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MGMT **Promoter Methylation Cutoff with Safety Margin for Selecting Glioblastoma Patients into Trials Omitting Temozolomide. A Pooled Analysis of Four Clinical Trials.**

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Translational Relevance

MGMT-testing is disputed, which hinders stratified therapy and clinical trials omitting temozolomide. It is therefore of importance to determine the clinically relevant cutoff(s) defining the *MGMT* promoter methylation status for glioblastoma that allows safe clinical decision making and patient selection into trials omitting temozolomide.

The pooled analysis of quantitative *MGMT* MSP (methylation-specific PCR) data from 4041 glioblastoma patients screened or randomized in four clinical trials allowed determination and validation of an unsupervised cutoff and a lower cutoff supervised by outcome. The latter defines a “grey zone” comprising patients with low *MGMT* methylation who performed significantly better than truly unmethylated patients. This lower safety margin is suitable for selecting truly unmethylated patients for stratified therapy to spare patients unnecessary toxicity.

Abstract

Purpose: The methylation status of the O6-methylguanine DNA methyltransferase (*MGMT*) gene promoter is predictive for benefit from temozolomide in glioblastoma. A clinically optimized cutoff was sought allowing patient selection for therapy without temozolomide, while avoiding to withhold it from patients who may potentially benefit.

Experimental Design: Quantitative *MGMT* methylation-specific PCR data were obtained for newly diagnosed glioblastoma patients screened or treated with standard radiotherapy and temozolomide in four randomized trials. The pooled dataset was randomly split into a training and test dataset. The unsupervised cutoff was obtained at a 50% probability to be (un)methylated. Receiver operating characteristics (ROC) analysis identified an optimal cutoff supervised by overall survival (OS).

Results: For 4041 patients valid *MGMT* results were obtained, whereof 1725 were randomized. The unsupervised cutoff in the training dataset was 1.27 ($\log_2$[1000x(*MGMT*+1)/*ACTB*]), separating unmethylated and methylated patients. The optimal supervised cutoff for unmethylated patients was -0.28 (AUC=0.61), classifying “truly unmethylated” (≤-0.28) and “grey zone” patients (>-0.28, ≤1.27), the latter comprising ~10% of cases. In contrast, for *MGMT* methylated patients (>1.27) more methylation was not related to better outcome. Both methylated and grey zone patients performed significantly better for OS than truly unmethylated patients (HR=0.35, 95% CI: 0.27-0.45, p<0.0001; HR=0.58, 95% CI: 0.43-0.78, p<0.001), validated in the test dataset. The MGMT assay was highly reproducible upon retesting of 218 paired samples (R^2=0.94).

Conclusions: Low *MGMT* methylation (grey zone) may confer some sensitivity to temozolomide treatment, hence the lower safety margin should be considered for selecting unmethylated glioblastoma patients into trials omitting temozolomide.

Introduction

A predictive role has been shown for the methylation status of the O^6-methylguanine DNA methyltransferase (*MGMT*) gene promoter for benefit from Temozolomide (TMZ) in newly diagnosed glioblastoma patients (1-4). Consequently, the *MGMT* methylation status is used as a stratification factor in trials comprising TMZ treatment. All contemporary trials have confirmed the strong prognostic role of the MGMT status in glioblastoma patients treated with the combination of radiation concurrent with TMZ, followed by maintenance TMZ (TMZ/RT→TMZ) (1, 5-10).The lack of efficacy of TMZ in *MGMT* unmethylated glioblastoma warrants replacement with an agent with a different mechanisms of action, or omission of TMZ to avoid futile therapy and associated toxicity. Trials specifically designed to selecting only *MGMT* unmethylated glioblastoma patients and replacing TMZ in the experimental arm are becoming a common strategy in clinical research (11-14) (CheckMate 498, NCT02617589; N^2M^2, NCT03158389). However, the best way of assessing the *MGMT* promoter methylation status remains strongly debated (15). It remains unclear which pattern and extent of *MGMT* promoter methylation is required to prevent MGMT mediated DNA repair that sensitizes glioblastoma patients to alkylating agent chemotherapy. A correlation of the extent of *MGMT* promoter methylation with outcome in patients treated with TMZ chemo-radiotherapy has been suggested (16, 17). Accurate and reproducible assays with clinically relevant cutoffs are required, in order not to withhold TMZ from patients who may potentially benefit, while sparing others from unnecessary toxicity and cost.

In the present analysis we aim at revisiting the *MGMT* methylation cutoff using the pooled datasets of recent prospective randomized clinical trials, which had used the same quantitative, methylation specific PCR (qMSP) MGMT assay (18, 19) and had delivered the identical backbone treatment of TMZ/RT→TMZ to newly diagnosed glioblastoma patients. These combined datasets provide the unique opportunity to explore and validate the relationship between the extent of *MGMT* promoter methylation and overall survival (OS).

The specific goals of this research project are (i) re-evaluation of the technical (unsupervised) cutoff that discriminates methylated and unmethylated patients, whereby patients have a 50% probability to be methylated or unmethylated, (ii) definition of an optimal cutoff for glioblastoma patients, supervised by overall survival (OS), (iii) validation of the findings in an independent test dataset, (iv) evaluation of the assay reproducibility, and finally (v) comparison to the current assay-based classification used in routine diagnostics. The overarching goal is to provide one or more cutoffs that allow treatment decisions for personalized therapy and appropriate selection of patients into clinical trials omitting TMZ.

Patients and methods

Data Selection

Quantitative *MGMT* promoter methylation data was obtained from four trials for newly diagnosed glioblastoma, with central MGMT testing by the same qMSP assay, applying the same cutoff [1 in $\log_2$ space; $\log_2\left(\frac{MGMT}{ACTB} \times 1000\right)$] (18, 19), and using the standard TMZ/RT→TMZ schedule as backbone treatment (5). Patients with available MGMT classification (n=4458) had been randomized into (i) the control arm of the phase III AVAGlio trial (n=472, NCT00943826) (8), (ii) the control or experimental arm of the RTOG 0825 phase III trial (n = 621, NCT00884741) (7), (iii) the control or experimental arm of the CENTRIC (phase III) or CORE (phase II) trials that selected patients with a methylated or unmethylated *MGMT* promoter, respectively (n = 545, CENTRIC NCT00689221; n = 265, CORE NCT00813943) (18, 20); or (iv) patients who were screened, but neither randomized in CENTRIC (n = 2328) nor CORE (n = 227) (n=3365). All four selected trials failed to demonstrate improvement in overall survival of the experimental arm based on hazard ratios as reported. For randomized patients survival data and baseline information with respect to age, extent of surgery, and performance status were available. Data can be applied for via the following weblink: http://www.eortc.be/services/forms/erp/request.aspx.

Constitution of Training and Test Cohort

For the present analysis, only samples passing the quality threshold for providing a "valid" test result (≥1250 copies of the normalizer gene β-actin, *ACTB*) were considered. This all patients (all-P) population included both randomized and screened patients, whereas the randomized patients (rand-P) population was a subset of the all-P population. The data was randomly split into a *training* and a *test* cohort, stratified for trial, extent of resection (complete resection, partial resection, biopsy only, other), and performance status (PS=0, PS≥1). The all-P training cohort was used for the unsupervised analyses, while the rand-P training cohort was used for the supervised analyses of the relationship between the extent of *MGMT* methylation and OS. Validation of the findings was performed in the all-P and rand-P test cohorts, respectively.

Retest Dataset

A cohort of patients was selected randomly among patients screened but not randomized for CENTRIC. Retest tissue sections had been set aside for this purpose as of protocol, if enough tissue was available. Sample identifiers of retest tissue sections were blinded (relabeled). The initial MGMT testing was performed at the certified MDxHealth site in Liège, Belgium and retesting took place at their laboratory in Irvine, CA, USA. Only samples with valid ACTB results in both the original and retest data were selected.

All protocols were approved by the local ethics committees or institutional review boards and competent authorities, and patients provided written informed consent for trial participation and/or participation in marker screening including retesting. The trials were performed according to the guidelines of Helsinki (21).

Quantitative Methylation-Specific PCR MGMT Assay

The qMSP *MGMT* test was performed and evaluated essentially as described (18, 19) and is commercially available (PredictMDx test). In brief, DNA was isolated from sections of macro-

dissected FFPE tumor tissue. After bisulfite treatment the copy numbers of methylated *MGMT* and the reference gene *ACTB* were determined by quantitative PCR. A valid test required a minimum of 1250 *ACTB* copies measured.

For this study the calculation of the ratio of the *MGMT* and *ACTB* copy numbers was slightly modified as compared to the original procedure (19) by adding one *MGMT* copy to the numerator: $\log_2\left(\frac{MGMT+1}{ACTB} \times 1000\right)$. The result is termed corrected MGMT $\log_2$ ratio hereafter. Samples with zero *MGMT* copies would otherwise be lost after logarithmic transformation. For the calculations the original *MGMT* values were used, ignoring the technical limit of detection of the assay set at ≥ 10 copies of methylated *MGMT*.

Determination of the Unsupervised Cutoff and MGMT Methylation Status

We applied a bimodal Gaussian mixture distribution to model the corrected MGMT $\log_2$ ratio. The unsupervised cutoff in the all-P training cohort, defined as the 50% probability to be (un)methylated, was used to classify patients as unmethylated (≤ cutoff point) or methylated (> cutoff point). The same cutoff was used to classify the patients in the test cohort.

Determination of the Optimal Cutoff Supervised by OS

To identify an optimal cutoff point supervised by OS in both unmethylated and methylated patients in the rand-P training cohort, time-dependent Receiver Operating Characteristic (ROC) analysis with nearest neighbor estimation was used (22). OS predictions at two years were made in both groups. The optimal supervised cutoff point was chosen as the value that maximized the Youden's index, if the area under the curve (AUC) was >0.6, otherwise no cutoff point was determined. The optimal supervised cutoff point was used to classify patients further, both in the training and test populations.

Statistical Analysis

The all-P and rand-P training and test cohorts were compared using descriptive statistics. Categorical variables are presented as frequencies and percentages. Continuous variables are described by their median and interquartile range (IQR). Initial comparison of OS by MGMT status was performed using Kaplan Meier (KM) plots accompanied by a log-rank test. A univariate Cox model assessed the effect of MGMT methylation status on OS, whereas a multivariate Cox model was used for sensitivity purposes. All survival analyses were stratified by trial. Statistical significance was determined at the 2-sided 5% significance level.

Assay MGMT methylation status reproducibility and comparison with the original procedure was quantified using Cohen's Kappa coefficient. The original procedure uses the uncorrected *MGMT* $\log_2$ ratio with a cutoff of 1 and a lower safety margin of -0.75. This cutoff was based on 602 patient samples from CENTRIC, and the lower safety margin was set at the lower bound 95% CI of being unmethylated as described (12). A limit of detection of the diagnostic assay was also applied that sets <10 methylated *MGMT* copies to unmethylated.

All analyses were carried out in R version 3.3.0.

Results

Descriptive Analyses

Valid qMSP MGMT results were available for 4041 patient samples (all-P population; 90.6% of all available samples), consisting of 2316 patients screened only (57.3%) and 1725 randomized patients (rand-P population, 42.7%) (Fig. 1). Only for the latter full treatment and survival outcome data were available. The All-P population was randomly split into a training and test cohort stratified for trial and clinical factors, respectively comprising 2021 and 2020 patients. The rand-P training and test cohorts contained 863 and 862 patients, respectively.

The origin of the patients (trial) and baseline characteristics are summarized in Table 1 and were balanced between cohorts.

Assay Reproducibility

The reproducibility of the assay was evaluated in 218 paired sample sets with ACTB copies ≥ 1250 in both the original and retest data. Retest values for the corrected MGMT $\log_2$ ratio were plotted in function of the original values (Fig. 2). The coefficient of determination (R^2) was >93%, indicating that most of the variability in the retest data could be explained by the original data.

Unsupervised Technical Cutoff

The unsupervised cutoff for the corrected MGMT $\log_2$ ratio, separating methylated and unmethylated samples, was equal to 1.27 on the $\log_2$ scale (Fig. 3A). After assignment of the MGMT methylation status there were 1332 unmethylated patients (65.9%) and 689 methylated patients (34.1%) in the all-P training cohort.

Association between MGMT Methylation Status and OS

The median OS from randomization in the whole rand-P training cohort was 19.3 months (95% CI, 17.5–20.7). Baseline characteristics were balanced between the 460 *MGMT* unmethylated patients (53.3%) and 403 methylated patients (46.6%) (supplementary Table S1). Median OS was 14.5 months (95% CI, 14.0-15.3) and 26.5 months (95% CI, 25.1-30.2), respectively (Fig. 3B). *MGMT* methylated patients had a significantly longer OS compared to unmethylated patients (log-rank test, p<0.0001; HR=0.39, 95% CI, 0.30-0.50). Similar results were obtained in the multivariate analysis (supplementary Table S2).

Supervised Optimal Cutoff

For the unmethylated patients in the rand-P training cohort a time-dependent ROC curve with an AUC equal to 0.61 was obtained, resulting in an optimal cutoff point of -0.28 on the

log_2 scale (Supplement Fig. S1A). This corresponds to a 96% probability of being unmethylated as visualized in Figs 4A and 4B. In contrast, for the methylated patients the ROC curve yielded an AUC of 0.50, suggesting no association between extent of methylation and outcome (supplementary Fig. S1B).

Lower Safety Margin and OS

The optimal supervised cutoff of -0.28 obtained in the unmethylated rand-P training subset was applied as a lower safety margin in the entire rand-P training cohort. The grey zone comprised 82 patients (9.5%), while 378 patients (43.8%) were labeled as truly unmethylated. The KM plot is displayed in Fig. 4C and the survival curves differed significantly according to the log rank test ($p<0.0001$). Univariate Cox regression analysis resulted in a HR of 0.35 (95% CI: 0.27- 0.45, $p<0.0001$) for the methylated patients, and a HR of 0.58 for patients in the grey zone (95% CI, 0.43-0.78, $p<0.001$), respectively, when compared to the truly unmethylated patients. Similar results were obtained in the multivariate analysis (Table 2).

Validation of Unsupervised Cutoff and Supervised Safety Margin and OS in the Independent Test Cohort

There were 375 truly unmethylated patients (43.5%), 70 grey zone patients (8.1%), and 417 methylated patients (48.4%) in the rand-P test cohort. The median OS in the whole rand-P test cohort was 17.7 months (95% CI, 16.7-19.3). *MGMT* methylated patients had a significantly longer OS compared to unmethylated patients (supplementary Table S2, supplementary Fig. S2A). When including the lower safety margin, the survival curves differed significantly (log rank test, $p<0.0001$, Fig. S2B in the Supplement). The univariate Cox model resulted in a HR of 0.38 (95% CI, 0.29-0.49, $p<0.0001$) for the methylated patients, and a HR of 0.70 for patients in the grey zone (95% CI, 0.51- 0.96, $p=0.03$), both compared to the truly unmethylated patients. Similar results were obtained in the multivariate model (Table 2).

Good classification in retest dataset

Application of the 1.27 unsupervised cutoff to the retest dataset of 218 paired samples yielded 8 methylation status mismatches (3.7%; supplementary Table S3). Cohen's Kappa coefficient for inter-rater agreement was 0.93 (95% CI, 0.88-0.98) indicating almost perfect agreement between the original and retest methylation status. After also applying the lower safety margin the Kappa value was 0.80 (95% CI, 0.73-0.88), still indicating almost perfect agreement (supplementary Table S4). A Kappa-value of 0.89 (95% CI, 0.83- 0.95) was obtained, when in addition applying the limit of detection of the diagnostic qMSP assay that considers <10 copies of methylated *MGMT* below the limit of detection and classifies them as unmethylated by default (supplementary Table S5).

Comparison of the validated new classification with the original procedure

When comparing our classification method to the original procedure and cutoff (18, 19) in both the all-P training and test cohort, Cohen's Kappa coefficients of respectively 0.93 (95% CI: 0.92 – 0.94) and 0.95 (95% CI, 0.94-0.96) were obtained, indicating almost perfect agreement (supplementary Table S6). When the limit of detection of the diagnostic assay was applied, the comparison between the original and new classification method (supplementary Table S7) resulted in a Cohen's Kappa value of 0.98 (95% CI, 0.97-0.99) in the both the all-P training and test cohorts.

Discussion

We aimed at determining a clinically relevant cutoff for the qMSP MGMT assay that is most widely used in clinical trials for patient stratification and, more importantly, for treatment strategies omitting TMZ in patients with unmethylated glioblastoma. The technical cutoff of the MGMT assay used has been defined as the value where the probability of being methylated or unmethylated is 50% (18, 19). The uncertainty regarding the methylation status close to the cutoff is high. Our pooled analysis from four randomized trials allowed

determination and validation of the technical cutoff as well as a clinically relevant cutoff, supervised by OS in a large pooled dataset of patients treated uniformly with the current standard of care (TMZ/RT → TMZ).

This supervised optimal cutoff (-0.28, corrected MGMT log_2 ratio) was situated below the technical cutoff obtained (1.27) and represents a lower safety margin which defines a grey zone of "low" methylation (Fig. 2). Patients whose MGMT value was situated in this grey zone did significantly better than those classified as "truly" *MGMT* unmethylated (<-0.28). Application of the lower safety margin in trials comparing schedules of TMZ/RT → TMZ to RT only (4) may shed new light on the interpretation of the apparent "low benefit" from TMZ in the "*MGMT* unmethylated" population. Consequently, grey zone patients may benefit from TMZ treatment and should not be considered for treatments withholding TMZ.

In contrast, among patients classified as *MGMT* methylated (>1.27, above the technical cutoff), a higher extent of methylation was not associated with a further gain in OS. This may suggest that detection of *MGMT* methylation in GBM using this assay is indicative of the second hit, completely inactivating *MGMT*. The first hit is the GBM characteristic loss of one copy of chromosome 10 on which *MGMT* resides (10q26) (23). For tumor types retaining both copies of *MGMT* other clinical cutoffs may apply predicting sensitivity to TMZ/alkylating agents as we have recently reported for IDH mutated grade II glioma treated with TMZ or RT in the EORTC-22033 randomized phase III trial (24, 25).

Comparison of the here presented OS-supervised MGMT classification (methylated, grey zone or unmethylated) with the original classification and cutoffs (12, 18) revealed a high level of agreement. In the original classification procedure we had defined the safety margin as the 95% probability to be unmethylated (12) based on theoretical considerations as it is unknown which methylation pattern and how much methylation is required for complete silencing of *MGMT* expression in glioblastoma (26). This safety margin was applied for

patient selection into trials omitting TMZ (12, 13). This boundary is very similar to the safety margin determined with the OS supervised analysis in this study that corresponds to a 96% chance of being unmethylated. Thus, our study now demonstrates the *clinical* importance of respecting a grey zone by providing the respective supporting outcome data. Implementation of the safety margin essentially groups methylated and grey zone results into the TMZ requiring patient population and selects the truly unmethylated patients as suitable for treatment without TMZ. This needs to be taken into account for clinical trial planning.

Despite the large dataset and high reproducibility of the assay (R^2=0.94) our study suffers from some limitations. All analyses were retrospective, which might have caused patient selection and cannot guarantee that training and test cohorts were balanced in terms of unmeasured confounders. In addition, no survival data was available for screened patients only, reducing the sample size for supervised and subgroup analyses. Yet, no better datasets to address this important issue for clinical practice and future clinical trial design is likely to become available.

It is important to note that the extent of methylation as measured and quantified by different MGMT tests may not necessarily have the same biological significance. Distinct tests use different principles (15) and/or interrogate different CpGs that do not all have the same impact on *MGMT* silencing (26-28), which is the principle mechanism for sensitizing patients to TMZ. Consequently, cutoffs and corresponding safety margins need to be determined and validated for each assay (17, 29-31).

In conclusion, the present analysis demonstrates that the qMSP assay is robust and technically reproducible, and confirms the strong impact of *MGMT* methylation on outcome in a large clinical trial populations treated with TMZ/RT→TMZ. The re-establishment of the cutoffs in a large dataset with a slightly different calculation model and using outcome information, yielded almost identical classification into methylated, grey zone, and truly

unmethylated patients as compared to the original procedure described (12). The clinically relevant cutoff informed by OS defined a grey zone with a safety margin that identifies patients who perform significantly better than truly unmethylated patients and may have some benefit from TMZ. This grey zone could be validated in an independent dataset indicating that these patients should not be selected for treatment schemes avoiding TMZ.

With this study we aim to encourage stratified TMZ treatment for glioblastoma patients implementing a safety margin for guiding treatment decisions. This should facilitate testing new treatment paradigms without TMZ in *MGMT* unmethylated GBM patients who direly need better treatments.

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Authors' Contributions

MEH, EG, TG, and MW designed the study. MEH, GJ, WVW, GJ, and JS, were involved in design, coordination of testing, and re-testing. GJ coordinated testing and validation in all trials and transferred the original data. RS, OC, MRG, LBN, and MW were coordinators of the trials and provided data. EG performed the statistical analyses supervised by TG. All authors were involved in data interpretation, manuscript writing or reviewing and commenting, and approval of the final version of the manuscript.

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Figure Legends

Figure 1. Flow of patient samples through the study. all-P, all patients population; rand-P, randomized patient population; *ACTB,* β-actin gene.

Figure 2. Reproducibility of qMSP MGMT assay.

The Original and Retest dataset (corrected MGMT $\log_2$ ratios, [$\log_2$ ($\frac{MGMT+1}{ACTB} \times 1000$)]) from 218 paired samples are visualized in a scatter plot. The R-squared was 93%. Retests were performed using a second set of FFPE tumor sections in a different laboratory blinded to the original results.

Figure 3. Unsupervised *MGMT* promoter methylation cutoff and OS.

The unsupervised cutoff of 1.27 obtained in the all-P training cohort is indicated in green in the bimodal distribution of the corrected MGMT $\log_2$ ratio values [$\log_2$ ($\frac{MGMT+1}{ACTB} \times 1000$)] (A). The Kaplan Meyer plot visualizes OS in the rand-P training cohort separated into patients with *MGMT* promoter-methylated and -unmethylated tumors (p<0.0001, logrank test). The shaded area represents the 95% confidence interval.

Figure 4. Optimal *MGMT* promoter methylation cutoff and OS.

The position of the optimal cutoff point of -0.28 [corrected MGMT $\log_2$ ratio value, $\log_2$ ($\frac{MGMT+1}{ACTB} \times 1000$)] is indicated in orange in the bimodal distribution of the entire all patients (all-P) training cohort (A). It corresponds to a 96% chance to be unmethylated (4% chance to be methylated) as illustrated in the posterior probability plot (B) and defines the lower bound of the “grey zone“ (-0.28, and ≤1.27). The Kaplan Meyer plot visualizes (C) the outcome of patients in the randomized patient (rand-P) training cohort separated into *MGMT* promoter methylated (<1.27), grey zone (-0.28, and ≤1.27), and truly unmethylated patients (<-0.28) (p<0.0001, logrank test). The shaded area represents the 95% confidence interval.

Table 1. Patient origin (trial) and baseline characteristics of split datasets

	TRAINING cohort (%)		TEST cohort (%)	
	all-P [a] (n=2021)	**rand-P [b] (n=863)**	**all-P [a] (n=2020)**	**rand- P [b] (n=862)**
Trial				
CENTRIC (randomized)	264 (13.1)	264 (30.6)	262 (13.0)	262 (30.4)
CENTRIC (screened)	1058 (52.4)	NA	1058 (52.4)	NA
CORE (randomized)	132 (6.5)	132 (15.3)	133 (6.6)	133 (15.4)
CORE (screened)	100 (4.9)	NA	100 (5.0)	NA
AVAGlio	170 (8.4)	170 (19.7)	170 (8.4)	170 (19.7)
RTOG 0825	297 (14.7)	297 (34.4)	297 (14.7)	297 (34.5)
Baseline Characteristics				
Performance status (randomized patients only)				
PS = 0	NA	516 (59.8)	NA	514 (59.6)
PS ≥ 1	NA	347 (40.2)	NA	345 (40.0)
Missing	NA	0 (0)	NA	3 (0.4)
Extent of resection (randomized patients only)				
Complete resection	NA	454 (52.6)	NA	453 (52.6)
Partial resection	NA	394 (45.7)	NA	392 (45.5)
Biopsy only	NA	6 (0.7)	NA	7 (0.8)
Other	NA	8 (0.9)	NA	8 (0.9)
Missing	NA	1 (0.1)	NA	2 (0.2)
Age in years (randomized patients only)				
Median (Q1,Q3)	NA	57 (50, 63)	NA	57.5 (50, 64)
Corrected $\log_2$ MGMT ratio				
Median (Q1,Q3)	-0.58 (-2.00, 3.24)	0.63 (-1.97, 4.77)	-0.58 (-1.96, 3.54).	1.06 (-1.79, 4.94)

[a] all-P, all patients population; [b] rand-P, randomized patients population

Table 2. Outcome by MGMT promoter methylation status in the rand-P training & test cohorts.

Methylation status	N (%)	Observed Events	Median survival [months]	HR (95% CI)	P-value	Adj. HR (95% CI)[a]	Adj. P-value
Training cohort							
Truly unmethylated	378 (43.8)	302	14.0 (13.1 – 14.7)	1.00		1.00	
Grey zone	82 (9.5)	50	18.5 (16.2 – 25.0)	0.58 (0.43 – 0.78)	<0.001	0.64 (0.47 – 0.87)	<0.01
Methylated	403 (46.7)	203	26.5 (25.1 – 30.2)	0.35 (0.27 – 0.45)	<0.0001	0.32 (0.25 – 0.42)	<0.0001
Test cohort							
Truly unmethylated	375 (43.5)	299	13.6 (12.9 – 14.7)	1.00		1.00	
Grey zone	70 (8.1)	46	16.5 (13.8 – 20.6)	0.70 (0.51 – 0.96)	0.03	0.71 (0.52 – 0.97)	0.03
Methylated	417 (48.4)	219	25.6 (23.2 – 28.4)	0.38 (0.29 – 0.49)	< 0.0001	0.36 (0.28 – 0.46)	< 0.0001

[a] adjusted for age, ECOG performance status, and extent of resection

Figure 1

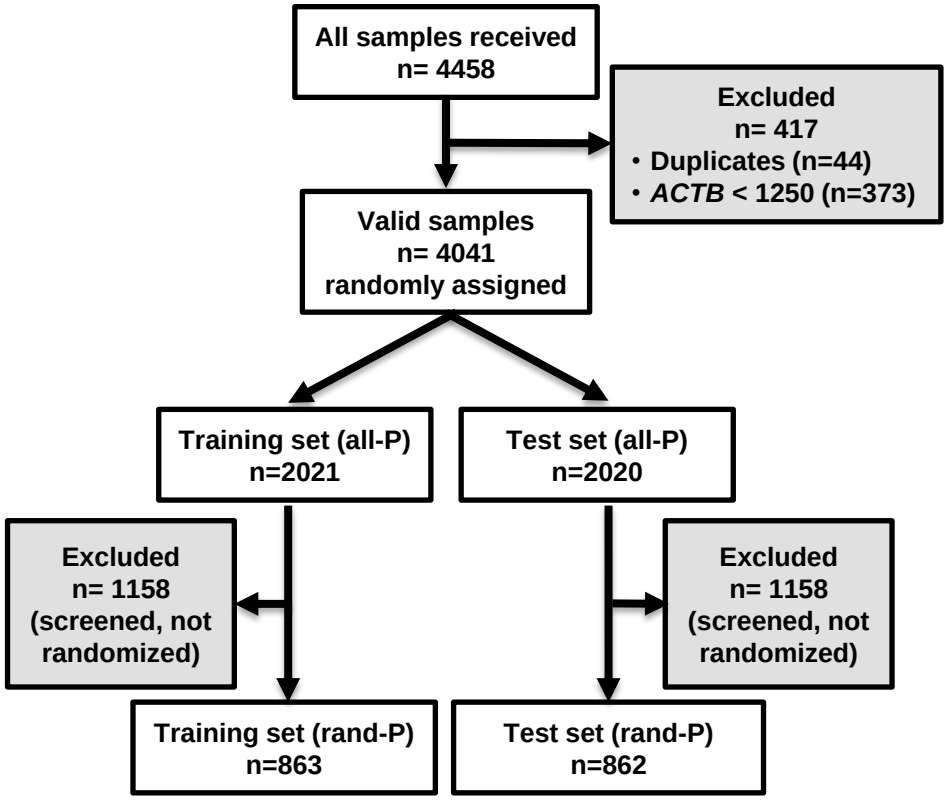

All samples received
n= 4458
Excluded
n= 417
• Duplicates (n=44)
• *ACTB* < 1250 (n=373)
Valid samples
n= 4041
randomly assigned
Training set (all-P)
n=2021
Test set (all-P)
n=2020
Excluded
n= 1158
(screened, not
randomized)
Excluded
n= 1158
(screened, not
randomized)
Training set (rand-P)
n=863
Test set (rand-P)
n=862

Figure 2

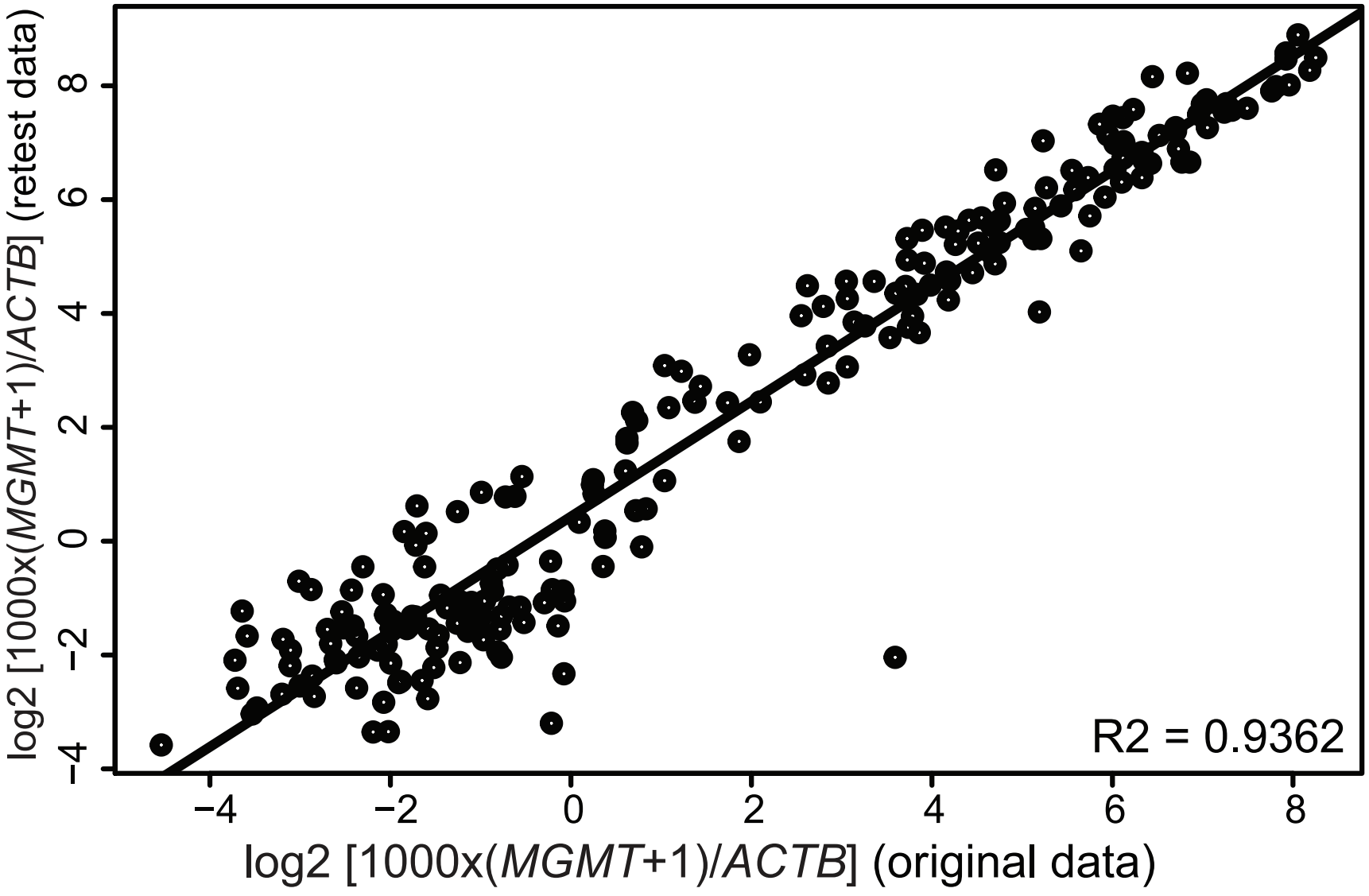

log2 [1000x(*MGMT*+1)/*ACTB*] (retest data)
log2 [1000x(*MGMT*+1)/*ACTB*] (original data)
R2 = 0.9362
−4
−2
0
2
4
6
8

Figure 3

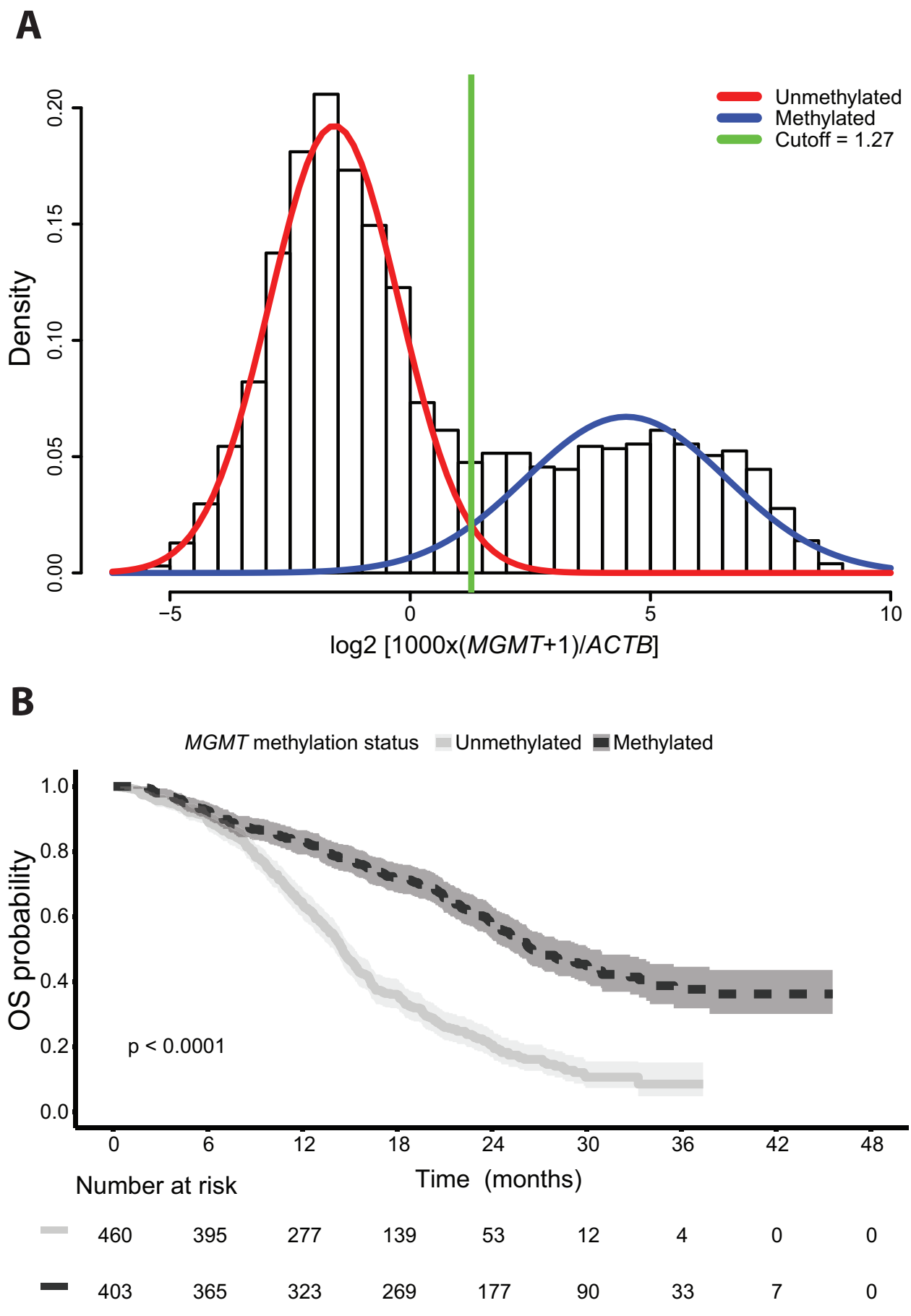
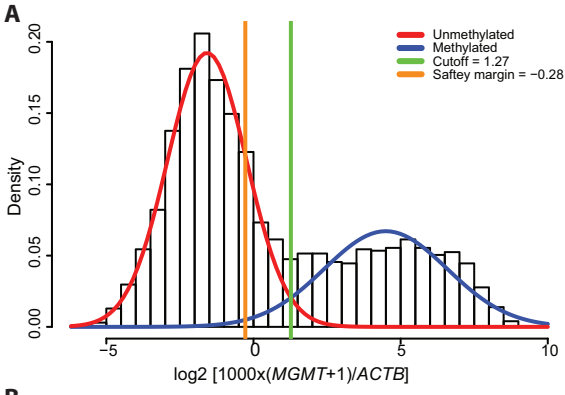
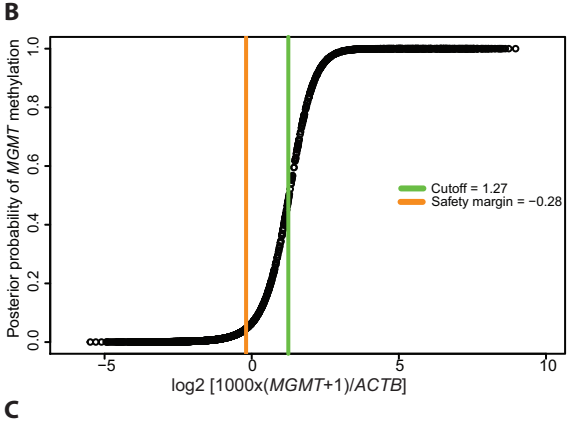
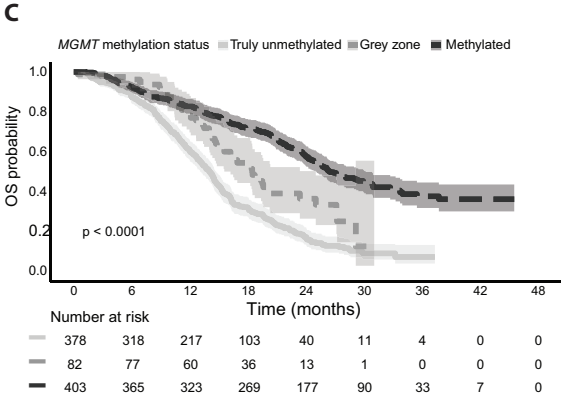

A
Unmethylated
Methylated
Cutoff = 1.27
Density
0.00
0.05
0.10
0.15
0.20
−5
0
5
10
log2 [1000x(MGMT+1)/ACTB]
B
MGMT methylation status
Unmethylated
Methylated
OS probability
0.0
0.2
0.4
0.6
0.8
1.0
p < 0.0001
0
6
12
18
24
30
36
42
48
Time (months)
Number at risk
460 395 277 139 53 12 4 0 0
403 365 323 269 177 90 33 7 0

Figure 4


A
Unmethylated
Methylated
Cutoff = 1.27
Saftey margin = −0.28
Density
0.00
0.05
0.10
0.15
0.20
−5
0
5
10
log2 [1000x(MGMT+1)/ACTB]


B
Posterior probability of MGMT methylation
0.0
0.2
0.4
0.6
0.8
1.0
Cutoff = 1.27
Safety margin = −0.28
−5
0
5
10
log2 [1000x(MGMT+1)/ACTB]


C
MGMT methylation status
Truly unmethylated
Grey zone
Methylated
OS probability
0.0
0.2
0.4
0.6
0.8
1.0
p < 0.0001
0
6
12
18
24
30
36
42
48
Time (months)
Number at risk
378 318 217 103 40 11 4 0 0
82 77 60 36 13 1 0 0 0
403 365 323 269 177 90 33 7 0