Supplementary Material

Supplementary Materials and Methods

rs16906252 SNP genotyping

High-resolution melting analysis were performed on the LightCycler® 480 (Roche Life Science) using previously published primers[1]. The real-time PCR cycling protocol started with one cycle of 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 20 s, 72°C for 20 s. The melting step was performed from 65°C to 95°C after a denaturation step of 1 min at 95°C and a hybridization step of 40°C for 1 min. For the reaction mixtures the LightCycler® 480 High Resolution Melting Master (Roche) was used at a final concentration at 1×. Final $MgCl_2$ concentration was 2.5 mM and the final primer concentrations were 200 nM of each primer, and 25 ng of DNA was used as template. Sanger sequencing was performed by the commercial services of Eurofins® using the same forward primer as in the pyrosequencing assay and the following reverse primer: 3'-AGCATGGAAGGGTAGGAAGAAC-5'.

Quantitative- and allelic methylation analyses by qMSP-pyrosequencing

Sodium bisulfite conversion of the samples was performed using the EZ DNA Methylation kit (Zymo Research) according to the manufactures' instructions, with slight modifications; samples were incubated at 42°C for 30 minutes instead of 37°C for 15 minutes. For the bisulfite reaction the alternative incubation conditions described in the appendix were used. The LightCycler® 480 (Roche Life Science) was used for real-time PCR and melting analysis. The real-time PCR cycling protocol started with one cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 20 s, 70°C for 20 s, 72°C for 20 s. The melting step was performed from 65°C to 95°C after a denaturation step of 1 min at 95°C and a hybridization step of 40°C for 1 min. For the reaction mixtures the SYBR Green Master Mix (Roche) was used at a final concentration at 1×. Final primer concentrations were 200 nM of each primer, and 25 ng of DNA was used as template. The final reaction volume was 20 µL. Primer sequences have been published previously[2]. The Alu assay used for normalization was used without the TaqMan probe using an intercalating fluorescent dye instead as previously described[1]. Pyrosequencing was performed on the PyroMark Q24 (Qiagen) using the PyroMark Gold Q24 reagents (Qiagen), according to the manufactures' instructions.

Standard pyrosequencing (Therascreen MGMT Pyro kit)

Sodium bisulfite conversion of the samples was performed using the Epitect bisulfite kit (Qiagen, Hilden, Germany) according to the manufactures' instructions. PCR and pyrosequencing was performed using the Therascreen MGMT Pyro kit (Qiagen) according to the manufactures' instructions, with slight modifications; 1 µl of the component "streptavidin sepharose high

performance" was used instead of 2 μl. For each pyrosequencing run three controls were included: One NTC from PCR, one methylated DNA control provided with the kit and one sample of DNA from an anonymous healthy blood donor or whole genome amplified DNA (unmethylated DNA) prepared as previously described[3].

References

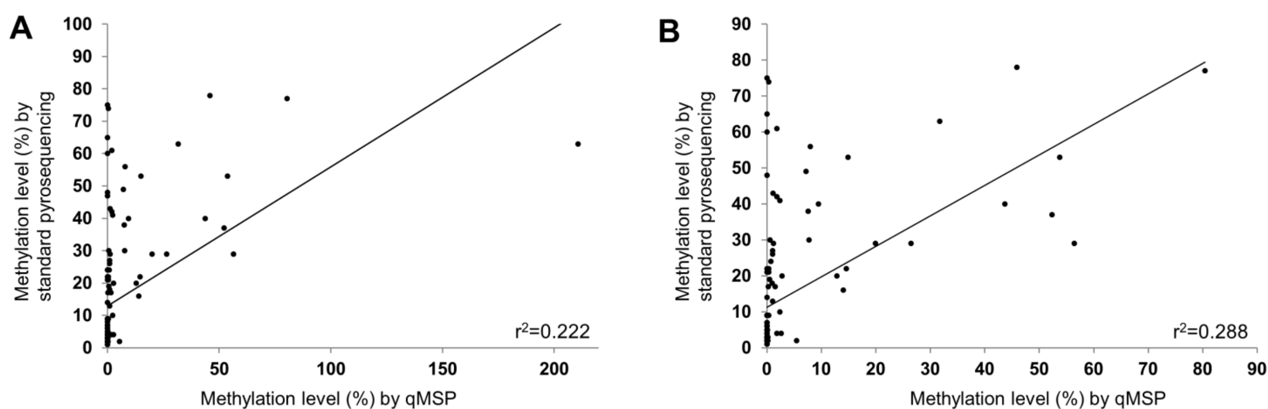
1. Kristensen LS, Nielsen HM, Hager H, Hansen LL. Methylation of MGMT in malignant pleural mesothelioma occurs in a subset of patients and is associated with the T allele of the rs16906252 MGMT promoter SNP. *Lung Cancer.* 2011; 71(2):130-136.

2. Kristensen LS, Treppendahl MB, Asmar F, et al. Investigation of MGMT and DAPK1 methylation patterns in diffuse large B-cell lymphoma using allelic MSP-pyrosequencing. *Scientific reports.* 2013; 3:2789.

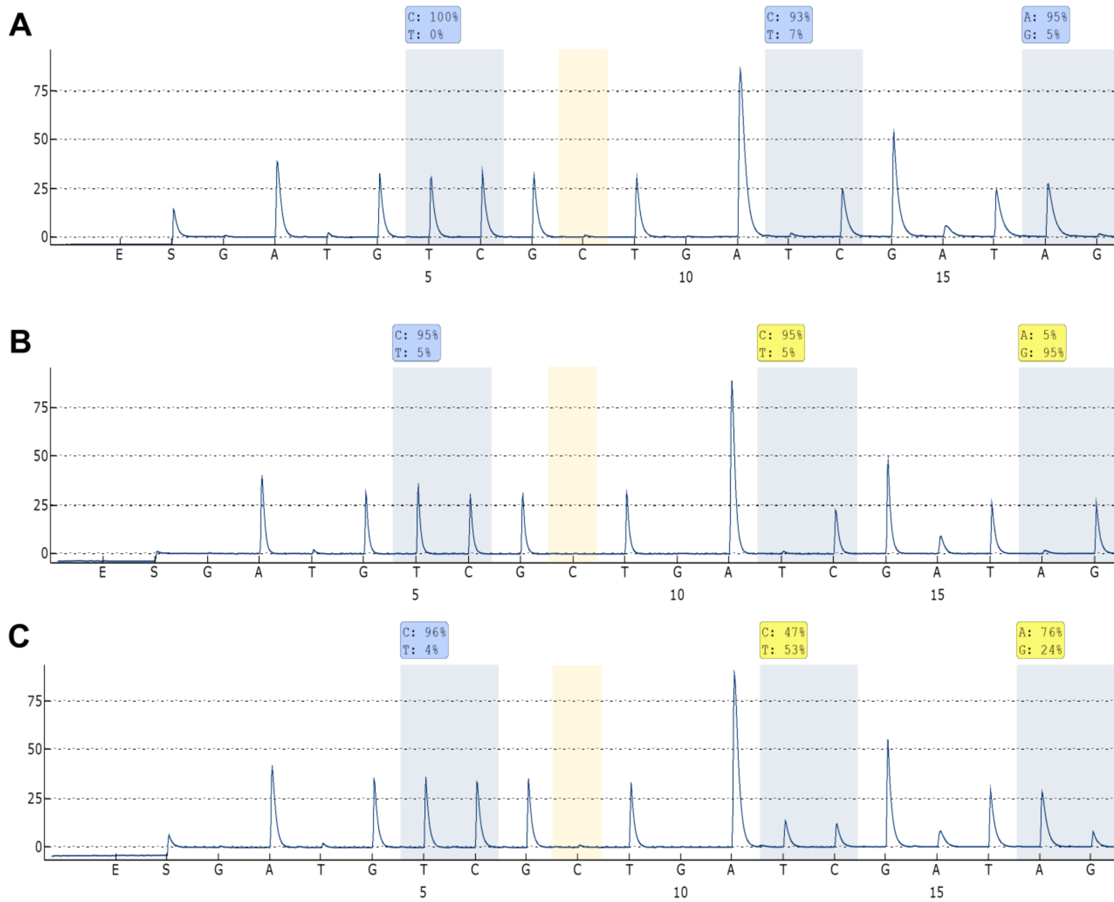
3. Kristensen LS, Mikeska T, Krypuy M, Dobrovic A. Sensitive Melting Analysis after Real Time-Methylation Specific PCR (SMART-MSP): high-throughput and probe-free quantitative DNA methylation detection. *Nucleic acids research.* 2008; 36(7):e42.

Supplementary Figures

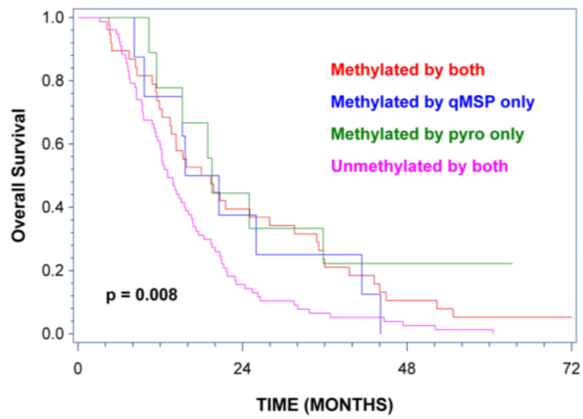
Supplementary Fig. 1. Genomic location of the qMSP-pyrosequencing primers. The MSP primers are indicated as black arrows and the sequencing primer used for pyrosequencing is indicated as a blue arrow. The reverse MSP primer is labeled with biotin. The rs16906252 SNP is in bold. CpG sites are in red. The CpG sites analyzed by the standard pyrosequencing assay are in bold red and underlined.



Supplementary Fig. 2. Comparison of qMSP and standard pyrosequencing data. (A) Methylation levels determined by qMSP plotted against methylation levels determined by standard pyrosequencing. (B) The same data as in (A) after removal of an outlier. Relatively weak correlations were observed.



Supplementary Fig. 3. Allelic methylation analyses. (A) Pyrogram of a sample being methylated only at the A-allele. (B) Pyrogram of a sample being methylated only at the G-allele. (C) Pyrogram of a sample being methylated at both alleles. High methylation levels of the CpG sites indicate that amplification occurred from methylated template, and it can be observed that the bisulfite conversion of the non-CpG C is essentially complete. Thus, these methylation results are regarded as true positive results. The antisense strand was analyzed as C and T cannot be distinguished after bisulfite treatment.



EVENTS	PATIENTS AT RISK		
36	38	15	4
8	8	3	.
7	9	4	2
77	77	12	2

Supplementary Fig. 4. Overall survival according to methylation status determined by qMSP and standard pyrosequencing. Patients being positive for only one of the two methods are separated according to which of the methods they tested positive for.

Supplementary Tables

Supplementary Table 1. Patient demographics and clinical data.

Age (years), median (range)	59.0 (22–74)
Gender, *n* (%)	
Female	56 (37.1)
Male	95 (62.9)
ECOG performance status, *n* (%)	
0	90 (59.6)
1	42 (27.8)
2	13 (8.6)
Missing	6 (4.0)
Diagnosis, *n* (%)	
Primary Glioblastoma	145 (96.0)
Secondary Glioblastoma	6 (4.0)
Multifocal Disease, *n* (%)	
Yes	14 (9.3)
No	136 (90.1)
Missing	1 (0.7)
Extent of tumor resection, *n* (%)	
Biopsy	5 (3.3)
Partial resection	82 (54.3)
Gross total resection	62 (41.1)
Missing	2 (1.3)
Corticosteroid therapy at initiation of RT/TMZ therapy, *n* (%)	
Yes	110 (72.8)
No	39 (25.8)
Missing	2 (1.3)
No. of TMZ cycles following initial RT/TMZ therapy, *n* (%)	
Median	3
0	23 (15.2)
1	7 (4.6)
2	36 (23.8)
3	10 (6.6)
4	7 (4.6)
5	16 (10.6)
6	52 (34.4)
Best clinical response to initial RT/TMZ therapy, *n* (%)	
CR	0 (0)
PR	20 (13.2)
SD	64 (42.4)
PD	60 (39.7)
Missing	7 (5.9)

Reoperation, *n* (%)	
Yes	58 (38.4)
No	81 (53.6)
Missing	12 (7.9)
Number of palliative therapies	
No	68 (45.0)
1	48 (31.8)
2	19 (12.6)
3	3 (2.0)
Missing	13 (8.6)
Follow-up duration (months), median (range)	94.0 (53–123)

Abbreviations: CR, complete response; PD, progressive disease; PR, partial response; RT, radiotherapy; SD, stable disease; TMZ, temozolomide.

Supplementary Table 2. Semi-quantitative comparison of qMSP and standard pyrosequencing.

	Low methylation (MIP-pyrosequencing)	High methylation (MIP - pyrosequencing)	Total
Low methylation (qMSP)	10	4	14
High methylation (qMSP)	5	19	24
Total	15	23	38

Supplementary Table 3. Comparison of DNA methylation data assessed by qMSP and IHC data

	No methylation	Low methylation	Medium methylation	High methylation	Total
No protein expression	55	22	10	7	94
Low protein expression	21	4	1	1	27
Medium protein expression	11	2	1	0	14
High protein expression	11	1	0	1	13
Total	98	29	12	9	148

Supplementary Table 4. Comparison of DNA methylation data assessed by standard pyrosequencing and IHC data

	No methylation	**Low methylation**	**Medium methylation**	**High methylation**	**Total**
No protein expression	43	13	15	10	81
Low protein expression	20	2	2	1	25
Medium protein expression	11	1	0	0	12
High protein expression	10	0	0	1	11
Total	84	16	17	12	129

Supplementary Table 5. *MGMT* methylation status by qMSP and standard pyrosequencing according to genotype.

	qMSP		
Genotype	**Methylation positive**	**Methylation negative**	**Total**
CC	42	96	138
CT or TT	9	4	13
Total	51	100	151
	Standard pyrosequencing		
	Methylation positive	**Methylation negative**	**Total**
CC	40	80	120
CT or TT	7	5	12
Total	47	85	132