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

Annals of Oncology 2015;

doi: 10.1093/annonc/mdv272

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

author(s) list: L. Barault [1], A. Amatu [2], F.E. Bleeker [3], C. Moutinho [4], C. Falcomatà [1], V. Fiano [5], A. Cassingena [2], G. Siravegna [1,6,7], M. Milione [8], P. Cassoni [5], F. De Braud [9], R. Rudà [10], R. Soffietti [10], T. Venesio [1], A. Bardelli [1,6], P. Wesseling [11,12], P. de Witt Hamer [13], F. Pietrantonio [8], S. Siena [2], M. Esteller [4,14,15], A. Sartore-Bianchi [2], F. Di Nicolantonio [1,6*]

affiliation(s) list:

[1] *Experimental Clinical Molecular Oncology, Candiolo Cancer Institute-FPO, IRCCS, Candiolo (Torino), Italy;*

[2] *Niguarda Cancer Center, Ospedale Niguarda Ca' Granda, Milan, Italy;*

[3] *Department of Clinical Genetics, Academic Medical Center, University of Amsterdam, The Netherlands;*

[4] *Cancer Epigenetics and Biology Program (PEBC), Bellvitge Biomedical Research Institute (IDIBELL), Barcelona, Catalonia, Spain;*

[5] *Department of Medical Sciences, University of Turin, Citta` della Salute e della Scienza Hospital, Turin, Italy;*

[6] *Department of Oncology, University of Torino, Candiolo (Torino), Italy;*

[7] *FIRC Institute of Molecular Oncology (IFOM), Milan, Italy;*

[8] *Department of Pathology and Laboratory Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy;*

[9] *Department of Medical Oncology Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy;*

[10] *Department of Neuroscience, University of Turin and Città della Salute e della Scienza Hospital, Turin, Italy;*

[11] *Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands*

[12] *Department of Pathology, Radboud University Medical Center, Nijmegen, The Netherlands*

[13] *Department Neurosurgery, Neurosurgical Center Amsterdam, VU University Medical Center, Amsterdam, The Netherlands*

[14] *Department of Physiological Sciences II, School of Medicine, University of Barcelona, Catalonia, Spain;*

[15] *Institucio Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Catalonia, Spain*

full address for correspondence

**corresponding author:*

Dr Federica Di Nicolantonio

Department of Oncology, University of Torino

Strada Provinciale, 142 km 3,95

10060 Candiolo, Torino-Italy

Phone: +39 011 993 3837

Fax: +39 011 993 3225

(Email: federica.dinicolantonio@unito.it)

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.

Acknowledgements

We are grateful to Mrs. Kirsten Mooij, Dept. of Pathology and Biobank, VU University Medical Center, Amsterdam, The Netherlands, for her excellent support in getting the samples for the GBM-validation cohort ready for further analysis. We are also grateful to Ms. Stefania Giove, Core Facilities, Candiolo Cancer Institute-FPO, IRCCS, Candiolo, Italy, for her wonderful support in preparing the paraffin section for the GBM-validation cohort.

Funding

This study was supported by 5 per mille 2009 MIUR—from Fondazione Piemontese per la Ricerca sul Cancro—ONLUS [“Farmacogenomica” to F.D.N.]. Work in the authors’ laboratories was also supported by the European Community’s Seventh Framework Programme [602901 MErCuRIC to A.B. and F.D.N., 259015 COLTHERES to A.B., F.D.N., M.E. and S.S.]; Associazione Italiana per la Ricerca sul Cancro IG [12812 to A.B.]; Associazione Italiana per la Ricerca sul Cancro MFAG [11349 to F.D.N.]; Associazione Italiana per la Ricerca sul Cancro 2010 Special Program Molecular Clinical Oncology 5 per mille [9970 to A.B. and S.S.]; Ministero dell’Istruzione, dell’Università e della Ricerca [PRIN 2010-2011 to A.B.]; grant from Oncologia Ca’ Granda (OCGO) Fondazione [to S.S.]; grant from Regione Lombardia and Ministero Salute [Identification and monitoring of gene mutations in peripheral blood and urine as a diagnostic tool for patients with solid tumors to S.S.]; Fondazione Piemontese per la Ricerca sul Cancro-ONLUS 5 per mille 2010 and 2011 Ministero della Salute [to F.D.N. and A.B.].

Disclosure

Ludovic Barault was the recipient of a post-doctoral fellowship from Fondazione Umberto Veronesi in 2013 and 2015.

All remaining authors have declared no conflicts of interest.

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: e262-265.
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.

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.

Table S1: Impact on overall survival (OS) or progression free survival (PFS) of *MGMT* status in GBM or mCRC patients, according to the different assays employed to assess gene methylation. All survival data are expressed in months.

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.

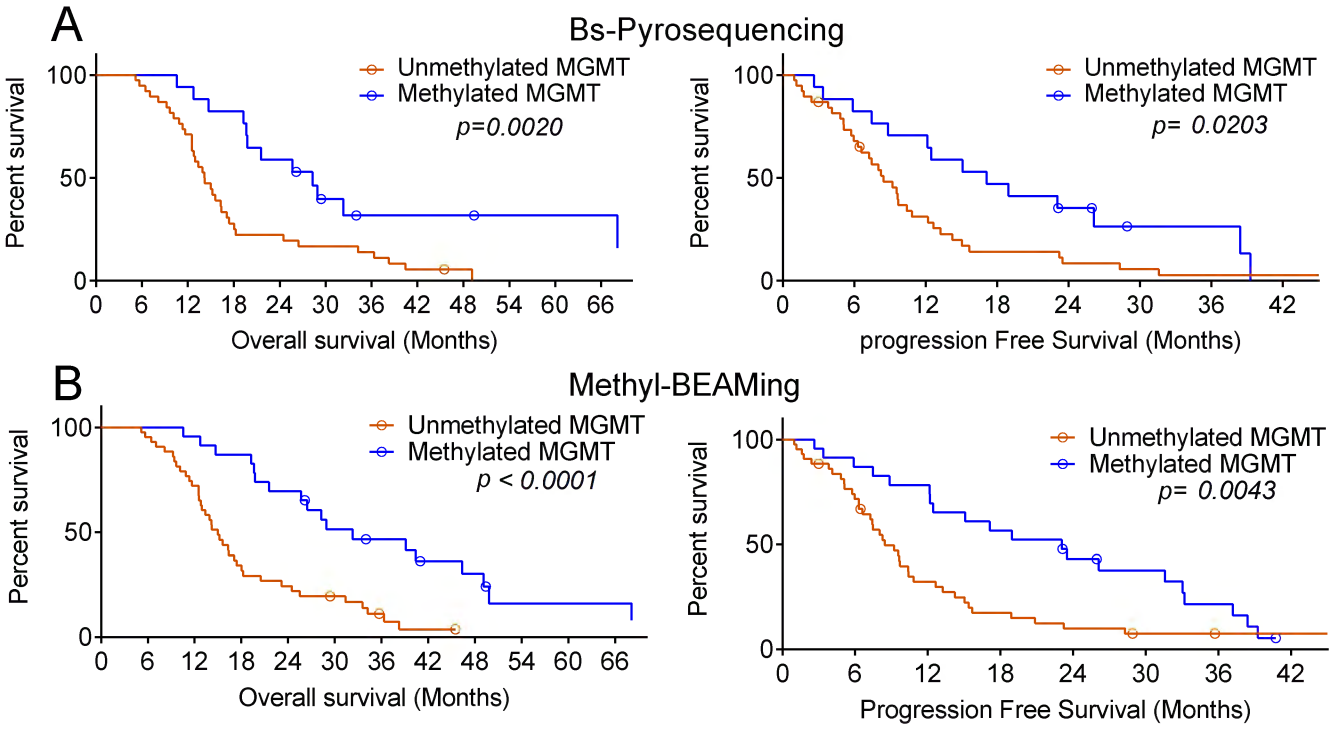
Figure 2B: Predictive value of *MGMT* methylation status by Bs-Pyrosequencing in mCRC tissue. Waterfall plot indicates response to dacarbazine.

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.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Bs-Pyrosequencing
Unmethylated MGMT
Methylated MGMT
p=0.0020
Percent survival
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Overall survival (Months)
Unmethylated MGMT
Methylated MGMT
p= 0.0203
Percent survival
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Overall survival (Months)
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Methylated MGMT
p= 0.0043
Percent survival
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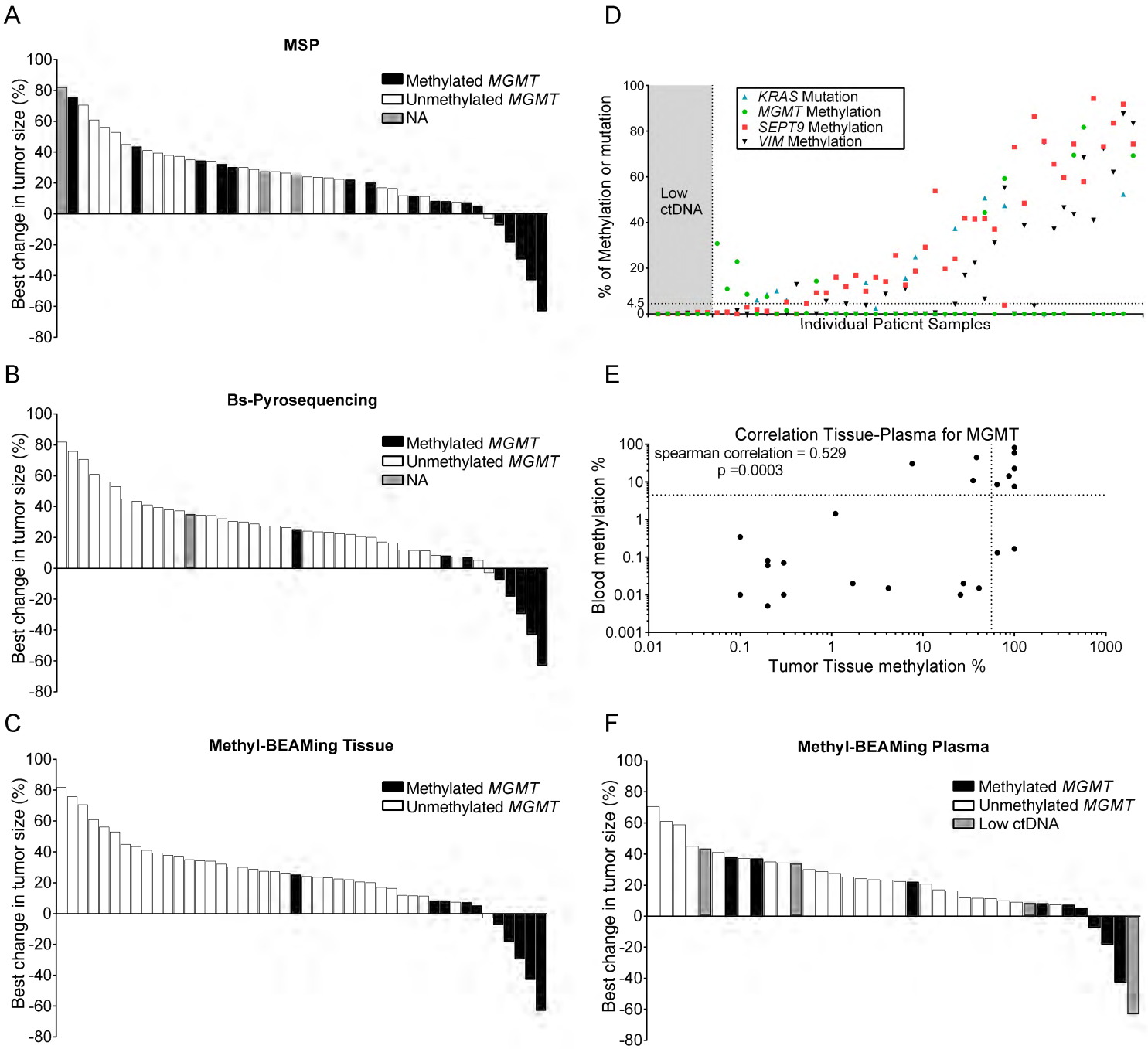
0 6 12 18 24 30 36 42
Progression Free Survival (Months)


A
MSP
Methylated MGMT
Unmethylated MGMT
NA
Best change in tumor size (%)
B
Bs-Pyrosequencing
Methylated MGMT
Unmethylated MGMT
NA
Best change in tumor size (%)
C
Methyl-BEAMing Tissue
Methylated MGMT
Unmethylated MGMT
Best change in tumor size (%)
D
KRAS Mutation
MGMT Methylation
SEPT9 Methylation
VIM Methylation
Low ctDNA
% of Methylation or mutation
Individual Patient Samples
E
Correlation Tissue-Plasma for MGMT
spearman correlation = 0.529
p =0.0003
Blood methylation %
Tumor Tissue methylation %
F
Methyl-BEAMing Plasma
Methylated MGMT
Unmethylated MGMT
Low ctDNA
Best change in tumor size (%)

Data S1.

Additional Methods

Patients

GBM training-set samples were obtained from 98 patients who had undergone brain surgery at the Academic Medical Center (AMC) in Amsterdam, the Netherlands, between 1988 and 2006 and for which follow-up and tissue were available. Oral consent for removal of the tissue and its storage in the tumor bank for research purposes was obtained and documented in patients' medical charts. Research was performed on "waste" material and stored in a coded fashion. Consent for this project was reviewed and waivered by the Medical Ethics Review Committee of the Academic Medical Center and University of Amsterdam (reference number W14_224 # 14.17.0286). Frozen samples were stored in the tumor bank maintained by the Departments of Neurosurgery and Neuropathology at the AMC. Tumor samples were included only if there was at least 80% of cancer cells, verified by hematoxylin and eosin staining. More details about the enrollment procedure and the patients' characteristics can be found in the previous study [1].

The GBM validation-set consisted of 66 tissue samples from patients with newly-diagnosed GBM, who had surgery and chemoradiation with a follow-up of at least two years at the VU University Medical Center in Amsterdam. Inclusion criteria consisted of (1) adult patients older than 17, (2) a new histopathological diagnosis of supratentorial GBM between 2005 and 2011, verified by an independent neuropathologist, (3) no prior brain tumor treatment to exclude dedifferentiated glioma, (4) pre- and postoperative MRI within 3 days of surgery, (5) standard adjuvant therapy consisting of 30x2 Gy radiotherapy and concomitant temozolomide, followed by six monthly cycles of adjuvant temozolomide. All patients had resective surgery, except two patients who had a biopsy procedure only, because of eloquent location, with subsequent chemoradiation [2].

CRC training-set samples were obtained from 68 patients enrolled in the DETECT-01 Trial [3] at Ospedale Niguarda Ca' Granda, Milan, Italy, between May 2011 and March 2012. In the DETECT-01 trial, patients with chemorefractory mCRC were treated with dacarbazine (250 mg/m2) intravenously day 1-4 every 21 days. *MGMT* promoter methylation was retrospectively assessed by MSP [3]. All patients met the following inclusion criteria: age

18 years or more, Eastern Cooperative Oncology Group performance status of ≤ 1, histologically confirmed mCRC. Written informed consent was obtained from each patient. The study was performed according to the Declaration of Helsinki and good clinical practice, being approved by the ethics committee of Ospedale Niguarda Ca' Granda. Among 68 patients enrolled in the study, 61 cases (90%) had remaining material and were reassessed for methylation status at IRCCS in Candiolo, Italy. DNA was extracted from paraffin-embedded block from archival tumor tissue of primary and/or metastases and tumor content was ascertained by hematoxylin and eosin staining (possible in 60 cases only). Forty nine of the 60 patients also had a blood sample withdrawn prior to treatment with dacarbazine in which the methylation status could also be performed in the plasma. Plasmas were stored at -80°C until DNA extraction. Response rate to dacarbazine was assessed according to Response Evaluation Criteria in Solid Tumors (RECIST1.1) criteria. An objective response (partial response) was defined as a reduction of at least 30 percent in the sum of all target lesions. Progressive disease was defined as at least a 20% increase in the sum of diameters of target lesions. Stable disease was defined as shrinkage neither sufficient to qualify for partial response nor sufficient increase to qualify for progressive disease. Further details can be found in the original clinical trial report [3].

CRC validation-set samples were obtained from 32 patients enrolled in the phase II study at the Department of Medical Oncology of the Fondazione IRCCS Istituto Nazionale dei Tumori of Milan, previously published by Pietrantonio and colleagues [4]. Between August 2012 and July 2013, 32 patients with advanced, chemorefractory CRC were included the study. Patients with histologically confirmed MGMT-methylated metastatic CRC and measurable disease were eligible. The study was conducted according to Good Clinical Practices and was approved by the local ethics committee. All subjects provided written informed consent. Further details can be found in the original clinical trial report [4]. Among 32 patients enrolled in the study, 23 cases (72%) had remaining material and were reassessed for methylation status at IRCCS in Candiolo, Italy.

All survival data were blinded until completion of molecular analyses.

Tumor and Plasma Sample Preparation

DNA was collected and extracted from GBM training-set samples, as previously described [1]. One hundred nanograms of DNA were used for bisulfite conversion using the Epitect

bisulfite kit (Qiagen) according to manufacturer's protocol. Elution was performed using 80 µl of elution buffer to improve DNA recovery (2x40 µl). Methyl-BEAMing [5] was originally developed using this kit since its protocol contains a specific reagent (RNA carrier) expecting to allow conversion of samples of low quality and quantity.
For the GBM validation-set samples, three slices of 10um were cut from FFPE block and DNA was extracted at the University of Torino, using the QIAamp DNA FFPE Tissue Kit (Qiagen). For each samples, a minimum of 500ng of DNA was bisulfite converted using the EZ DNA Methylation-Gold Kit (Zymo research) according to manufacturer's protocol. Elution was performed using 40 µl of elution buffer to improve DNA recovery (2x20 µl).For mCRC training-set tissue samples, DNA was previously extracted and bisulfite converted at IDIBELL (Barcelona) as described [3]. In instances of insufficient material, slides were cut from another FFPE block and DNA was re-extracted at the University of Torino, using the QIAamp DNA FFPE Tissue Kit (Qiagen).

DNA was collected and extracted from the mCRC validation-set samples, as previously described [4]. For each sample, a maximum of 250ng of DNA was bisulfite converted using the EZ DNA Methylation-Gold Kit (Zymo research) according to manufacturer's protocol. Elution was performed using 40 µl of elution buffer to improve DNA recovery (2x20 µl).

Forty-nine plasma samples withdrawn prior to treatment were available from the DETECT trial. cfDNA was extracted at the University of Torino from 1ml of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) following manufacturer protocol. We originally tested the Epitect bisulfite kit (Qiagen), as previously described in the original methyl-BEAMing protocol [5], but we were not able to consistently recover DNA from this procedure (no amplification). Therefore bisulfite conversions of cfDNA were performed using the EZ DNA Methylation-Gold Kit (Zymo research) which allowed recovery of DNA in all samples. Twenty microliters of cfDNA were converted according to manufacturer's protocol. Elution was performed using twice 10 µl of M-Elution Buffer. For five patients only plasma samples were available as tissue had been previously exhausted by other tests thus preventing comparison between sample types.

MGMT Methylation Assay Controls

Ultramer oligomers of 250bp (corresponding to the fully methylated or fully unmethylated bisulfite converted template) were used as positive controls. Controls were considered as amplified products until achievement of the working concentration (1pM). Specificity and sensitivity of each technique were verified using an artificial scale of methylation made by mixing the two positive controls (Figure S1 and supplemental data 2). Each batch of amplifications was performed in presence of two positive controls (unmethylated and methylated) and one negative (no template) control.

Methylation specific PCR

Amplification was performed as previously described [6]. Amplification products were analyzed by agarose gel electrophoresis and quantified using the ImageJ software after background subtraction. The methylation ratio was calculated dividing the methylated specific signal by the sum of methylated plus unmethylated specific signal.

Bs-Pyrosequencing

Amplification was carried out using the Platinum® Taq (Life technologies) (conditions and primers in table S2). PCR products were purified on the PyroMark Q24 Vacuum Workstation according to manufacturer protocol and annealed with the sequencing primer before being run on the PyroMark Q24 (Qiagen). Pyrograms were analyzed using PyroMark Q24 Software, average of the 6 CpG sites methylation values was used for further analyses.

Methyl-BEAMing assay

BEAMing analysis is a multistep digital PCR based technique published by Diehl and colleagues [7]. Its application for methylation is named Methyl-BEAMing and has been previously described to detect methylation of the *VIM* gene [5]. A first amplification that allows the enrichment of the locus of interest was carried out using the Platinum® Taq (Life technologies) (conditions and primers in table S2). PCR products were diluted (1/20000 for DNA of tissue origin, 1/6000 for cfDNA) and reamplified in an emulsion PCR allowing physical separation and independent amplification of the different templates. PCR mixes were prepared according to conditions in table S2, seventy microliters of Emulsifire oil were added. Emulsion was performed by repetitive pipetting. Afterwards, PCR emulsion breaking and hybridization (sequences in table S2) were carried out using Inostics

reagents and following published protocol [7]. Fluorescence was assessed on a CyAN flow cytometer (Beckam-Coulter) using the filters previously established with controls (scale of methylation). The percentage of methylation was calculated dividing the methylated specific signal by the sum of methylated plus unmethylated specific signal.
Micro-dissection of CRC tissue was not performed prior to DNA extraction. Consequently, methylation values in CRC tissue were corrected for tumor cellularity by dividing the percentage of methylation with the percentage of tumor content evaluated by hematoxylin and eosin stain. Percentages were maximized at 100% when the ratio was over this value. Tumor content was available for 60 out of the 61 cases in the CRC training-set, and for all samples in the validation-set.

Droplet digital PCR analysis

Isolated circulating free DNA was amplified using ddPCR™ Supermix for Probes (Bio-Rad) using KRAS (PrimePCR™ ddPCR™ Mutation Assay, Bio-Rad) as previously described [8]. ddPCR was then performed according to manufacturer's protocol and the results reported as percentage or fractional abundance of mutant DNA alleles to total (mutant plus wild type) DNA alleles. 8 to 10 µl of DNA template was added to 10 µl of ddPCR™ Supermix for Probes (Bio-Rad) and 2 µl of the primer and probe mixture. This reaction mix was added to a DG8 cartridge together with 60 µl of Droplet Generation Oil for Probes (Bio-Rad) and used for droplet generation. Droplets were then transferred to a 96 well plate (Eppendorf) and then thermal cycled with the following conditions: 5 minutes at 95°C, 40 cycles of 94°C for 30s, 55°C for 1 minute followed by 98°C for 10 minutes (Ramp Rate 2°C/sec). Droplets were analyzed with the QX200™ Droplet Reader (Bio-Rad) for fluorescent measurement of FAM and HEX probes. Gating was performed based on positive and negative controls, and mutant populations were identified. The ddPCR data were analyzed with QuantaSoft analysis software (Bio-Rad) to obtain Fractional Abundance and Copy Number Variations of the mutant or amplified DNA alleles in the wild-type or normal background. The quantification of the target molecule was presented as number of total copies (mutant plus WT) per sample in each reaction. ddPCR analysis of normal control plasma DNA (from cell lines) and no DNA template controls were always included.

Table S2: Assays primers and conditions

		5'→3' or reference	primer concentration (mM)	Assay Annealing temperature	Assay [Mg] mM
Bs-Pyrosequencing					
MGMT	Forward	GTTTAGGATATGTTGGGATAGT	10		
	Reverese	GGACACCGCTGATCGTTTAAACCACCCAAACACTCACCAA	1	58	1.5
	Universal	GGGACACCGCTGATCGTTTA	9		
	Sequencing	GTTTTTAGAAYGTTTTGYGTTT	4		
Methyl-BEAMing 1st PCR					
MGMT	Forward	TCCCGCGAAATTAATACGACGTTTAGGATATGTTGGGATAGT	10	50	1.5
	Reverse	GCTGGAGCTCTGCAGCTAAACCACCCAAACACTCACCAA	10		
SEPT9	Forward	TCCCGCGAAATTAATACGACGGATTTAGAAGGTGGGTGTTGG	10	54	1
	Reverse	GCTGGAGCTCTGCAGCTACCAAACCCACCCCCAAAATCCTCTC	10		
Methyl-BEAMing Emulsion PCR					
	Forward	TCCCGCGAAATTAATACGAC	10	Ref [7]	Ref [7]
	Reverse	GCTGGAGCTCTGCAGCTA	100		
Methyl-BEAMing Hybridization					
MGMT	Unmethylated	CACAAACAATACACACCACAA	0.2		
	Methylated	CGCAAACGATACGCACCGCGA	0.2	NA	NA
	Universal	CCCAAACACTCACCAAA	0.2		
SEPT9	Unmethylated	CCACAACCACAACAACC	0.2		
	Methylated	CCGCGACCGCAACAACC	0.2	NA	NA
	Universal	CCCCCAAAATCCTCTCCAAC	0.2		
Digital Droplet PCR					
KRAS	G12V	dHsaCP2000005			
	G12D	dHsaCP2000001			
	G12C	dHsaCP2000007	NA	NA	NA
	G12S	dHsaCP2000011			
	G13D	dHsaCP2000013			

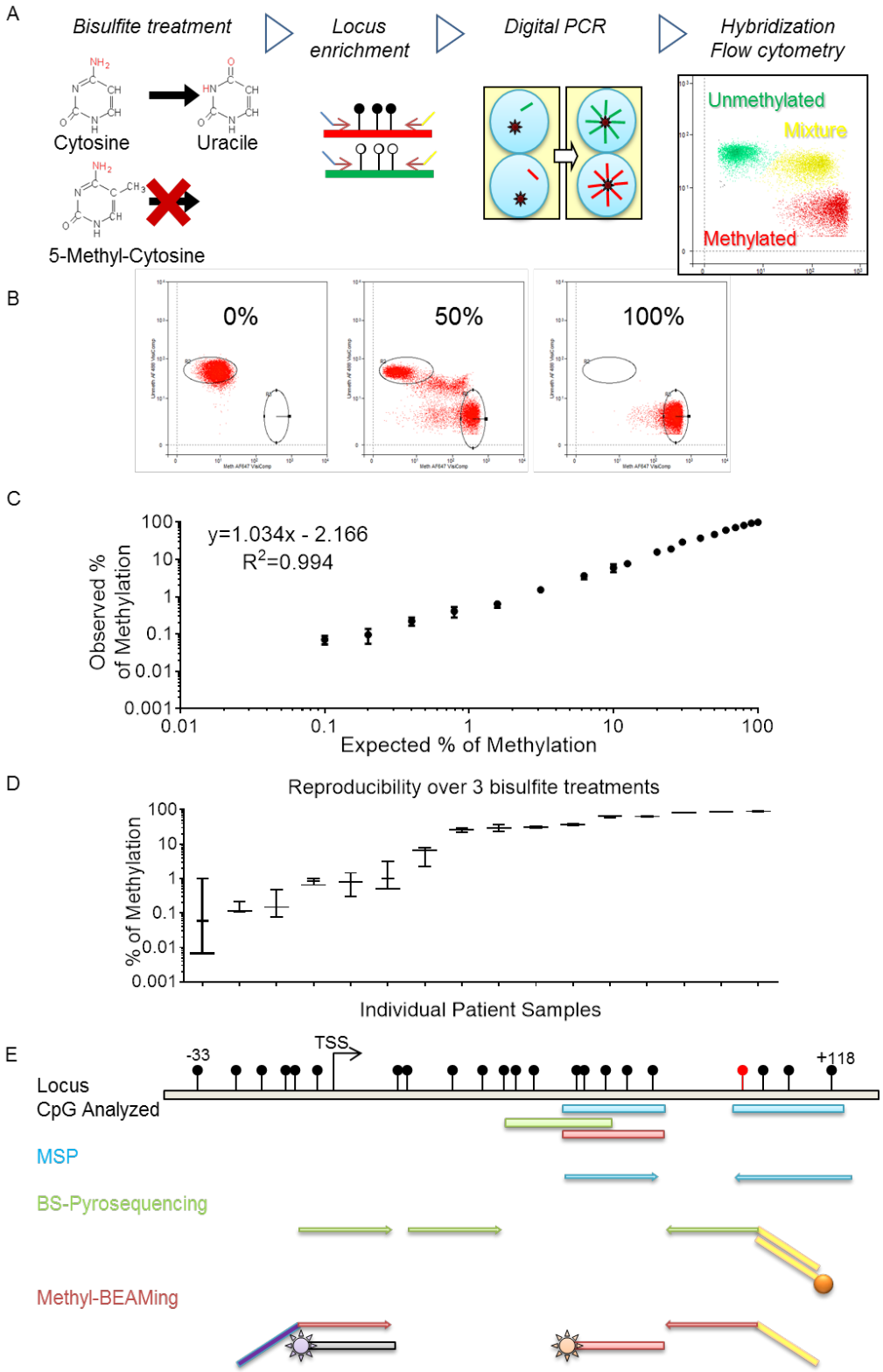


Figure S1: A) Workflow of Methyl-BEAMing assay. B) Flow cytometer output for 0%, 50% and 100% of methylation. C) Linearity of quantification of ultramer oligonucleotide mixture. D) Reproducibility of the Methyl-BEAMing assay across three independent bisulfite treatments in 16 GBM samples. E) *MGMT* locus with CpG analyzed, position of primers and probes for each technique. TSS= Transcription start site. The Red CpG site corresponds to cg12981137, identified by Bady *et al.* as associated with TMZ response in GBM [9].

References relative Supplemental Data S1

1. 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.

2. Stupp R, Mason WP, van den Bent MJ et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005; 352: 987-996.

3. 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.

4. 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.

5. Li M, Chen WD, Papadopoulos N et al. Sensitive digital quantification of DNA methylation in clinical samples. Nat Biotechnol 2009; 27: 858-863.

6. 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.

7. Diehl F, Li M, He Y et al. BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. Nat Methods 2006; 3: 551-559.

8. Siravegna G, Mussolin B, Buscarino M et al. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. Nat Med 2015.

9. 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.

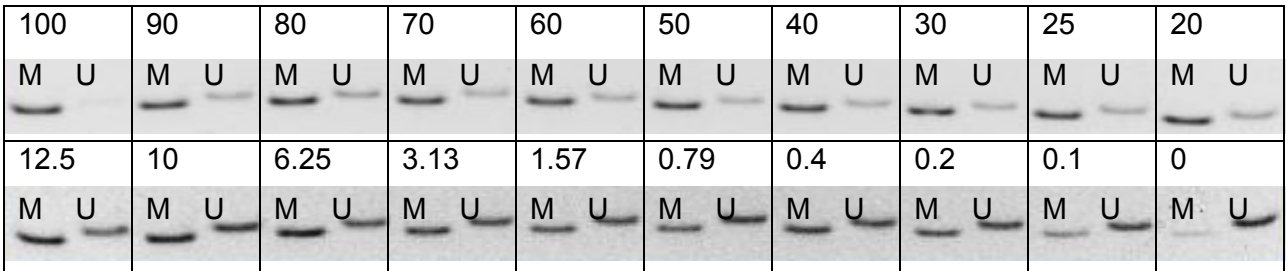
Data S2

Sensitivity, reproducibility and specificity of the MGMT Methyl-BEAMing assay

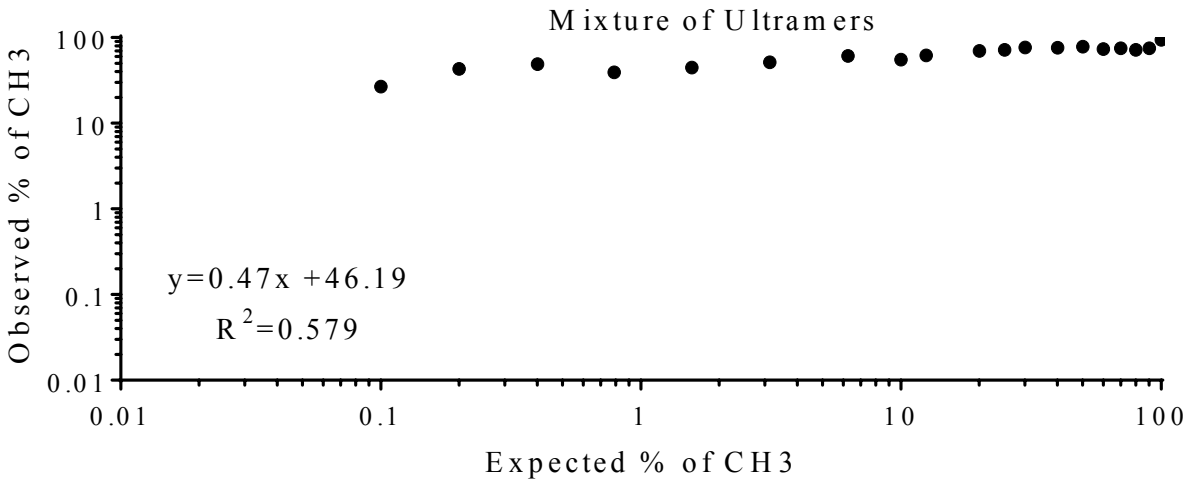
Methyl-BEAMing (Figure 1A) is a multistep process starting from the bisulfite conversion of the DNA followed by the enrichment of the locus of interest, reamplification by emulsion PCR in presence of magnetic beads. Beads covered with amplicons are purified, hybridized with sequence specific fluorescent probes and detected by flow cytometry. Quantification ability of the Methyl-BEAMing assay was tested with a scale made of template corresponding to the fully methylated or unmethylated bisulfite converted sequence. Flow cytometer analysis filters were calibrated using the 0, 50 and 100% methylation samples to obtain an observed value closest to the expected one (Figure 1B). This step allowed correcting the discrepancy between template specific amplification and probe efficiency. The linearity between observed and expected value was then assessed (Figure 1C) with the whole scale. Methyl-BEAMing conserved a linearity of amplification throughout the whole range of methylated mixture ($y= 1.034x -2.166$; $R^2= 0.994$). The sensitivity of detection was as good as 1 methylated copy out of 1000 unmethylated copies. Reproducibility of *MGMT* Methyl-BEAMing was tested using three independent bisulfite treatments of 16 GBM samples (Figure 1D). The highest deviation observed was of 6.3%. Average standard deviation across samples was 1.8%.

MSP and Bs-Pyrosequencing assessing closely related CpG sites were also tested (Figure 1E) using the scale of methylation (Supplemental Data 2). MSP showed high sensitivity but could not discriminate high from low methylated template. Pyrosequencing demonstrated a good reliability for quantification of methylated fraction from 5% and more but lack of specificity to discriminate samples with percentage of methylation below 5%.

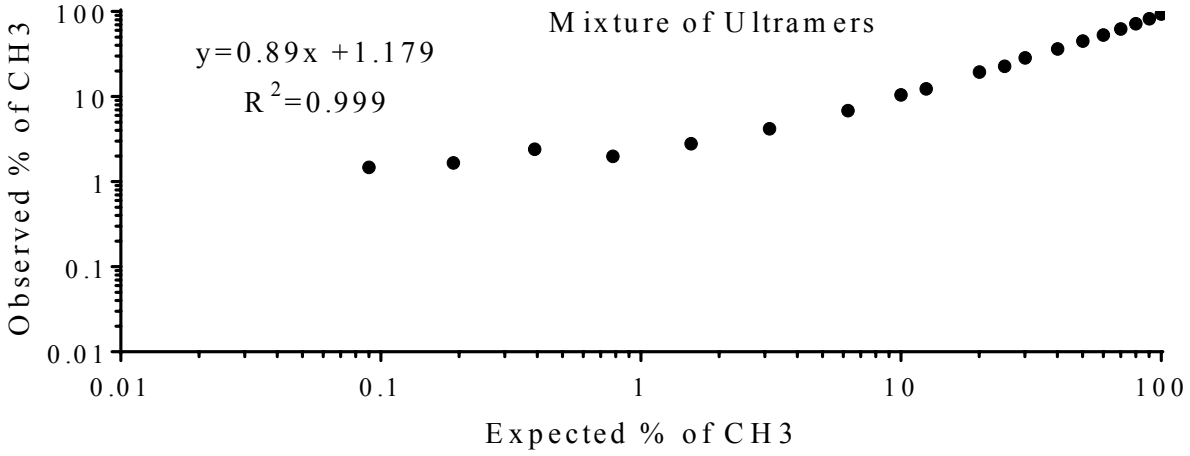
Data S2A) Methylation scale by MSP using mixture of ultramer oligonucleotides. Values above gel correspond to expected percentage, M correspond to methylated specific band, U to unmethylated specific band.



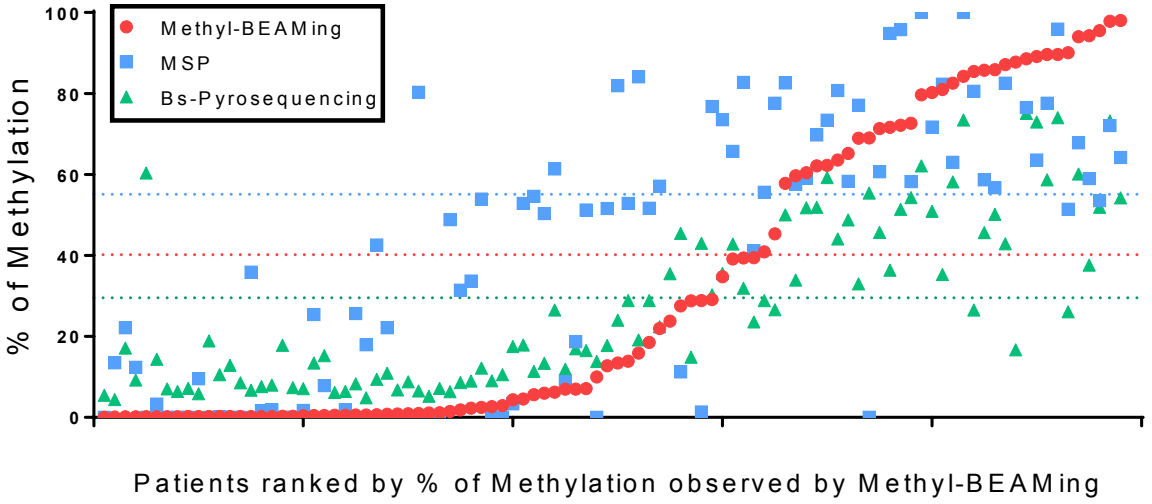
Data S2B) Methylation scale by MSP using mixture of ultramer oligonucleotides. Observed values obtained through densitometry:



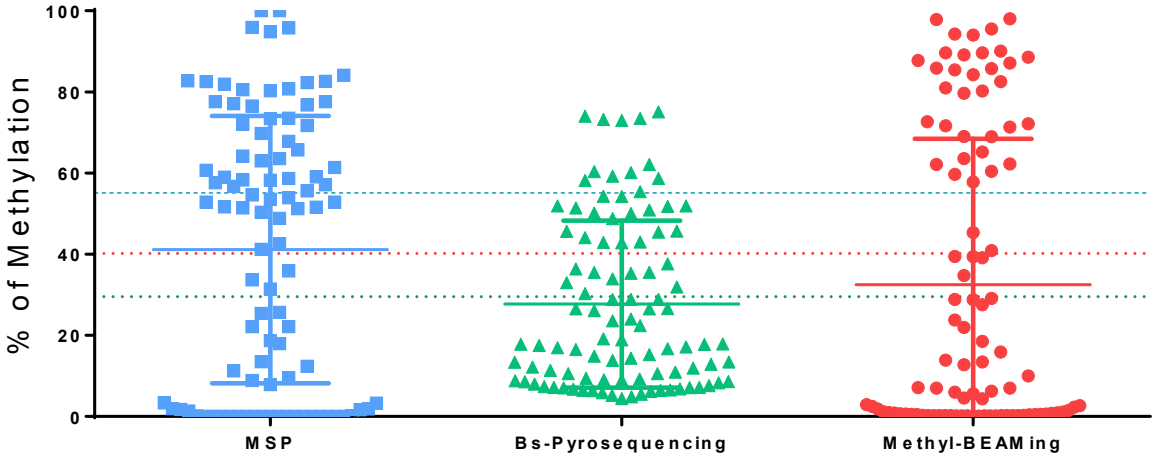
Data S2C) Methylation scale by Bs-Pyrosequencing using mixture of ultramer oligonucleotides. Below 5% of expected methylation Bs-Pyrosequencing does not allow accurate quantification of methylation.



Data S3A) Distribution of methylation value assessed by all three assays in the GBM training set and sorted by % of methylation by Methyl-BEAMing. Assay-specific thresholds evaluated by ROC analyses are plotted as dot lines with the specific color of each method.

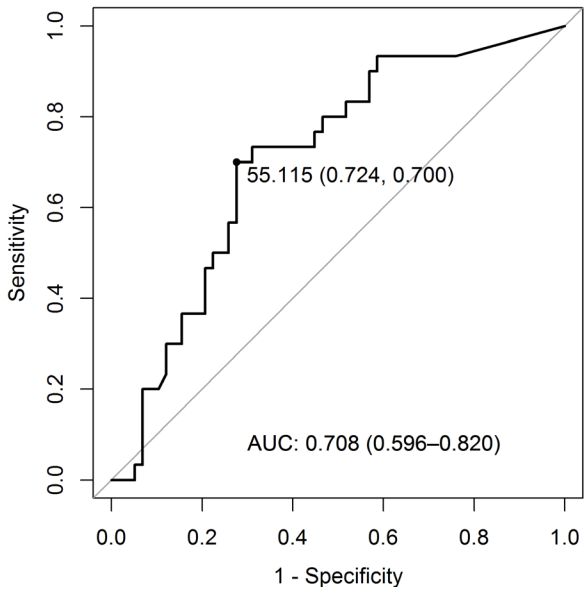


Data S3B) Distribution of methylation value assessed by all three assays in the GBM cohort. Assay-specific thresholds evaluated by ROC analyses are plotted as dot lines with the specific color of each method.



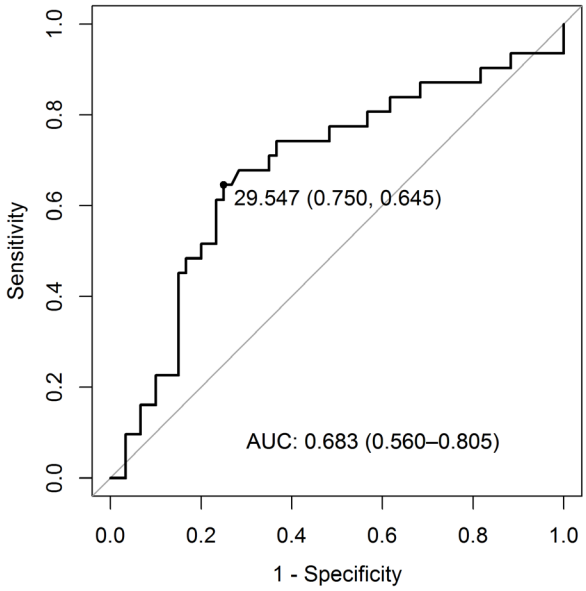
Data S3C) ROC Analyses for Overall survival at 1 year in the GBM training cohort according to each assay.

- **MSP**



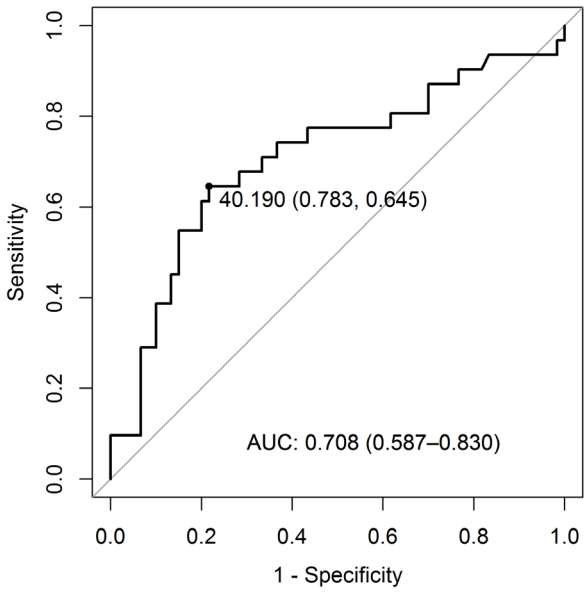
threshold	55.12
specificity	0.72
sensitivity	0.70
npv	0.82
ppv	0.57

- **Bs-Pyrosequencing**



threshold	29.55
specificity	0.75
sensitivity	0.65
npv	0.80
ppv	0.57

- **Methyl-BEAMing**



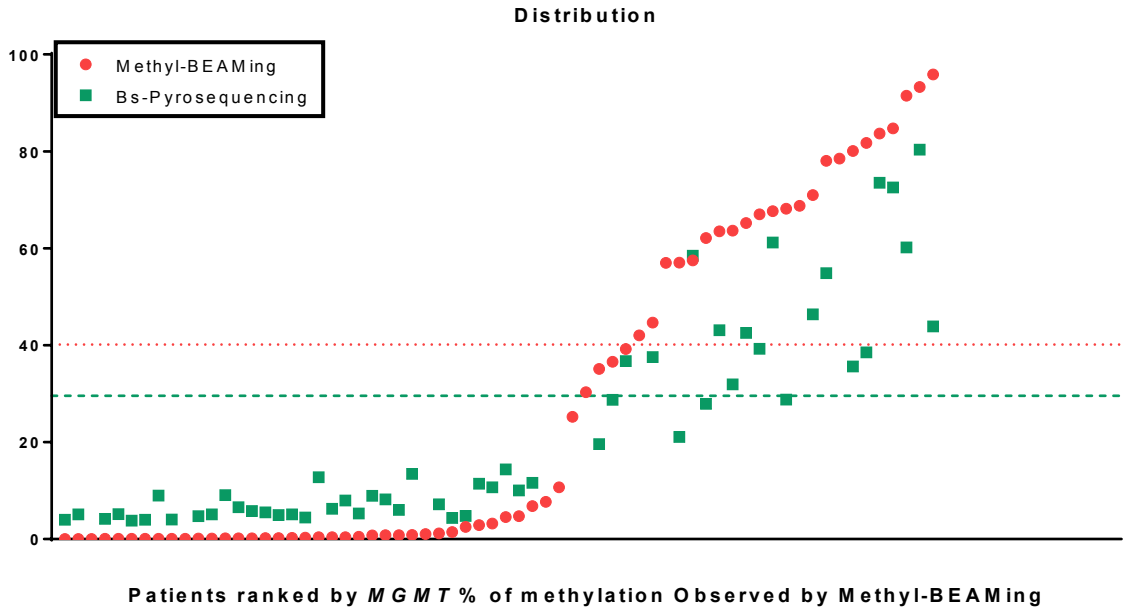
threshold	40.19
specificity	0.78
sensitivity	0.65
npv	0.81
ppv	0.61

Data S3D) Assay concordance in the GBM training cohort.

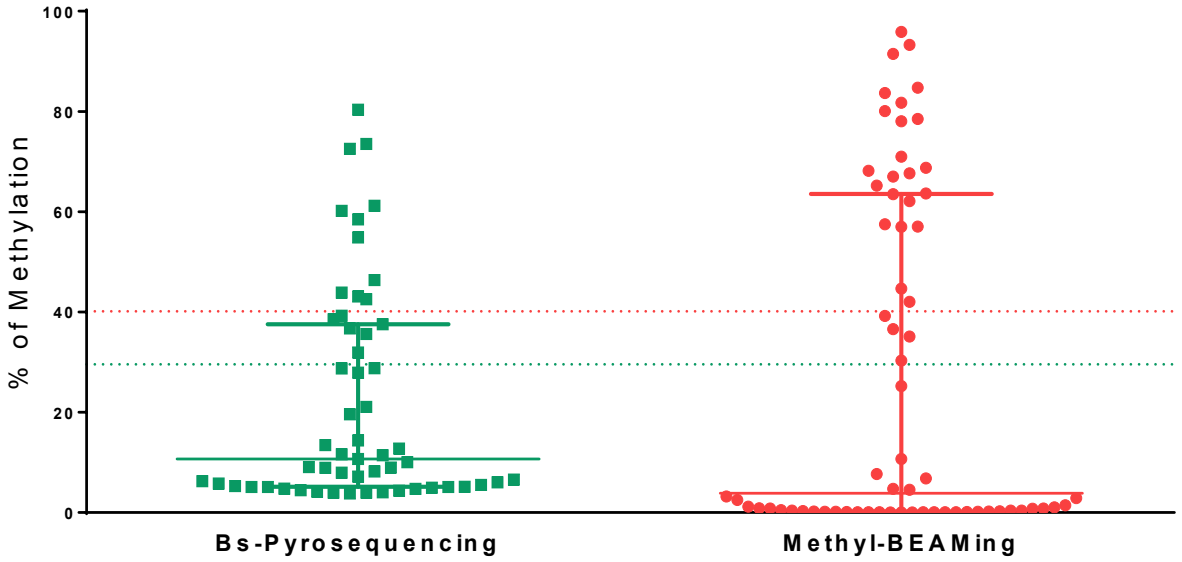
		Methyl-BEAMing			**Bs-Pyrosequencing**		
		Methylated	Unmethylated	NA	Methylated	Unmethylated	NA
MSP	Methylated	31	9	0	32	8	0
	Unmethylated	3	52	0	5	50	0
	NA	1	2	0	1	2	0
Bs-Pyrosequencing	Methylated	30	8	0			
	Unmethylated	5	55	0			
	NA	0	0	0			

	Kappa	% concordance
Methyl-BEAMing vs. MSP	0.690 [0.551- 0.829]	84.7
Methyl-BEAMing vs. Bs-Pyrosequencing	0.717 [0.574-0.859]	86.7
Bs-Pyrosequencing vs. MSP	0.672 [0.530-0.815]	83.7

Data S3E) Distribution of *MGMT* methylation values assessed by the indicated assays in the GBM validation set and sorted by % of methylation by Methyl-BEAMing. Assay-specific thresholds evaluated by ROC analyses are plotted as dot lines with the specific color of each method.



Data S3F) Distribution of methylation value assessed by the two techniques in the GBM validation cohort and sorted by techniques. Assay-specific thresholds evaluated by ROC analyses are plotted as dot lines with the specific color of each method.

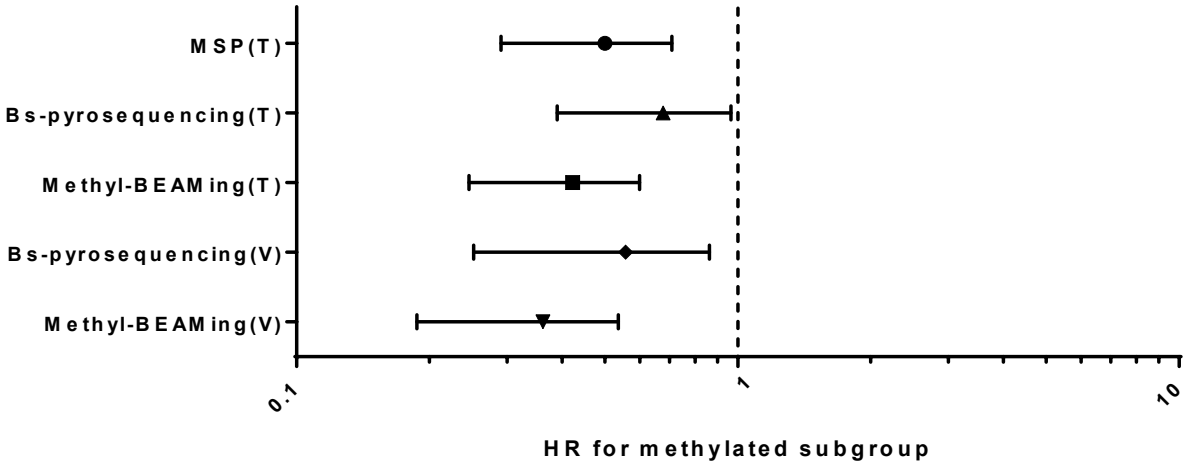


Data S3G) Concordance between Methyl-BEAMing and Bs-pyrosequencing in the GBM validation cohort.

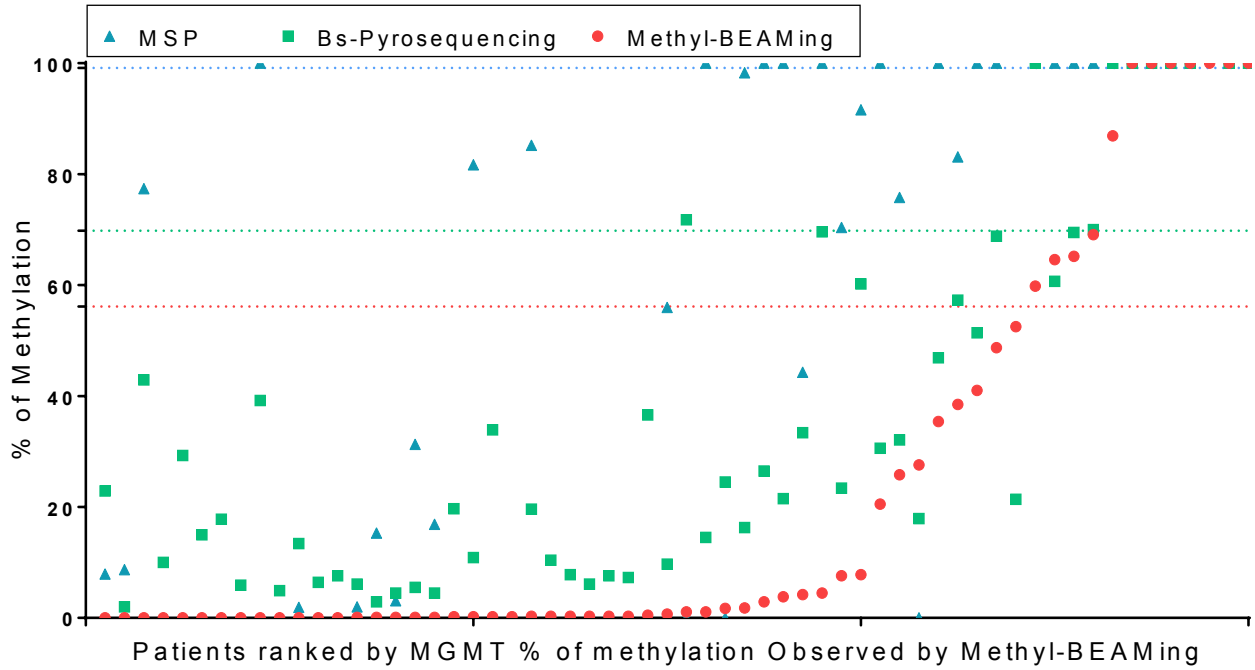
		Methyl-BEAMing		
		Methylated	Unmethylated	NA
Bs-Pyrosequencing	Methylated	18	2	0
	Unmethylated	2	36	0
	NA	4	7	0

	Kappa	% of agreement
Methyl-BEAMing vs. Bs-Pyrosequencing	0.597 [0.442-0.753]	78.26

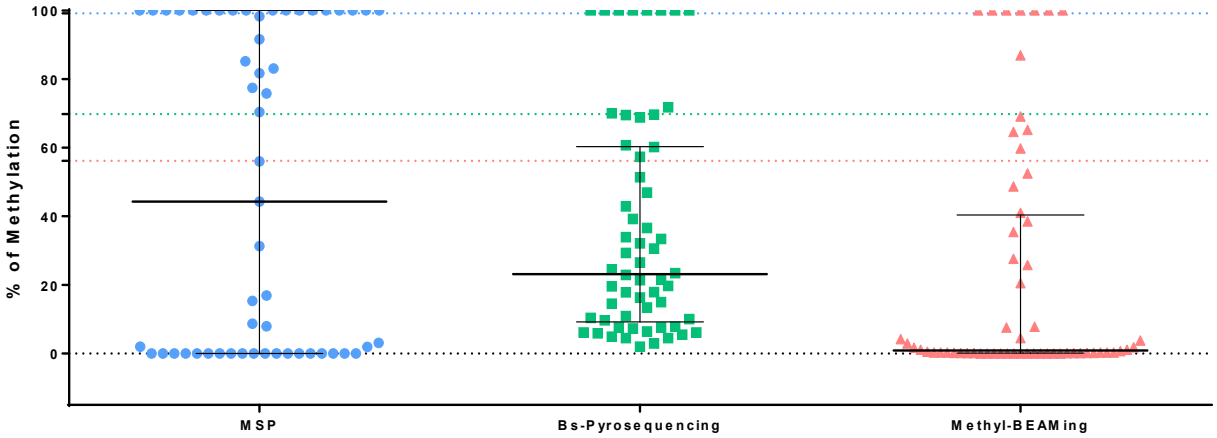
Data S3H) Comparison of hazard ratio for death (overall survival) of the methylated subgroup evaluated by the different methods in the training cohort (T) and validation cohort (V).



Data S4A) Distribution of methylation value assessed by all three assays in the DETECT mCRC cohort and sorted by % of methylation by Methyl-BEAMing. Assay-specific thresholds evaluated by ROC analyses are plotted as dot lines with the specific color of each method.

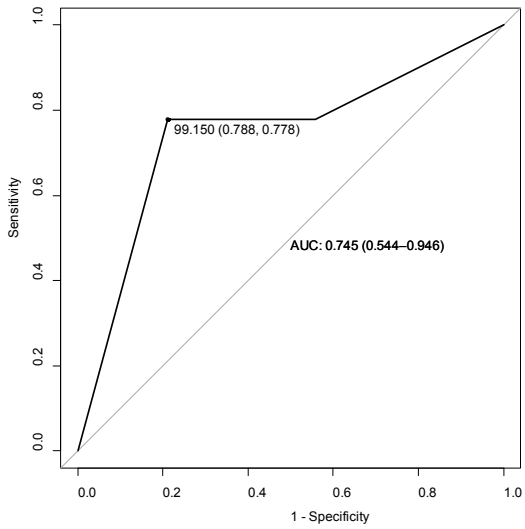


Data S4B) Distribution of methylation values assessed by all three assays in the DETECT mCRC cohort. Assay-specific thresholds evaluated by ROC analyses are plotted as dot lines with the specific color of each method.



Data S4C) ROC Analyses for PFS at 12 weeks in the DETECT mCRC cohort according to each assay.

- **MSP**



threshold 99.15

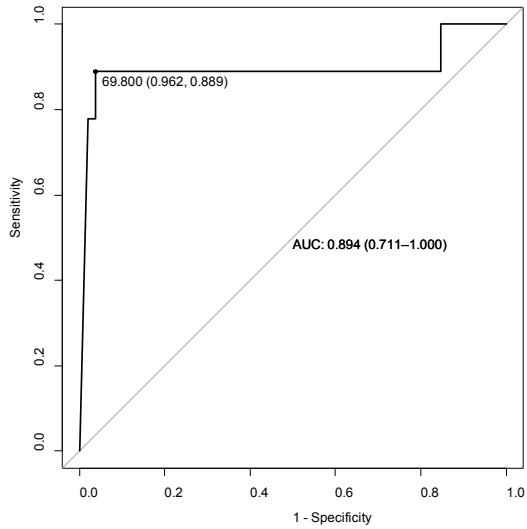
specificity 0.79

sensitivity 0.78

npv 0.95

ppv 0.39

- **Bs-Pyrosequencing**



threshold 69.80

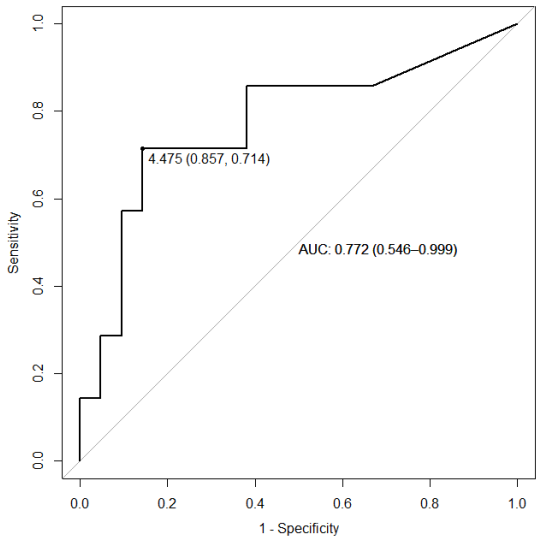
specificity 0.96

sensitivity 0.89

npv 0.98

ppv 0.80

- **Methyl-BEAMing**



threshold 56.15

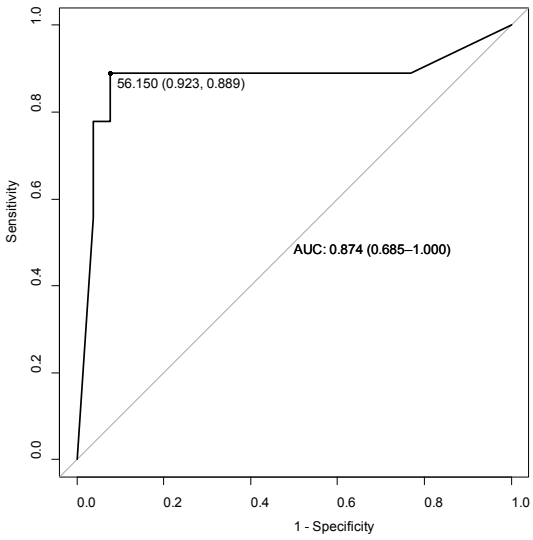
specificity 0.92

sensitivity 0.89

npv 0.98

ppv 0.67

- **Methyl-BEAMing Plasma**



Threshold	4.48
Specificity	0.86
Sensitivity	0.71
Npv	0.95
Ppv	0.45

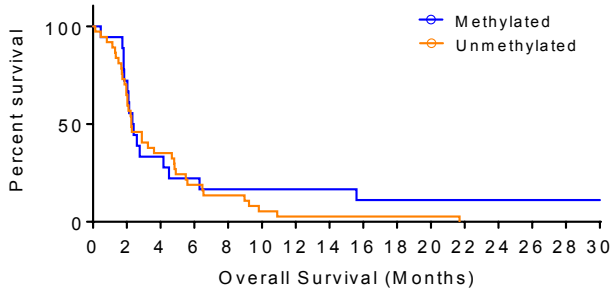
Data S4D) Assay concordance between in the DETECT mCRC cohort.

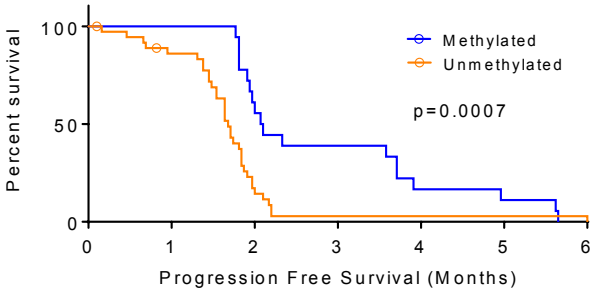
		Methyl-BEAMing Tissue			Bs-Pyrosequencing		
		Methylated	Unmethylated	NA	Methylated	Unmethylated	NA
MSP	Methylated	9	9	0	7	11	0
	Unmethylated	0	37	0	1	35	1
	NA	3	2	0	2	2	1
Bs-Pyrosequencing	Methylated	9	1	0			
	Unmethylated	2	46	0			
	NA	1	1	0			
Methyl-BEAMing Plasma	Methylated	6	3	0			
	Unmethylated	3	31	0			
	NA	0	0	0			

	Kappa [95% CI]	% of agreement
Methyl-BEAMing vs. MSP	0.478 [0.281-0.674]	76.67
Methyl-BEAMing vs. Bs-Pyrosequencing	0.745 [0.544-0.946]	91.67
Bs-Pyrosequencing vs. MSP	0.376 [0.165-0.587]	71.67
Methyl-BEAMing Tissue vs. Methyl-BEAMing Plasma	0.578 [0.277-0.880]	86.05

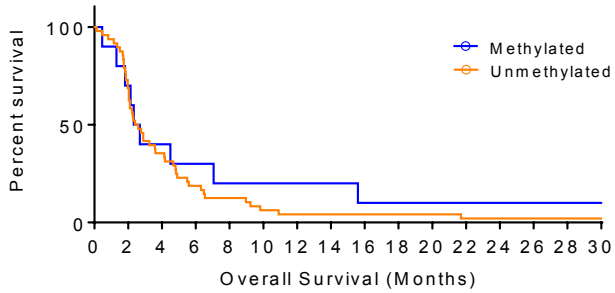
Data S4E) Overall survival and Progression Free Survival in DETECT mCRC patients stratified according to *MGMT* methylation cut-off values calculated for each assay shown in Data S4C).

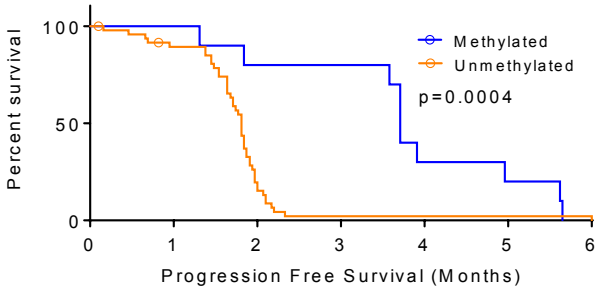
- MSP



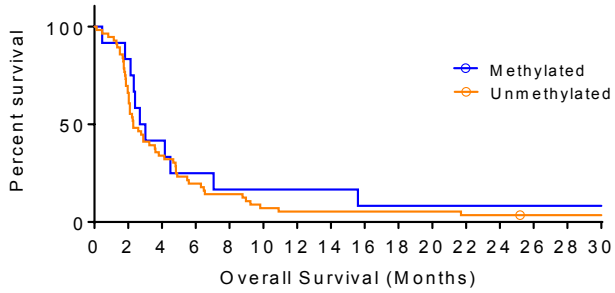


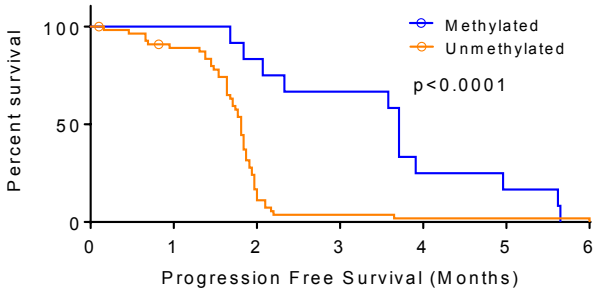
- Bs-Pyrosequencing



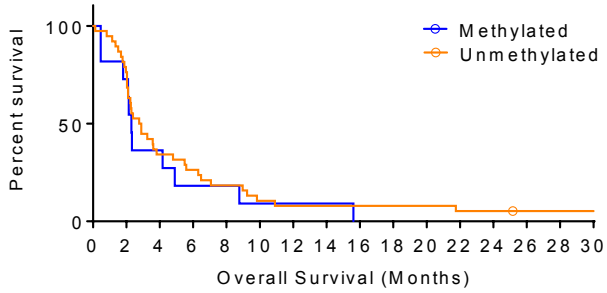


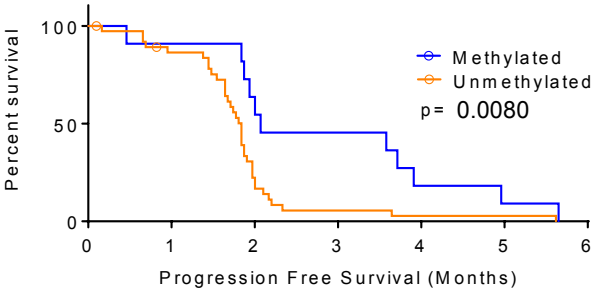
- Methyl-BEAMing Tissue





- Methyl-BEAMing Plasma





Data S4F) Contingency table for clinical benefit (information available in 58 cases for tissues, and 47 cases for plasmas) based on RECIST for DETECT mCRC patients according to each assay:

MSP

	Clinical benefit (SD+PR)	No Clinical benefit (PD)	total
Methylated	7	11	18
Unmethylated	1	34	35
NA	1	4	5
total	9	49	58

PPV	0.39
NPV	0.78

Bs-Pyrosequencing

	Clinical benefit (SD+PR)	No Clinical benefit (PD)	total
Methylated	8	2	10
Unmethylated	1	45	46
NA	0	2	2
total	9	49	58

PPV	0.8
NPV	0.89

Methyl-BEAMing Tissue

	Clinical benefit (SD+PR)	No Clinical benefit (PD)	total
Methylated	8	4	12
Unmethylated	1	45	46
total	9	49	58

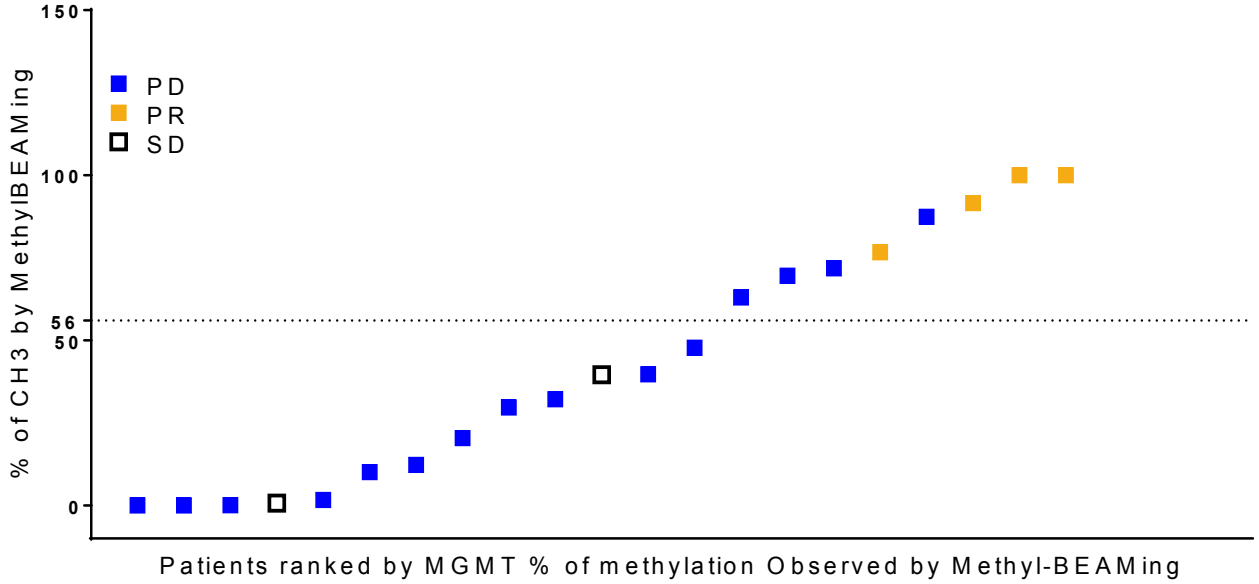
PPV	0.67
NPV	0.89

Methyl-BEAMing Plasma

	Clinical benefit (SD+PR)	No Clinical benefit (PD)	total
Methylated	5	6	11
Unmethylated	2	34	36
total	7	40	47

PPV	0.45
NPV	0.71

Data S4G) Distribution of the methyl-BEAMing value in the mCRC validation cohort and response status to treatment with TMZ. PD : Progressive Disease; PR: Partial Response; SD: Stable Disease.

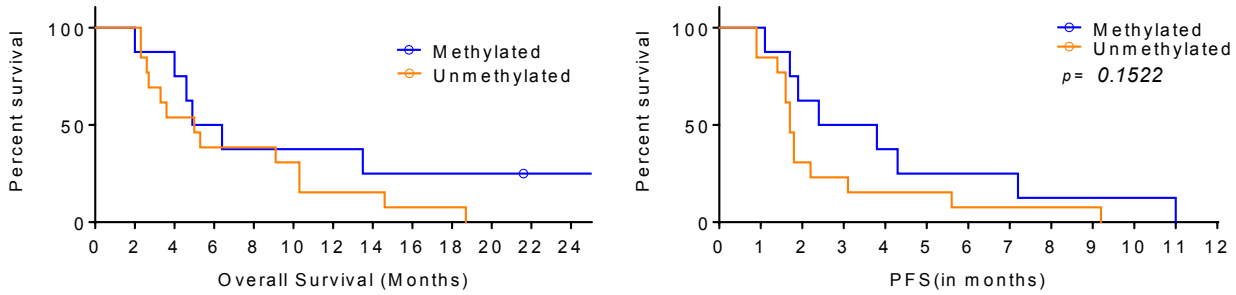


Methyl-BEAMing

	Clinical benefit (SD+PR)	No Clinical benefit (PD)	total
Methylated	4	4	8
Unmethylated	2	11	13
total	6	15	21

PPV	0.5
NPV	0.67

Data S4H) Overall and Progression Free Survival in the mCRC validation cohort by MGMT status evaluated by methyl-BEAMing:



Data S4I) Examples of pyrosequencing profiles in GBM and CRC tissue samples. GBM show a high intralocus heterogeneity of methylation across the MGMT promoter sequence which is not observed in CRC: