Digital PCR assessment of MGMT promoter methylation coupled with reduced protein expression optimises prediction of response to alkylating agents in metastatic colorectal cancer patients

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Running title: MGMT combined score in colorectal cancer

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Abstract

Background: O(6)-methylguanine-DNA-methyltransferase (MGMT) is a repair protein which deficiency makes tumors more susceptible to the cytotoxic effect of alkylating agents. Five clinical trials with temozolomide or dacarbazine have been performed in metastatic colorectal cancer (mCRC) with selection based on methyl-specific PCR (MSP) testing with modest results. We hypothesized that mitigated results are consequences of unspecific patient selection and that alternative methodologies for MGMT testing such as immunohistochemistry (IHC) and digital PCR could enhance patient enrollment.

Patients and methods: Formalin-fixed paraffin embedded archival tumor tissue samples from four phase II studies of temozolomide or dacarbazine in MGMT MSP-positive mCRCs were analyzed by IHC for MGMT protein expression and by methyl-BEAMing (MB) for percentage of promoter methylation. Pooled data were then retrospectively analyzed according to objective response rate, progression-free survival (PFS) and overall survival (OS).

Results: 105 patients were included in the study. Twelve had achieved partial response (PR) (11.4%), 24 stable disease (SD) (22.9%) and 69 progressive disease (PD) (65.7%). Patients with PR/SD had lower IHC scores and higher MB levels than those with PD. MGMT expression by IHC was negatively and MB levels positively associated with PFS

(p<0.001 and 0.004, respectively), but not with OS. By combining both assays, IHC low/MB high patients displayed an 87% reduction in the hazard of progression (p<0.001) and a 77% in the hazard for death (p=0.001).

Conclusion: In mCRC selected for MGMT deficiency by MSP, IHC and MB testing improve clinical outcome to alkylating agents. Their combination could enhance patient selection in this setting.

Word count using Ms-Word 2010:

2234 words

Introduction

MGMT is a repair protein that removes alkylating groups from the O^6-guanine in DNA. It protects normal and tumor cells from this type of DNA damage by moving the alkylating group to a cysteine residual within its own protein [1, 2]. Approximately 40% of metastatic colorectal cancer (mCRC) show silencing of the *MGMT* gene, which leads to absence of the corresponding protein [3]. Due to this deficiency, the tumor cell is not able to effectively repair O^6-methylguanine adducts, causing a higher frequency of G:C > A:T transitions and potentially enhancing the susceptibility to cytotoxic effect of alkylating agents such as temozolomide (TMZ) or dacarbazine [2-4]. MGMT deficiency can be assessed in tumor samples either as promoter hypermethylation by Methyl-Specific PCR (MSP) [1] and digital PCR-based methods such as MethylBEAMing (MB) [5] or lack of protein expression by immunohistochemistry (IHC) [6].

MGMT status is a clinically validated predictive biomarker for glioblastoma [7] and has been extensively studied for advanced melanoma [8], two malignancies where alkylating agents have been the backbone of systemic treatment for years. However, the same is not the case for mCRC where these drugs are not routinely used. Indeed, limited data are available regarding treatment of CRC with dacarbazine or TMZ based on MGMT methylation. Five phase II trials have assessed the clinical efficacy of alkylating agents in mCRC based on this biomarker [9-13]. In all these studies, selection was based on *MGMT* promoter methylation by Methyl-Specific PCR (MSP), a qualitative assay performed on formalin-fixed paraffin embedded archival tumor tissue. However, even in populations enriched for MSP-positive tumors, the clinical activity of alkylating agents was limited, with response rates ranging from 4 to 16%, making the role of this biomarker still unclear.

Therefore, even though MSP is a commonly used assay for melanoma [8] and glioblastoma [7], selection of patients according to this methodology seems to be a useful but not sufficient condition for achieving clinical benefit with alkylating agents in mCRC.

With the aim of better refining the molecular selection in this setting, we retrospectively analyzed MSP-positive tumor samples by IHC and digital PCR from 105 mCRC patients treated within four phase-II studies of TMZ or dacarbazine and evaluated association of combined MGMT protein status and MGMT gene promoter methylation on clinical outcome.

Materials and methods

Patient population – Formalin-fixed paraffin embedded archival tumor tissue samples from the following four clinical trials were obtained for analysis of tumor content, MGMT IHC score and MGMT promoter hypermethylation by MB: DETECT (EUDRACT 2011-002080-21) [10]; TEMECT-TEMozolomide Evaluation in ColorecTal cancer, EUDRACT number 2012-003338-17 [9]; INT Study n. 20/13 #1 [13] and INT Study 20/13 #2 [12] (**Figure A**). Pooled data from these trials were retrieved according to objective response rate (RECIST 1.1), progression-free survival (PFS) and overall survival as reported in original studies.

MGMT biomarker assays

Immunohistochemistry analysis of MGMT – Immunohistochemical expression of MGMT was assessed as previously described [12]. Briefly, IHC scoring was performed in a semi-quantitative fashion, taking into account both extension and intensity of staining. Positive MGMT staining (ranging from 0 to 3) was defined as the staining intensity of the majority of tumor cells similar to that of the adjacent endothelial cells (score = 3). Negative MGMT staining (IHC) was defined as tumor cells with no staining (score = 0) or with weaker staining than that of endothelial cells (score = 1). Extension of tumor immunoreactivity was divided in quartiles (0%; up to 25 %; 26–50 %; 51–75%; or 76–100 %). Final IHC score was obtained by multiplying intensity (0, 1, 2 or 3) by extension scores. For combined

score, tumors with values ≤75 were considered IHC-low, while those with values of 76-300 were considered IHC-high.

Methyl-BEAMing – Methylation assessment of MGMT promoter region was assessed using MB protocol as described previously [5]. Percent of methylation was then normalized on tumor content evaluated by hematoxylin and eosin staining to correct for stromal infiltration present in the original slide used for DNA extraction. For combined score, tumors with values below 63% were considered MB-low, while those with values above or equal to 63% were considered MB-high.

Both IHC and MB analyses were performed in a blinded fashion.

Statistical analysis

The present study was promoted by Niguarda Cancer Center and sample size was determined by the highest number of patients collected from clinical trials reported in **Figure A** based on availability of tumor specimens suitable for IHC and/or MB analysis. All data were first analyzed and described by mean and standard deviation or by median and range, according to their distribution. Binomial end points have been analyzed by means of univariate logistic regression; models as a whole were evaluated by likelihood ratio test and by their pseudo-R2 measure, whereas the significance of the single independent variables was evaluated by means of the Wald test. All statistical tests were two sided. Time-to-event analysis was performed using the Kaplan-Meier product-limit method; the equality of the survivor function was then evaluated using the log-rank test. OS was defined as the time from start of treatment with TMZ or dacarbazine until death from any cause, censoring patients who had not died at the date last known alive; PFS was defined as the time from start of treatment until tumor progression. Statistical significance was set at $p<0.05$ for each analysis; all analyses were carried out using STATA software (STATA

Corp, College Station, TX) running on a Windows 7 machine (Microsoft, Redmond, WA).

Results

As shown in **Table A**, a total of 105 patients were included in this analysis. Among these, 12 achieved partial response (PR) (11.4%), 24 stable disease (SD) (22.9%) and 69 progressive disease (PD) (65.7%) as best response in their respective clinical trials. The IHC evaluation of MGMT could be performed in 75 cases (71.4%), while digital PCR (MethylBEAMing) in 97 samples (92.4%; among which three cases could not be evaluated for tumor content making normalization impossible) (Suplementary Table **A**). There was no linear correlation (Pearson's R=-0.2, p=0.08) between MGMT protein expression and promoter silencing of the gene (**Supplementary Figure A**), indicating that paired tests in individual patients were not inversely correlated as expected.

When biomarkers were correlated to objective response rates, we found that patients with PR or SD had lower IHC scores and higher MB percentage levels than those with PD (**Figure B**). A Cox regression univariate model was then applied to study association of these biomarkers with survival (**Table B**), showing that MGMT expression by IHC was negatively associated with PFS ($p<0.001$). Similarly, but with an inverse effect as expected, MGMT promoter methylation % values were positively associated with PFS ($p=0.004$). The same effect was numerically observed with regard to OS, although not reaching statistical significance. Finally, in order to overcome limits of either test alone, we tested if a combined score could enhance the predictive value of MGMT. To this aim, we dichotomized IHC score and promoter methylation % values according to the lower and higher tertiles, respectively (**Table S1**). Applying this combined score in a multivariate model, patients with IHC low/MB high displayed 87% reduction in the hazard of progression ($p<0.001$) and a 77% in the hazard for death ($p=0.001$). Accordingly, when Kaplan-Meier analysis of survival was performed based on these categories, we observed

that both PFS and OS were higher in those patients displaying IHC low/MB high as compared with the other two groups ($p<0.0001$ and 0.006, respectively) (**Figure C**).

Discussion

MGMT promoter methylation status is a validated biomarker of response to TMZ in glioblastoma, and because of initial reports of activity of TMZ in CRC, it has been adopted for selecting patients in phase II trials also in this tumor type despite lack of validation. In this regard, since epigenetic silencing by promoter hypermethylation is the only mechanism accounting for MGMT loss, its detection at the molecular level by methyl-specific PCR (MSP) has been identified as the reference method. Several trials applied this assay for patient selection in mCRC, achieving a response rate around 10% in the setting of chemorefractory disease [9-13], and to date MGMT hypermethylated status by MSP has not been yet validated in this tumor type. MGMT status can be ascertained by means of other assays, such as detection of intact protein by IHC [6, 14] or quantification of epigenetic silencing by digital PCR methylation assays (methyl-BEAMing) [5]. Therefore, we elected to exploit clinical data and pathology samples from 4 phase II studies of alkylating agents (TMZ or dacarbazine) conducted in heavily pretreated mCRC patients in order to investigate the relative contribution of these assays in the prediction of clinical benefit. In glioblastoma, for which MGMT MSP methylated status alone is of prognostic value, the combination of promoter methylation with IHC expression analysis optimized the assessment of MGMT status [15]. We therefore reasoned that combined assessment of promoter methylation assessed by digital-PCR and IHC expression could further refine the role of MGMT status as a predictive biomarker in mCRC.

In the present retrospective analysis of 105 patients, accounting for the largest molecularly selected population of MGMT MSP-positive mCRC patients treated with alkylating agents, we found a potential role as biomarkers for MGMT status detected by either IHC or MB,

and a combined effect of these assays. Based on these findings, results of previous clinical studies should be critically re-interpreted. The current study indicates that detection of MGMT promoter methylation by MSP may be useful but not sufficient condition for achieving best results from TMZ/dacarbazine treatment, and integration with IHC and digital PCR based approached could enhance patient selection. Nevertheless, our data should be interpreted with caution because this analysis was a pooled retrospective study with intent to generate a new testable hypothesis which will requires further validations. However, to our knowledge (and from data available from clinicaltrials.gov), no study but one [11], with limited number of mCRC cases, would fit this setting, preventing the current possibility for validation. An indirect way to confirm the results might be with trials using TMZ in combination with other agents (such as Capecitabine), for which one of the institute involved in the study has recently started enrollment (NCT02414009).

We also recently demonstrated that MGMT status changes over time in archival tumor samples *versus* biopsies taken at initiation of treatment with TMZ [9], and the present study was performed on archival tissues only. Combined MGMT assessment in the current analysis might have allowed identification of cases for which clonality of tumor sample was restricted to MGMT silenced cells and which were less likely to diverge between diagnosis and start of treatment. Also, tumor heterogeneity regarding MGMT status may lead to mixed responses and progressions at different metastatic sites that sometimes may be observed and considered as treatment failures in clinical trials.

In line with our previous study [5], high levels of MGMT promoter hypermethylation as quantified by MB were positively associated with improved PFS also in this pooled dataset. However, even if gene silencing by methylation is assessed by quantitative methods, refinement of patient selection by digital PCR based approaches appears still suboptimal [9], suggesting that other factors could modulate response to alkylating agents in CRC.

It is well known that in solid tumors inconsistencies may occur between protein expression and alterations of the corresponding oncogenes, such as for PTEN or ALK [16, 17]. It has been recently reported that there are discrepancies between lack of expression of MGMT and promoter methylation of the gene in CRC [18]. In line with these findings, we did not observe a correlation between IHC score and the percentage of promoter methylation by MB in our pooled series. These data imply that other transcriptional and post-transcriptional mechanisms may be involved in MGMT expression in CRC cells, as hypothesized elsewhere [19, 20]. Such mechanisms may impact on its role as a biomarker and may not be entirely captured by each assay alone. Based on these considerations, we adopted a combined MGMT score integrating IHC and MB that ended up in the identification of a patient subpopulation with different PFS and OS upon treatment, with those with low IHC/high MB displaying the best outcome.

While the present study encompasses the vast majority of available clinical data, it should be acknowledged that a possible limitation is the lack of a clinical dataset of MGMT MSP-negative patients as a control; however clinical and ethical constraints limit possibilities of treating patients not selected according to the current reference method. We also note that there are limitations linked to the subjective assessment of the observer when scoring MGMT IHC, and lack of accurate quantification of protein expression. This could be encompassed by proteomic analyses in the future.

In conclusion, five phase II clinical trials have previously assessed the clinical efficacy of alkylating agents in mCRC [9-13] and, despite some evidence of improved disease control rate, the role of MGMT assessment as a biomarker in this tumor type has remained unclear. In this retrospective analysis of MGMT MSP-positive CRC samples from most of the patients treated in these trials, we report that there is a predictive role for combined

protein-gene MGMT testing, such as IHC detection of protein expression and MB quantification of promoter methylation. Future studies of alkylating agents for CRC should not rely on a single assay at the protein or gene level, but rather combine both assessments of MGMT status for refining patient selection. To this regard, we propose an algorithm based on preliminary IHC assessment followed by methyl-BEAMing in case of detection of IHC low MGMT expression.

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References

1. 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.

2. Esteller M, Herman JG. Generating mutations but providing chemosensitivity: the role of O6-methylguanine DNA methyltransferase in human cancer. Oncogene 2004; 23: 1-8.

3. Esteller M, Toyota M, Sanchez-Cespedes M et al. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumorigenesis. Cancer Res 2000; 60: 2368-2371.

4. Ju HX, An B, Okamoto Y et al. Distinct profiles of epigenetic evolution between colorectal cancers with and without metastasis. Am J Pathol 2011; 178: 1835-1846.

5. Barault L, Amatu A, Bleeker FE et al. Digital PCR quantification of MGMT methylation refines prediction of clinical benefit from alkylating agents in glioblastoma and metastatic colorectal cancer. Ann Oncol 2015; 26: 1994-1999.

6. Shima K, Morikawa T, Baba Y et al. MGMT promoter methylation, loss of expression and prognosis in 855 colorectal cancers. Cancer Causes Control 2011; 22: 301-309.

7. 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.

8. 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.

9. Amatu A, Barault L, Moutinho C et al. Tumor MGMT promoter hypermethylation changes over time limit temozolomide efficacy in a phase II trial for metastatic colorectal

cancer. Ann Oncol 2016; 27: 1062-1067.

10. 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.

11. 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.

12. Pietrantonio F, de Braud F, Milione M et al. Dose-Dense Temozolomide in Patients with MGMT-Silenced Chemorefractory Colorectal Cancer. Target Oncol 2015.

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. Miyazaki M, Nishihara H, Terasaka S et al. Immunohistochemical evaluation of O6 - methylguanine DNA methyltransferase (MGMT) expression in 117 cases of glioblastoma. Neuropathology 2014; 34: 268-276.

15. 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.

16. Castillo-Martin M, Thin TH, Collazo Lorduy A, Cordon-Cardo C. Immunopathologic Assessment of PTEN Expression. Methods Mol Biol 2016; 1388: 23-37.

17. Ilie MI, Bence C, Hofman V et al. Discrepancies between FISH and immunohistochemistry for assessment of the ALK status are associated with ALK 'borderline'-positive rearrangements or a high copy number: a potential major issue for anti-ALK therapeutic strategies. Ann Oncol 2015; 26: 238-244.

18. Shen L, Kondo Y, Rosner GL et al. MGMT promoter methylation and field defect in

sporadic colorectal cancer. J Natl Cancer Inst 2005; 97: 1330-1338.

19. Zhang L, Zeng J, Zeng Z et al. MGMT in colorectal cancer: a promising component of personalized treatment. Tumour Biol 2016.

20. Zhao W, Soejima H, Higashimoto K et al. The essential role of histone H3 Lys9 di-methylation and MeCP2 binding in MGMT silencing with poor DNA methylation of the promoter CpG island. J Biochem 2005; 137: 431-440.

Table A – Characteristics of patients

Number of patients	105
Sex (M/F)	61/44
Age (median, range)	62 (37-85)
Baseline CEA (ng/mL) (median, range)	68.14 (1 – 39557)
N. previous lines of treatment (median, range)	3 (2-8)
Objective response rate (RECIST)	*N* (%)
PR	12 (11.4%)
SD	24 (22.9%)
PD	69 (65.7%)

Trials	*N* (%)
DETECT	25 (23.8)
TEMECT	29 (27.6)
INT1	23 (21.9)
INT2	28 (26.7)

Table B – Cox regression analysis assessing the impact of clinical variables and MGMT biomarkers on survival after treatment with alkylating agents. In the MGMT combined score, immunohistochemistry (IHC) and promoter methylation assessed by methyl-BEAMing (MB) were categorized as follows: IHC high: IHC score ≥4; IHC low: IHC score <4; MB low: promoter methylation <63%; MB high: promoter methylation ≥63%.

Variable	PFS			OS		
Univariate analysis						
	HR	***p***		**HR**	***p***	
Age	1.0000	*0.996*		0.9953	*0.622*	
Performance status (ECOG):						
0	1.0000			1.0000		
1	1.6147	*0.030*	*	1.9015	*0.004*	*
2	3.3116	*0.006*	*	11.5655	*<0.001*	*
Baseline CEA	1.0003	*0.107*		1,0005	*0.002*	*
Number of previous treatments	0.9987	*0.988*		1.0501	*0.607*	
MGMT IHC Score	1.1351	*<0.001*	*	1.0511	*0.058*	
MGMT promoter methylation (%)	0.9917	*0.004*	*	0.9945	*0.071*	
Multivariate analysis						
(ECOG Performance status):						
0	1.0000			1.0000		
1	1.1294	*0,657*		1.0786	*0.788*	
2	4.0153	*0.006*	*	13.7939	*<0.001*	*
Baseline CEA	1.0006	*0.008*	*	1.0005	*0.012*	*
MGMT combined score:						
IHC high/MB low	1.0000			1.0000		
IHC high/MB high or IHC low/MB low	0.4511	*0.007*	*	0.7980	*0.431*	
IHC low/MB high	0.1374	*<0.001*	*	0.2383	*0.001*	*

MB: methyl BEAMING; IHC: immunohistochemistry; * statistically significant (Wald test)

Supplementary Table A – Results of immunohistochemistry and methyl-BEAMing analysis of MGMT

Immunohistochemistry	
Number of patients	*75*
IHC score	*N* (%)
Low (0-3)	*32*(43)
Intermediate-High (4-12)	*43*(57)
Methyl-BEAMing	
Number of patients	*94**
Median value % (range)	29.7 (0 - 100)
Low (<63%)	*69* (73)
High (≥63%)	*25* (27)

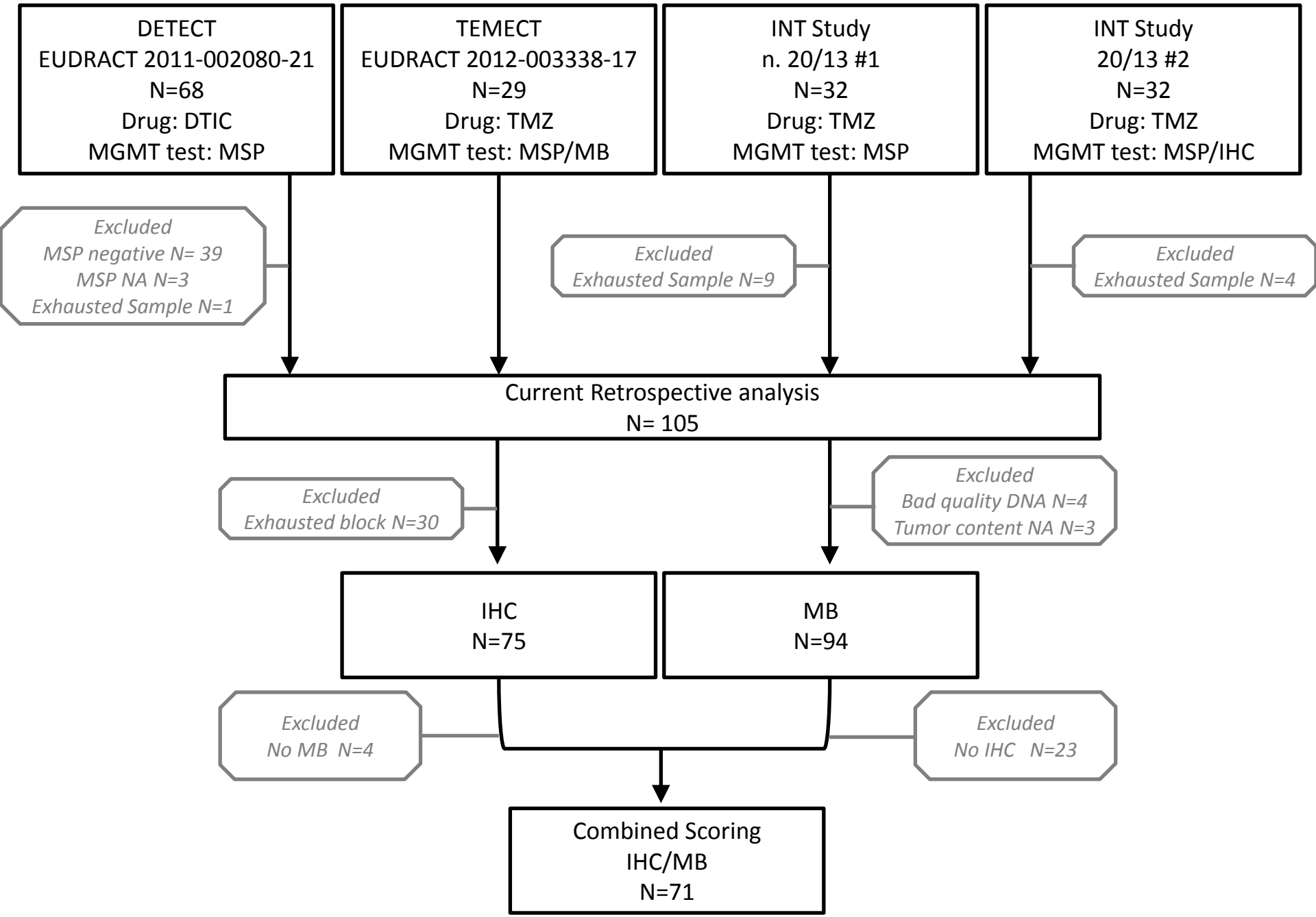
* 97 patients were successfully assessed but three could not be normalized due to absence of tumor content evaluation

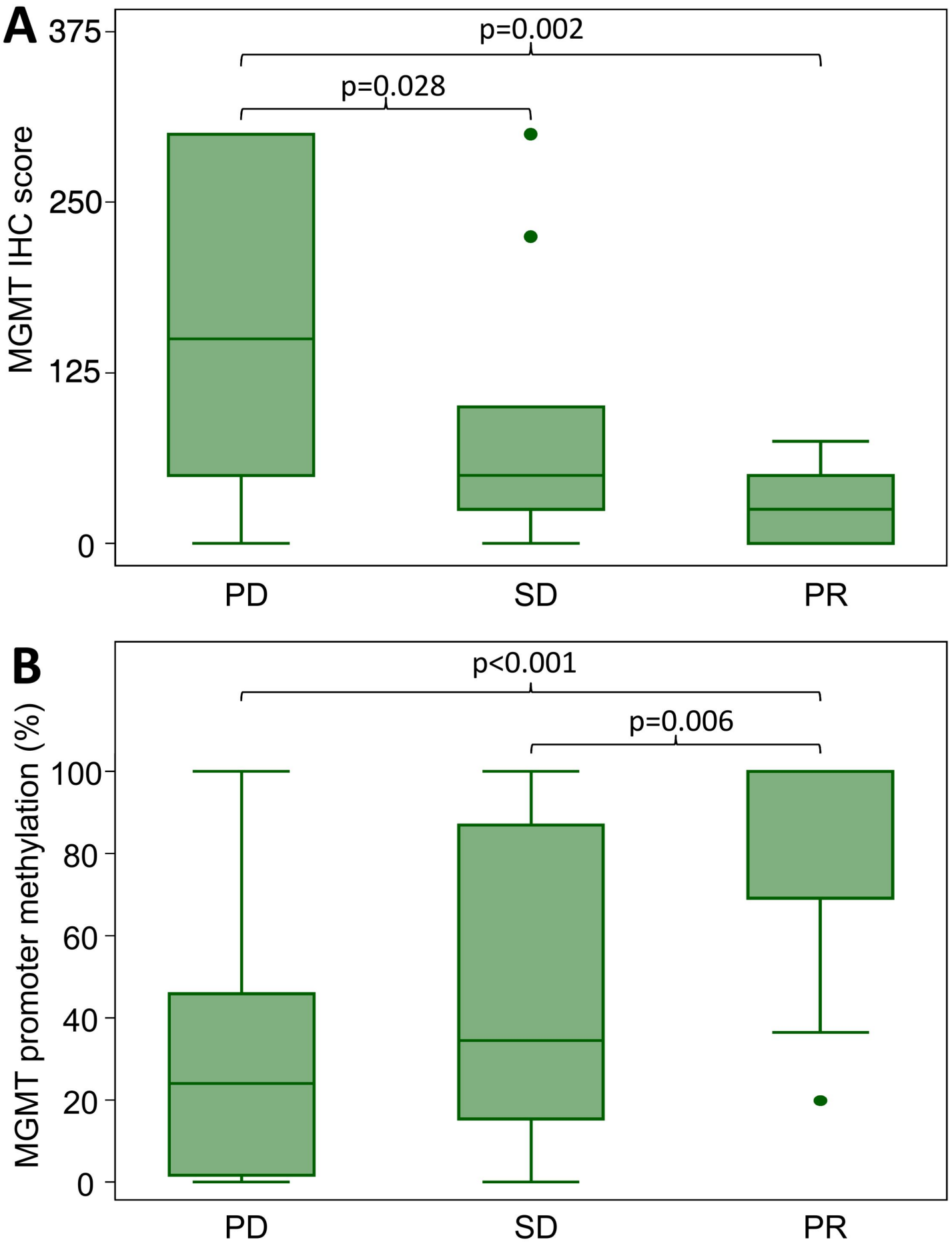
Figure A – Consort Diagram of the study. Four clinical trials involving alkylating agent in mCRC preselected for MGMT-MSP positive status were reanalyzed retrospectively with IHC and MB. DTIC: Dacarbazine; TMZ: Temozolomide; MSP: Methylation Specific PCR; IHC : immunohistochemistry; MB: methyl-BEAMing.

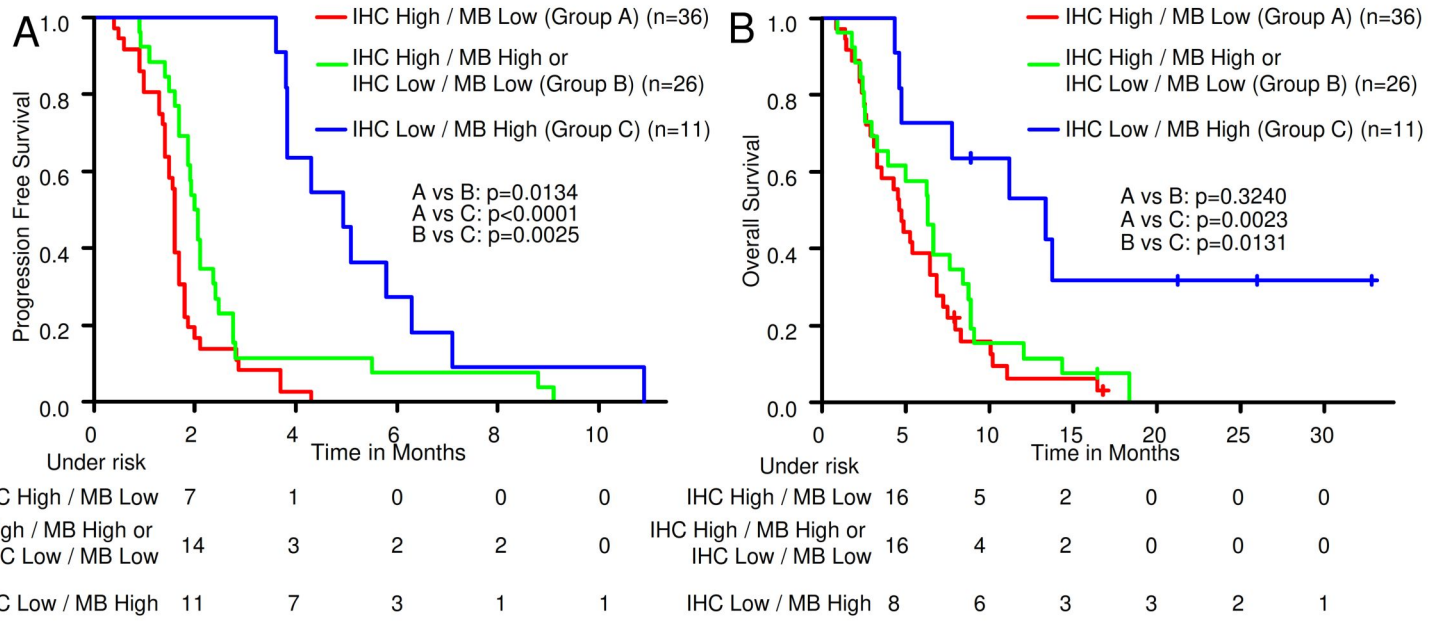
Figure B – Box plot graphic depicting distribution of immunohistochemistry MGMT score (**A.1**) and percentage of MGMT promoter methylation by methyl-BEAMing (**A2**) according to best objective response of patients studied in the pooled analysis. PD: progressive disease, SD: stable disease; PR: partial response, MB: methyl-BEAMing.

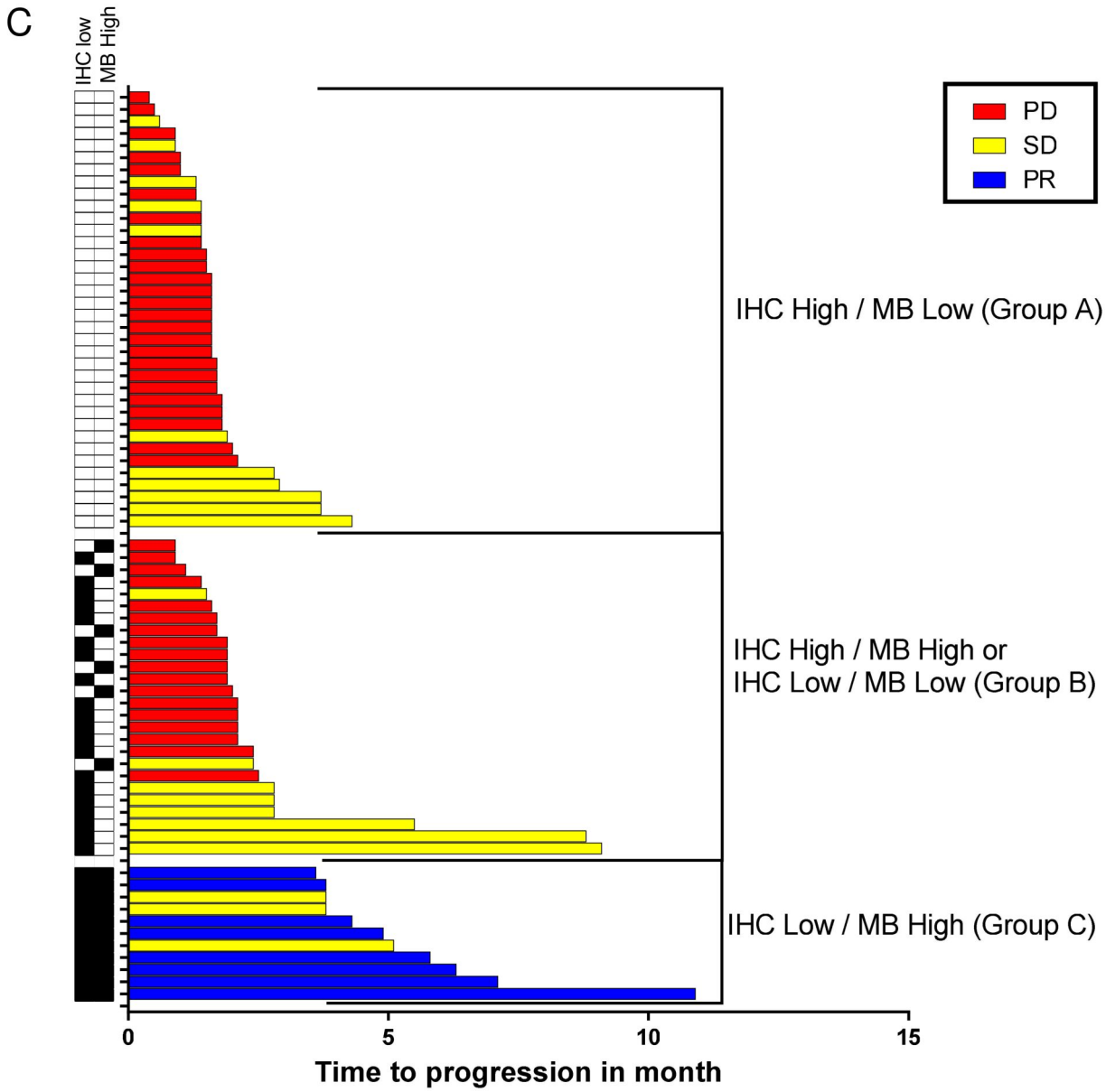
Figure C – Kaplan-Meier analysis of progression-free (**B1**) and overall (**B2**) survival according to the combined score of MGMT. (B3) Individual progression-free survival according to combined score of MGMT and best RECIST.

Supplementary Figure A – Correlation between immunohistochemistry MGMT score and percentage of MGMT promoter methylation by methyl-BEAMing.


DETECT
EUDRACT 2011-002080-21
N=68
Drug: DTIC
MGMT test: MSP
TEMECT
EUDRACT 2012-003338-17
N=29
Drug: TMZ
MGMT test: MSP/MB
INT Study
n. 20/13 #1
N=32
Drug: TMZ
MGMT test: MSP
INT Study
20/13 #2
N=32
Drug: TMZ
MGMT test: MSP/IHC
Excluded
MSP negative N= 39
MSP NA N=3
Exhausted Sample N=1
Excluded
Exhausted Sample N=9
Excluded
Exhausted Sample N=4
Current Retrospective analysis
N= 105
Excluded
Exhausted block N=30
Excluded
Bad quality DNA N=4
Tumor content NA N=3
IHC
N=75
MB
N=94
Excluded
No MB N=4
Excluded
No IHC N=23
Combined Scoring
IHC/MB
N=71


A
MGMT IHC score
375
250
125
0
p=0.002
p=0.028
PD
SD
PR
B
MGMT promoter methylation (%)
100
80
60
40
20
0
p<0.001
p=0.006
PD
SD
PR


A
Progression Free Survival
1.0
0.8
0.6
0.4
0.2
0.0
IHC High / MB Low (Group A) (n=36)
IHC High / MB High or
IHC Low / MB Low (Group B) (n=26)
IHC Low / MB High (Group C) (n=11)
A vs B: p=0.0134
A vs C: p<0.0001
B vs C: p=0.0025
0 2 4 6 8 10
Time in Months
Under risk
IHC High / MB Low 7 1 0 0 0
IHC High / MB High or IHC Low / MB Low 14 3 2 2 0
IHC Low / MB High 11 7 3 1 1
B
Overall Survival
1.0
0.8
0.6
0.4
0.2
0.0
IHC High / MB Low (Group A) (n=36)
IHC High / MB High or
IHC Low / MB Low (Group B) (n=26)
IHC Low / MB High (Group C) (n=11)
A vs B: p=0.3240
A vs C: p=0.0023
B vs C: p=0.0131
0 5 10 15 20 25 30
Time in Months
Under risk
IHC High / MB Low 16 5 2 0 0 0
IHC High / MB High or IHC Low / MB Low 16 4 2 0 0 0
IHC Low / MB High 8 6 3 3 2 1


C
IHC low
MB High
PD
SD
PR
IHC High / MB Low (Group A)
IHC High / MB High or
IHC Low / MB Low (Group B)
IHC Low / MB High (Group C)
0
5
10
15
Time to progression in month

Supplementary Table A – Results of immunohistochemistry and methyl-BEAMing analysis of MGMT

Immunohistochemistry	
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