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

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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 with 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 (GBM) patients. The prognostic and the predictive value of MGMT methylated status was validated in a second cohort of 66 GBM patients treated with temozolomide in which methyl-BEAMing displayed a better specificity than the other techniques. Cutoff values of MGMT methylation specificity 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 preselected 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

introduction

Alkylating agents such as dacarbazine and temozolomide (TMZ) are currently used in the clinical management of lymphomas, melanomas and as first-line treatment of 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 pretreated 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-Beam assay [14]. We validated the predictive and 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 with commonly used methods, including methylation-specific PCR (MSP) [15] and bisulfite pyrosequencing (Bs-pyrosequencing) [16]. Finally, we evaluated the ability of the methyl-beam assay to detect tumor methylation status directly from plasma samples to allow selection of CRC patients 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 2 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 supplementary Data S1, available at Annals of Oncology online. 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-Beam. Analyses were carried out in a blinded fashion without prior knowledge of MGMT methylation status. Cell-free circulating DNA (cfDNA) was assessed by methyl-beam. All the assays targeted CpGs within the differentially methylated region number 2 previously associated with TMZ response [18]. Detailed protocols are provided in supplementary Data S1, available at Annals of Oncology online.

Sensitivity, reproducibility and specificity of MGMT methyl-beam assays can be found in supplementary Data S1 and Data S2, available at Annals of Oncology online.

**Quality Control of cfDNA**

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

**Statistical Analysis**

Survival analyses and kappa statistics were carried out using Prism 6.01 for Windows (GraphPad Software). Differences in survival were tested by the log-rank test (Mantel–Cox). ROC analyses were carried out 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-beam, we employed tissue samples from a cohort of 98 patients with GBM diagnosed before TMZ was introduced as component of standard treatment of these tumors [17]. Methyl-beam was compared with two established techniques, namely MSP and Bs-pyrosequencing. For each method, ROC analysis was carried out to evaluate the threshold best fitting the overall survival (OS) at 1 year (supplementary Data S3A–C, available at Annals of Oncology online). Methylation classification for the three methods concurred in most of the cases with the best agreement between Bs-pyrosequencing and methyl-beam (86.7%) (supplementary Data S3D, available at Annals of Oncology online). All three methods identified a methylated subgroup of patients with better OS (P < 0.05 for all methods); however, quantitative techniques (Bs-pyrosequencing, methyl-beam) 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 supplementary Table S1 and Data S3E–G, available at Annals of Oncology online. 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-beam. Comparison of hazard ratios (supplementary Data S3H, available at Annals of Oncology online) showed a better stratification of the population with good prognosis and response to TMZ by methyl-beam.

**Prognostic and Predictive Value of MGMT Methylation in mCRC**

The DETECT-01 study evaluated dacarbazine treatment of 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...
mRNA expression levels. mRNA expression data were available for 58 patients (63.2%) belonging to the 20 cases with known G12 or G13 mutation in the corresponding tumor tissue, as well as the 24 cases with clinical benefit (Figure 2C), resulting in a PPV of 0.8 and a 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 cutoff 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) (supplementary Data S4G and H, available at Annals of Oncology online). ROC analysis was carried out to define the best threshold in cfDNA (supplementary Data S4C, available at Annals of Oncology online). Thirty-eight unmethylated cases (75.6%) were identified, 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; supplementary Data S4, available at Annals of Oncology online). 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).

In mCRC patients, MGMT methylation 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 supplementary Table S1, available at Annals of Oncology online. ROC analysis was carried out to define the best threshold in cfDNA (supplementary Data S4C, available at Annals of Oncology online). 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 2D) despite showing these alterations in the corresponding tissue (data not shown).

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%) (6 methylated and 31 unmethylated cases; Figure 2E; supplementary Table S1, available at Annals of Oncology online). 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 ctDNA was also associated with a significantly improved median PFS (2.1 versus 1.8 months for unmethylated group, P = 0.008, supplementary Table S1 and Data S4E, available at Annals of Oncology online). 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 2F, supplementary Data S4E, available at Annals of Oncology online). 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 assessed 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. 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 with 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 with Bs-pyrosequencing. Plasma samples were not available from 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 GBM patients receiving TMZ [23]. Further studies are therefore warranted to test whether liquid biopsy can be applied to 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 intralocus 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 (supplementary Data S4I, available at Annals of Oncology online). 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 may only contain DNA of non-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 cfDNA. Use of higher volume of plasma or exploitation of microvesicles, such as exosomes [28] could potentially solve this issue. Discrepancies between the plasma and tissue could be mainly explained by the low abundance of cfDNA. In the remaining cases, we speculate 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 carried out to investigate whether and to what extent MGMT methylated status is subjected to change 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 to allow 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 achieving clinical benefit from 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.

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**disclosure**

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**references**