Data S1).

Statistical Analyses

Survival analyses and kappa statistics were performed using Prism 6.01 for Windows (GraphPad Software). Differences in survival were tested by the log-rank test (Mantel-Cox). ROC analyses were performed with R bioconductor using the pROC package [19]. Hazard-Ratios were expressed using the log-rank test. All expressed *p*-values were calculated with two-tailed tests and were considered significant when $p<0.05$.

Results

Prognostic and predictive value of *MGMT* **methylation in GBM**

MGMT methylation is a well-known prognostic marker in GBM [8]. In order to establish the prognostic value of *MGMT* status assessed by Methyl-BEAMing, we employed tissue

samples from a cohort of 98 patients with GBM diagnosed before TMZ was introduced as component of standard treatment for these tumors [17]. Methyl-BEAMing was compared with two established techniques, namely MSP and Bs-Pyrosequencing. For each method, ROC analysis was performed to evaluate the threshold best fitting the overall survival (OS) at 1 year (Data S3A-C). Methylation classification for the three methods concurred in most of the cases with the best agreement between Bs-Pyrosequencing and Methyl-BEAMing (86.7%) (Data S3D). All three methods identified a methylated subgroup of patients with better OS ($p<0.05$ for all methods); however quantitative techniques (Bs-Pyrosequencing, Methyl-BEAMing) displayed a better specificity. Then, only quantitative methods were assessed in a validation cohort of 66 GBM treated with TMZ. Methylation ranges, status, and association with survival for both techniques are summarized in Table S1 and Data S3E-G. OS and PFS according to methylation status by both techniques are shown in Figure 1 and demonstrated better identification of long term responders with Methyl-BEAMing. Comparison of hazard-ratios (Data S3H), showed a better stratification of the population with good prognosis and response to TMZ by Methyl-BEAMing.

Prognostic and predictive value of *MGMT* methylation in mCRC

The *DETECT-01* study evaluated dacarbazine treatment for mCRC patients after failure of standard therapies. The original report determined *MGMT* methylation status via MSP and found that 44% of patients in the methylated subgroup achieved disease control as assessed by radiological methods, although no improvement in PFS was observed [11]. Archived FFPE tumor samples were available for 61 of the 68 patients originally enrolled in the trial. *MGMT* assessment was successful in 56 cases (91.8%) by MSP, 59 (96.7%) by Bs-Pyrosequencing and in all 61 cases by Methyl-BEAMing. Methylation values were normalized for 60 cases for which tumor content was available (Data S1). All techniques showed a bimodal distribution with similar range (Data S4A-B). *MGMT* methylation ranges,

status and association with survival are in Table S1. For each method ROC analysis was performed to evaluate the threshold best fitting the PFS at 12 weeks (Data S4C). When these cut-off values were applied, the number of cases classified as methylated by MSP, Bs-Pyrosequencing and Methyl-BEAMing was 18 (30%), 10 (17%) and 12 (20%), respectively. This resulted in 92% concordance between Bs-Pyrosequencing and Methyl-BEAMing, and 77% and 72% agreement between MSP and Methyl-BEAMing or Bs-Pyrosequencing respectively (Data S4D). No association with OS was observed with any of the techniques (Data S4E), suggesting that *MGMT* status might lack prognostic value in mCRC.

Response to dacarbazine was evaluated using RECIST criteria. Among the 61 available cases, nine patients showed disease control (two responders and seven individuals with stable disease; Data S4). MSP classified 18 cases as methylated, which included seven of the nine patients achieving clinical benefit, thereby displaying a positive predictive value (PPV) of 0.39 and a negative predictive value (NPV) of 0.88 (Figure 2A). Bs-Pyrosequencing achieved a PPV of 0.8 and NPV of 0.89, by classifying a total of 10 cases as methylated, of which eight patients with disease control (Figure 2B). Methyl-BEAMing identified 12 tumors as methylated, of which eight (67%) were from patients with clinical benefit (Figure 2C), resulting in a PPV of 0.67 and a NPV of 0.89.

Next, Bs-Pyrosequencing and Methyl-BEAMing were assessed in a validation cohort of 23 samples from mCRC patients treated with TMZ using the above identified cut-off values. Methyl-BEAMing was successful in 21 cases (91%) and identified 8 tumors as methylated, of which 4 (50%) were from patients with clinical benefit (all partial responders) (Data S4G-H), achieving a PPV of 0.5 and a NPV of 0.67. Methyl-BEAMing methylated subgroup also showed a trend for improved PFS. Bs-Pyrosequencing failed in 15 cases (65%) preventing further analyses.

Analysis of cfDNA in plasma from mCRC Patients

MGMT methylation of cfDNA was only assessed by Methyl-BEAMing assay. Evaluation

was successful in all 49 available samples. MGMT ranges, status and association with

survival are shown in Table S1. ROC analysis was performed to define the best threshold

in cfDNA (Data S4C). Thirty-eight unmethylated cases (75.6%) were identified. To verify

the presence of DNA from tumor origin (circulating tumor DNA, ctDNA), we assessed

KRAS mutational status for the 20 cases with known G12 or G13 mutation in the

corresponding tumor tissue, as well as *SEPT9* and *VIM* methylation in all samples.

Methylated *SEPT9* and *VIM* are two early markers of detection of intestinal disease

reported with over 85% prevalence in mCRC [14, 20]. Six samples were considered as low

ctDNA (four *KRAS* mutated and two wild type cases) since they displayed neither *KRAS*

mutation nor methylation in *SEPT9* or *VIM* in plasma (Figure 1D) despite showing these

alterations in the corresponding tissue (data not shown).

Out of the 49 available plasma samples only 43 had remaining matched tissue that could

be assessed for tumor content and *MGMT* methylation. Concordance was seen in 37

cases (86.1%) (six methylated and 31 unmethylated cases; Figure 1E; Data S4I).

Correlation between the *MGMT* methylation status in tissue and plasma samples indicates

that most of the methylated alleles present in the tissue were released in the blood

(Spearman correlation= 0.53, *p*=0.0003).

MGMT methylated status in cfDNA was also associated with a significantly improved

median PFS (2.1 months vs. 1.8 months for unmethylated group, *p*=0.008, Table S1, Data

S4E). Among the available plasma samples, seven were obtained from patients with

clinical benefit from dacarbazine treatment. *MGMT* methylated status was observed in 11

(22%) plasma and identified five of the seven patients achieving clinical benefit (Figure 1F,

Data S4F). Among the two unmethylated cases with clinical benefit, one did not have remaining tissue sample DNA and the second was considered as low ctDNA.

Discussion

MGMT methylation has been previously identified as a prognostic and predictive marker in GBM [2-5]. However its specificity for response prediction in GBM and other cancer types remains controversial. *MGMT* methylation status is usually evaluated by MSP or Bs-Pyrosequencing [15, 16]. Notably, recent phase II clinical trials in mCRC with alkylating agent therapies relied on MSP evaluation of *MGMT* [11-13] for patient selection or evaluation of response prediction. These studies demonstrated that up to 40% of heavily pretreated mCRC patients achieved some clinical benefit, indicating that drug repositioning could be helpful in this setting upon improved patient selection [21]. Here, we describe the use of Methyl-BEAMing, a highly sensitive and reproducible technique for the detection of *MGMT* methylation in tissue and plasma samples derived from cancer patients.

Prognostic significance of *MGMT* methylated status assessed by Methyl-BEAMing in GBM was improved compared to MSP or Bs-Pyrosequencing. Predictive value of *MGMT* methylated status for response to TMZ was also observed with a better stratification using Methyl-BEAMing compared to Bs-Pyrosequencing. Plasma samples were not available for the GBM patients, thereby preventing us from assessing the potential role of liquid biopsy in this setting. While the blood brain barrier may limit the amount of cfDNA in patients affected by CNS malignancies [22], it has been shown that real-time PCR can be used to detect *MGMT* methylation in the plasma of glioblastoma patients receiving TMZ [23]. Further studies are therefore warranted to clarify the role of liquid biopsy in GBM [24].

Our study retrospectively assessed two mCRC cohorts for which DNA was extracted from FFPE tissue. The three methods successfully evaluated most of the CRC samples in the first training-set yet with a better performance obtained with Methyl-BEAMing in terms of dynamic range. Both mCRC patient cohorts were enrolled in clinical trials with alkylating agents thus allowing the assessment of *MGMT* methylation as a predictive biomarker. Both Methyl-BEAMing and Bs-Pyrosequencing outperformed MSP in the CRC training-set strongly indicating that quantitative methods are needed to assess methylation markers in tissue. However, Bs-Pyrosequencing failed to reliably assess MGMT promoter methylation status in most of the samples of the validation cohort for which only limited amount of DNA was available. Consequently, although Bs-Pyrosequencing provides a robust quantification, its requirement for DNA with high quality and quantity could limit its use for specific sample types such as FFPE biopsies or cfDNA.

Of interest, a number of GBM cases showed intra-locus heterogeneity by Bs-Pyrosequencing (also described by Bady *et al.* using a methylation microarray platform [18]); while this pattern was rarely seen in the mCRC samples (Data S4I). As the current Bs-Pyrosequencing is the average of the six evaluated CpG sites, its accuracy might suffer from the heterogenous profiles observed in GBM as well as by incomplete bisulfite conversion. Therefore we hypothesize that this could explain the discrepancy of performance between Bs-Pyrosequencing and Methyl-BEAMing in the two tissue types.

It is also possible that *MGMT* methylation heterogeneity exists among individual tumor cells and that MGMT immunostaining could be used in combination with methylation based methods to better refine selection of patients [25]. However, so far, observer variability and lack of association with patient survival has hampered the use of immunohistochemistry as clinical biomarker in GBM [26, 27]. Studies that have addressed

the role of MGMT immunostaining as predictive biomarker of response in CRC are limited to case reports [10] and further investigations are needed in larger cohorts.

Plasma samples were only available for patients in the mCRC training cohort. We successfully assessed all cases via Methyl-BEAMing demonstrating high efficiency even with samples of poor quality and limited quantity. Reliability of the results was limited in a few instances by the observation that cfDNA samples are not only composed of DNA of tumor origin [22]. Therefore we evaluated *SEPT9* and *VIM* methylation, and *KRAS* mutation (when the tissue demonstrated an alteration) in cfDNA. Six samples out of 49 showed the absence of all these markers, strongly suggesting the absence of ctDNA. Use of higher volume of plasma or exploitation of micro-vesicles, such as exosomes [28] could potentially solve this issue. Discrepancies between the plasma and tissue could be mainly explained by the low abundance of ctDNA. In the remaining cases, we hypothesized that the tumor might have evolved between the time of diagnosis (tissue collection) and the treatment (plasma collection) since this period could have been longer than 10 years. An ongoing study including fresh biopsies is being performed to investigate whether and to what extent there is change of *MGMT* methylated status over time [29]. Nevertheless, the present comparison of plasma and tissue samples showed that cfDNA could be used as a good surrogate to tissue biopsies when the tumor load is controlled and normalized. To achieve this aim, optimization of house-keeping genes highly methylated in cancer and poorly methylated in blood is required. Development of such markers for each cancer type might be required and would enable a better use of alkylating agents across several malignancies.

In conclusion, regardless of the DNA origin (FFPE tissue or plasma) assessment of *MGMT* methylated status by Methyl-BEAMing selected a population highly enriched in patients

showing clinical benefit with dacarbazine or TMZ treatment. Our study therefore supports the clinical implementation of quantitative methods to measure *MGMT* methylation and improve selection of patients who could benefit from alkylating agent-based therapies.

Disclosure

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All remaining authors have declared no conflicts of interest.

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Figure 1: Overall (OS) and Progression Free Survival (PFS) of the GBM validation cohort by (A) Bs-pyrosequencing, and (B) Methyl-BEAMing. Methylated subgroup is in blue, Unmethylated in orange and censored cases are represented by circles.

Figure 2A: Predictive value of *MGMT* methylation status by MSP in mCRC tissue. Waterfall plot indicates response to dacarbazine.

Figure 2B: Predictive value of *MGMT* methylation status by Bs-Pyrosequencing in mCRC tissue. Waterfall plot indicates response to dacarbazine.

Figure 2C: Predictive value of *MGMT* methylation status by Methyl-BEAMing in mCRC tissue. Waterfall plot indicates response to dacarbazine.

Figure 2D: Distribution of methylation (*MGMT*, *SEPT9, VIM*) and mutational (*KRAS*) values in cfDNA. Individual samples were ranked according to average of *SEPT9* + *VIM* + *KRAS*. Grey area corresponds to cases in which no markers were detectable (<1%) and hence were considered to contain very low ctDNA. Threshold for MGMT methylated status is plotted as a dot line.

Figure 2E: Scatter plot of methylation values in tissue and plasma with Spearman correlation according to methylated status. Threshold for each type of tissue is indicated by a dot line.

Figure 2F: Predictive value of *MGMT* methylation status by Methyl-BEAMing in mCRC plasma. Waterfall plot indicates response to dacarbazine.