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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: O^6-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 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 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 O^6-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

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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-BEAMing 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-BEAMing 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-BEAMing. Analyses were carried out in a blinded fashion without prior knowledge of *MGMT* methylation status. Cell-free circulating DNA (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 supplementary Data S1, available at *Annals of Oncology* online. Sensitivity, reproducibility and specificity of MGMT methyl-BEAMing 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-BEAMing 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-BEAMing, 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-BEAMing 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-BEAMing (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-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 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-BEAMing. 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-BEAMing.

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

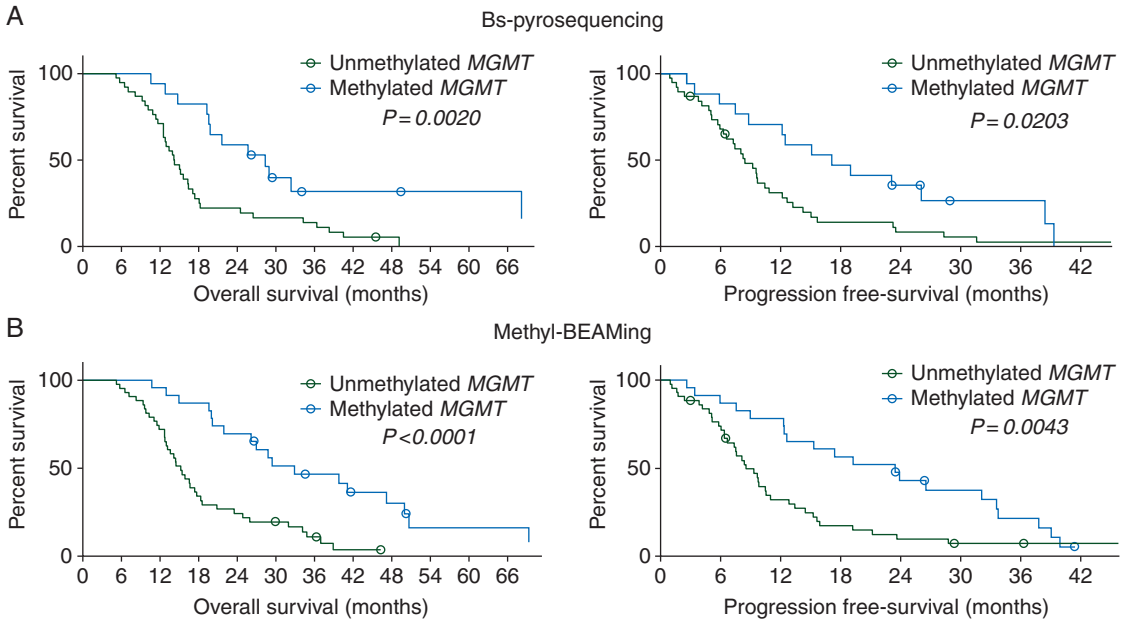

Figure 1. Overall (OS) and progression-free survival (PFS) of the GBM-validation cohort by (A) Bs-pyrosequencing and (B) methyl-BEAMing. Methylated subgroup is in grey (blue online), unmethylated in black (green online) and censored cases are represented by circles.

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 (supplementary Data S1, available at *Annals of Oncology* online). All techniques showed a bimodal distribution with similar range (supplementary Data S4A and B, available at *Annals of Oncology* online). *MGMT* methylation ranges, status and association with survival are in supplementary Table S1, available at *Annals of Oncology* online. For each method, ROC analysis was carried out to evaluate the threshold best fitting the PFS at 12 weeks (supplementary Data S4C, available at *Annals of Oncology* online). When these cutoff 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% or 72% agreement between MSP and methyl-BEAMing or Bs-pyrosequencing, respectively, (supplementary Data S4D, available at *Annals of Oncology* online). No association with OS was observed with any of the techniques (supplementary Data S4E, available at *Annals of Oncology* online), 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). 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 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), 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 in 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 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)

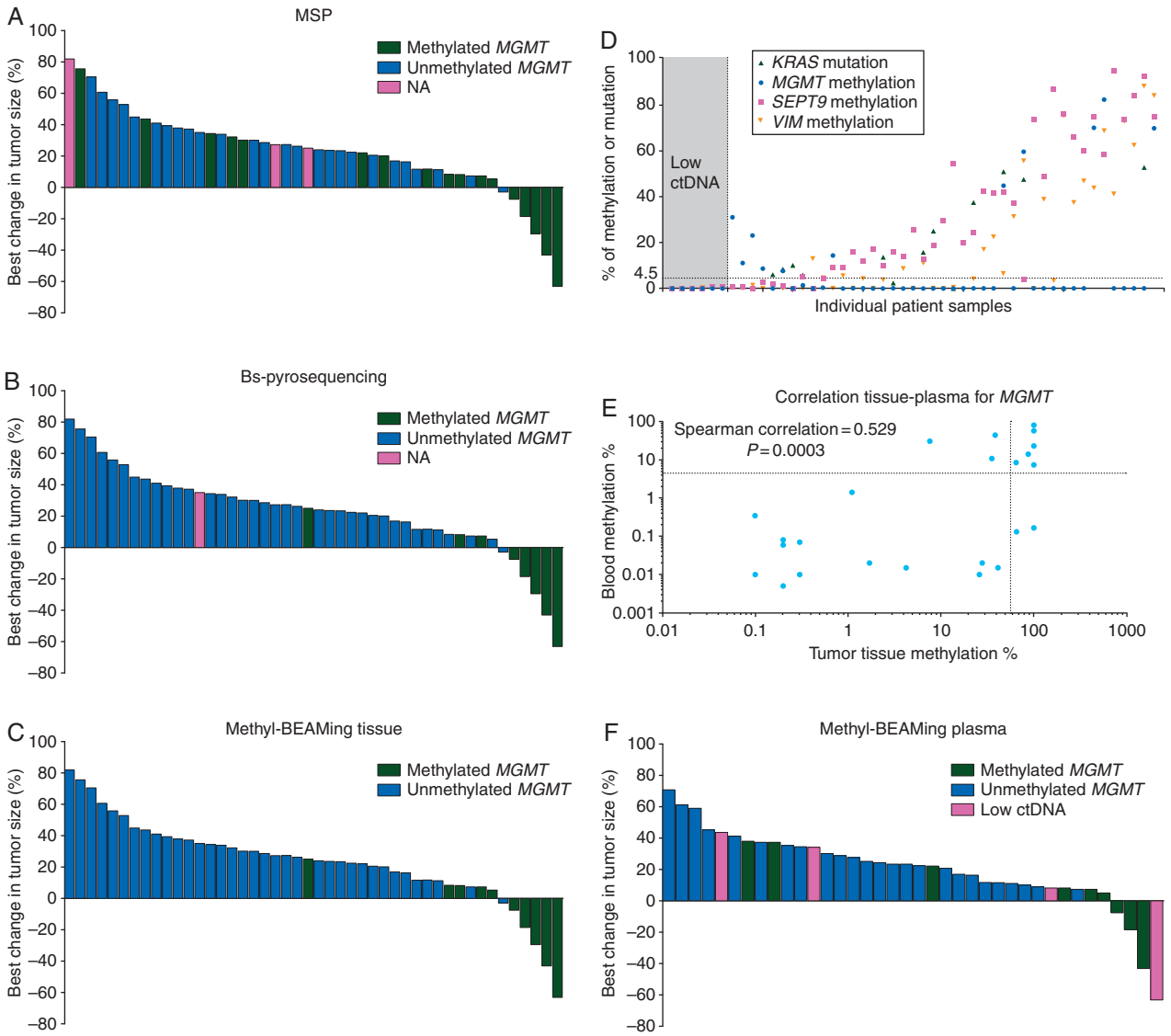

Figure 2. (A) Predictive value of *MGMT* methylation status by MSP in mCRC tissue. Waterfall plot indicates response to dacarbazine. (B) Predictive value of *MGMT* methylation status by Bs-pyrosequencing in mCRC tissue. Waterfall plot indicates response to dacarbazine. (C) Predictive value of *MGMT* methylation status by methyl-BEAMing in mCRC tissue. Waterfall plot indicates response to dacarbazine. (D) Distribution of methylation (*MGMT*, *SEPT9*, *VIM*) and mutational (*KRAS*) values in cfDNA. Individual samples were ranked according to average of *SEPT9* + *VIM* + *KRAS*. Grey (pink online) 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. (E) 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. (F) Predictive value of *MGMT* methylation status by methyl-BEAMing in mCRC plasma. Waterfall plot indicates response to dacarbazine.

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 Data S4I, 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 cfDNA 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 S4F, 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 ctDNA. 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 ctDNA. 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

1. Kaina B, Christmann M, Naumann S, Roos WP. MGMT: key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents. DNA Repair (Amst) 2007; 6: 1079–1099.
2. Esteller M, Garcia-Foncillas J, Andion E et al. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N Engl J Med 2000; 343: 1350–1354.
3. Hegi ME, Diserens AC, Gorlia T et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 2005; 352: 997–1003.
4. Kewitz S, Stiefel M, Kramm CM, Staege MS. Impact of O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation and MGMT expression on dacarbazine resistance of Hodgkin's lymphoma cells. Leuk Res 2014; 38: 138–143.
5. Tuominen R, Jewell R, van den Oord JJ et al. MGMT promoter methylation is associated with temozolomide response and prolonged progression-free survival in disseminated cutaneous melanoma. Int J Cancer 2015; 136: 2844–2853.
6. Gallego Perez-Larraya J, Ducray F, Chinot O et al. Temozolomide in elderly patients with newly diagnosed glioblastoma and poor performance status: an ANOCEF phase II trial. J Clin Oncol 2011; 29: 3050–3055.
7. Taal W, Dubbink HJ, Zonnenberg CB et al. First-line temozolomide chemotherapy in progressive low-grade astrocytomas after radiotherapy: molecular characteristics in relation to response. Neuro Oncol 2011; 13: 235–241.
8. Gerson SL. MGMT: its role in cancer aetiology and cancer therapeutics. Nat Rev Cancer 2004; 4: 296–307.
9. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. Cancer Res 2001; 61: 3225–3229.
10. Shacham-Shmueli E, Beny A, Geva R et al. Response to temozolomide in patients with metastatic colorectal cancer with loss of MGMT expression: a new approach in the era of personalized medicine? J Clin Oncol 2011; 29: e262–e265.
11. Amatu A, Sartore-Bianchi A, Moutinho C et al. Promoter CpG island hypermethylation of the DNA repair enzyme MGMT predicts clinical response to dacarbazine in a phase II study for metastatic colorectal cancer. Clin Cancer Res 2013; 19: 2265–2272.
12. Hochhauser D, Glynne-Jones R, Potter V et al. A phase II study of temozolomide in patients with advanced aerodigestive tract and colorectal cancers and methylation of the O6-methylguanine-DNA methyltransferase promoter. Mol Cancer Ther 2013; 12: 809–818.
13. Pietrantonio F, Perrone F, de Braud F et al. Activity of temozolomide in patients with advanced chemorefractory colorectal cancer and MGMT promoter methylation. Ann Oncol 2014; 25: 404–408.
14. Li M, Chen WD, Papadopoulos N et al. Sensitive digital quantification of DNA methylation in clinical samples. Nat Biotechnol 2009; 27: 858–863.
15. Yachi K, Watanabe T, Ohta T et al. Relevance of MSP assay for the detection of MGMT promoter hypermethylation in glioblastomas. Int J Oncol 2008; 33: 469–475.
16. Quillien V, Lavenu A, Karayan-Tapon L et al. Comparative assessment of 5 methods (methylation-specific polymerase chain reaction, MethyLight, pyrosequencing, methylation-sensitive high-resolution melting, and immunohistochemistry) to analyze O6-methylguanine-DNA-methyltranferase in a series of 100 glioblastoma patients. Cancer 2012; 118: 4201–4211.
17. Molenaar RJ, Verbaan D, Lamba S et al. The combination of IDH1 mutations and MGMT methylation status predicts survival in glioblastoma better than either IDH1 or MGMT alone. Neuro Oncol 2014; 16: 1263–1273.
18. Bady P, Sciuscio D, Diserens AC et al. MGMT methylation analysis of glioblastoma on the Infinium methylation BeadChip identifies two distinct CpG regions associated with gene silencing and outcome, yielding a prediction model for comparisons across datasets, tumor grades, and CIMP-status. Acta Neuropathol 2012; 124: 547–560.
19. Robin X, Turck N, Hainard A et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics 2011; 12: 77.
20. Potter NT, Hurban P, White MN et al. Validation of a real-time PCR-based qualitative assay for the detection of methylated SEPT9 DNA in human plasma. Clin Chem 2014; 60: 1183–1191.
21. Inno A, Fanetti G, Di Bartolomeo M et al. Role of MGMT as biomarker in colorectal cancer. World J Clin Cases 2014; 2: 835–839.
22. Bettegowda C, Sausen M, Leary RJ et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 2014; 6: 224ra224.
23. Fiano V, Trevisan M, Trevisan E et al. MGMT promoter methylation in plasma of glioma patients receiving temozolomide. J Neurooncol 2014; 117: 347–357.
24. Best MG, Sol N, Zijl S et al. Liquid biopsies in patients with diffuse glioma. Acta Neuropathol 2015; 129: 849–865.
25. Lalezari S, Chou AP, Tran A et al. Combined analysis of O6-methylguanine-DNA methyltransferase protein expression and promoter methylation provides optimized prognostication of glioblastoma outcome. Neuro Oncol 2013; 15: 370–381.
26. Preusser M, Charles Janzer R, Felsberg J et al. Anti-O6-methylguanine-methyltransferase (MGMT) immunohistochemistry in glioblastoma multiforme: observer variability and lack of association with patient survival impede its use as clinical biomarker. Brain Pathol 2008; 18: 520–532.
27. Melguizo C, Prados J, Gonzalez B et al. MGMT promoter methylation status and MGMT and CD133 immunohistochemical expression as prognostic markers in glioblastoma patients treated with temozolomide plus radiotherapy. J Transl Med 2012; 10: 250.
28. Thakur BK, Zhang H, Becker A et al. Double-stranded DNA in exosomes: a novel biomarker in cancer detection. Cell Res 2014; 24: 766–769.
29. Amatu A, Bencardino K, Barault L et al. Phase II study of temozolomide (TMZ) in metastatic colorectal cancer (mCRC) patients molecularly selected by MGMT promoter hypermethylation. J Clin Oncol 2015; 33 (Suppl 3): abstr 583.