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Original Research

Refining the selection of patients with metastatic colorectal cancer for treatment with temozolomide using proteomic analysis of O6-methylguanine-DNA-methyltransferase

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KEYWORDS
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Abstract ***Background:*** The repair enzyme O6-methylguanine-DNA-methyltransferase (MGMT) is a validated predictor of benefit from temozolomide (TMZ) in glioblastoma. However, only 10% of patients with *MGMT*-methylated metastatic colorectal cancer (mCRC) respond to TMZ.

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Temozolomide;
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Methods: Archived tumour samples (N = 41) from three phase II TMZ trials carried out in *MGMT*-methylated mCRC (assessed by methylation-specific polymerase chain reaction [PCR]) were stratified by MGMT status as assessed by three different methods: mass spectrometry, PCR/methyl-BEAMing and RNA-seq. The performance of each method was assessed in relation to overall response rate, progression-free survival (PFS) and overall survival (OS).

Results: Overall, 9 of 41 patients responded to TMZ. Overall response rates were 50% (9/18), 50% (6/12) and 35% (8/23) among patients determined likely to respond to TMZ by mass spectrometry, methyl-BEAMing and RNA-seq, respectively. Low/negative MGMT protein expressors by mass spectrometry had longer PFS than high MGMT expressors (3.7 vs 1.8 months; HR = 0.50, $P = 0.014$). Results for OS were similar but statistically non-significant (8.7 vs. 7.4 months; HR = 0.55, $P = 0.077$). No significant association between survival and MGMT status by methyl-BEAMing or RNA-seq could be demonstrated as comparable subgroups survival could not be confirmed/excluded. Specifically, the association of high versus low methyl-BEAMing MGMT hypermethylation with survival was HR = 0.783, $P = 0.46$ for PFS and 0.591, $P = 0.126$ for OS, while association of low versus high RNA-seq MGMT level with survival was HR = 0.697, $P = 0.159$ for PFS and HR = 0.697, $P = 0.266$ for OS.

Conclusions: Quantitative proteomic analysis of MGMT may be useful for refining the selection of patients eligible for salvage treatment with single-agent TMZ.

1. Introduction

Alkylating agents such as temozolomide (TMZ) are used to treat tumours since their ability to alkylate DNA causes DNA damage leading to tumour cell death. The repair enzyme O6-methylguanine-DNA-methyltransferase (MGMT) is involved in response to DNA damage caused by alkylating agents [1,2]. *MGMT* gene expression is epigenetically downregulated by hypermethylation of the promoter of CpG dinucleotides. This transcriptional silencing leads to absence of MGMT protein, thus impeding repair of chemotherapy-induced O6-alkylguanine adducts and potentially enhancing tumour susceptibility to alkylating drugs [2,3]. *MGMT* methylation status has been validated as predictor of benefit from TMZ in glioblastoma (GBM) patients [2,4–6].

MGMT silencing occurs in around 38% of colorectal carcinomas [7]. *MGMT* status as qualitatively assessed by methylation-specific polymerase chain reaction (MSP) was used to select patients with refractory metastatic colorectal cancer (mCRC) for five clinical trials of alkylating agents [8–13]. However, the activity of TMZ in heavily pre-treated mCRC patients selected by MSP is limited, with overall response rates about 3–16%. In attempts to improve the selection of mCRC patients, the predictive value of *MGMT* 'hyper'-methylation as quantitatively assessed by digital PCR/methyl-BEAMing (MB) was demonstrated [14,15]. This analysis corroborated reported discrepancies between MGMT protein expression by immunohistochemistry and alterations of matching genes in mCRC [16] and in other solid tumours [17,18].

Protein quantitation by mass spectrometry (MS) is widely considered the gold standard for biomarker measurement in biological samples [19–21]. MS-based assays can objectively quantify MGMT protein in formalin-fixed paraffin-embedded (FFPE) tumour tissues in an antibody-independent manner. Just as quantitative methylation overcomes the limitations of MSP (e.g. subjectivity of eye reading of the gel and lack of automation), MS-based protein quantitation avoids challenges inherent in immunohistochemical detection of MGMT protein such as high interobserver variability and lack of standard antibody types and scoring methods.

We hypothesised that tumour protein expression of MGMT as measured by MS would be a biomarker of resistance to TMZ and correlate with MGMT status by MB and by RNA sequencing (RNA-seq). We tested our hypothesis in the archived tumour samples of patients with refractory mCRC enrolled in three trials of TMZ [10,12,13], and we used predictive modelling to test which MGMT assessment method or their combination would most accurately identify patients responsible to TMZ.

2. Materials and methods

2.1. Patients and samples

This was a pooled analysis of archived tumour samples and clinical data from patients of three clinical trials of TMZ in refractory mCRC (EudraCT 2012-002766-13, INT 20/13 #1 and INT 20/13 #2) [10,12,13]. Patients met the following inclusion criteria: histologically confirmed mCRC; *MGMT* gene promoter methylation detected by MSP; at least one measurable lesion as defined by

Response Evaluation Criteria in Solid Tumours (RECIST) version 1.1 [22]; disease progression during or after treatment with standard chemotherapy and/or epidermal growth factor receptor (EGFR) inhibitor therapy; and Eastern Cooperative Oncology Group (ECOG) performance status $\leq$2. Patients received a standard TMZ regimen (150 mg/m^2/d for five consecutive days every 28 d) or a dose-dense regimen (75 mg/m^2/d, 21 d on/7 d off). Radiological assessments were conducted approximately every 8 weeks.

The present *post hoc* analysis included patients with an available archived tumour sample and treatment outcome data permitting evaluation of overall response rate (ORR), progression-free survival (PFS) and overall survival (OS). All samples and clinical data were anonymised, and this study was approved by the ethics committee at Fondazione IRCCS Istituto Nazionale dei Tumori of Milan in accordance with the Declaration of Helsinki. All patients had provided written informed consent to research use of their anonymised data.

2.2. Quantitative MGMT assessment

MGMT status was assessed by three methods: gene promoter methylation by MB, protein expression by MS and messenger RNA (mRNA) expression by RNA-seq. Methylation status was performed at IRCC Candiolo, Turin, Italy, as previously described [14]. Briefly, extracted and amplified DNA products from PCR were diluted and reamplified with emulsion PCR. Following emulsion breaking and hybridization, fluorescence was assessed via flow cytometry; the percentage of methylation was calculated as the ratio of the fluorescence from the methylated probe over the sum of methylated and unmethylated probe signals. MGMT hypermethylated status was defined as >63% cut-off [15].

MGMT protein was quantified with an MS assay as previously described [23]. Briefly, tumour areas of archived FFPE tissue sections were marked by a pathologist and microdissected using a non-contact laser method. The captured tumour cells were solubilised to tryptic peptides, and the total protein concentration of each tryptic peptide mixture was measured. Each sample was subjected to triplicate proteomic analysis using stable isotope-labelled internal standard peptides for quantitation of analytical targets. Proteomic expression analysis was performed with a TSQ Quantiva triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA) [24]. Data analysis was performed using Pinpoint (Version 1.3; Thermo Scientific) and Pinnacle software (Optys Tech Corporation, PA).

Patients were stratified into groups of 'low' and 'high' MGMT protein expression using a pre-specified threshold for MGMT of 200 amol/μg of total protein, based on the proteomic assay's limit of quantitation, which is determined from analyses of assay performance with respect to sensitivity and reproducibility.

RNA-seq was conducted by NantOmics as follows: RNA-seq libraries were prepared for the tumour sample using KAPA Stranded RNA-seq with RiboErase kit and sequenced on the Illumina sequencing platform. The resulting reads were aligned to RefSeq build 73 using Bowtie2 v2.2.6, Johns Hopkins University, then processed by RSEM v.1.2.25 [25] to estimate transcripts per kilobase million (TPM) and fragments per kilobase of exon per million fragments mapped (FPKM) for each isoform. Gene-level TPM and FPKM estimates are made using a weighted average of the isoform estimates, weighted by an RSEM-estimated percentage of each isoform's expression among all isoforms in the sample.

As a predetermined threshold for mRNA expression that correlates with response to TMZ has not been established, mRNA expression levels of all the cancer genome Atlas (TCGA) samples of colon and rectal cancers were plotted to find a natural break in the expression pattern that would match with the proteomic cut-off of 200 amol/μg. The distribution of MGMT TPM appeared bimodal with a natural break at 3.5 log2(TPM+1). This threshold was highly associated with the proteomic threshold (Fisher's exact test $P < 0.0008$) and was determined to be the optimal value for agreement between RNA-seq and proteomic values in Youden analysis. Expression levels of MGMT mRNA below this cut-off were considered indicative of likely response to TMZ.

Immunohistochemistry (IHC) analysis for MGMT was performed and scored as previously described [12].

2.3. Gene set analysis

In attempts to annotate the MGMT observed in this cohort with functional biological pathways or ontologies, genes significantly associated with either MGMT protein subgroups or MGMT mRNA expression were analysed using gene-set enrichment analysis. A total of five curated gene set databases were used in the analysis: KEGG, GO Molecular Functions, GO Biological Processes, BioCarta and WikiPathways. Genes associated with MGMT protein levels were identified by two-sample *t*-tests in gene expression between MGMT high ($\geq$200 amol/μg) versus MGMT low (<200 amol/μg) subgroups. As no genes were significant after Benjamini-Hochberg false discovery rate (FDR) correction, the top 50 most differentially expressed genes were studied for gene set enrichment. Genes significantly associated with MGMT mRNA expression levels were identified by correlation analysis, wherein the minimum R value for significance was 0.82 (one-sided, α = 2.6E-6, β = 0.99).

2.4. Statistical analysis

The performance of each MGMT assay was assessed using three patient endpoints: ORR according to RECIST version 1.1, PFS and OS. Cox proportional

hazard modelling and the Mantel–Cox log-rank test were used for survival comparisons. The Fisher's exact test was used to assess the relationship between MGMT status and patient response and the correlation between MGMT assessment methods.

2.5. Predictive modelling

The ability of the three MGMT assays (MS, MB and RNAseq) to predict patient response to TMZ was tested using leave-pair-out cross-validation. A predictive model was built using all samples except 2, and the model's performance was tested in one unseen positive sample and one unseen negative sample. This was repeated for all possible combinations of positive and negative samples. The average performance over all unseen test sets was the reported accuracy for a given predictive model.

3. Results

3.1. Patients and samples

Tumour samples from 41 TMZ-treated patients were available for analysis. These patients had a median age of 69 years and had received a median of three chemotherapeutic regimens prior to TMZ. Most patients had an ECOG status of 0 or 1 (85%); and at least two metastatic sites (56%), with liver as the most frequent. As expected in mCRC, all patients eventually progressed on TMZ. Twenty-six patients (63%) had progressive disease, nine had (22%) partial response and six (15%) had stable disease (Table 1).

Table 1
Characteristics of TMZ-treated patients with metastatic colorectal cancer (n = 41).

Characteristic	n (%)
Sex	
M	20 (49)
F	21 (51)
Age	
Median (range)	69 (48–85)
Clinical trial	
INT 20/13 #1	13 (27)
INT 20/13 #2	11 (32)
EudraCT 2012-002766-13	17 (41)
ECOG performance status	
0	15 (37)
1	20 (49)
2	6 (14)
RAS and BRAF mutational status	
Wild type (RAS and BRAF)	18 (44)
KRAS mutated	19 (46)
BRAF mutated	4 (10)
Primary tumour location	
Right-sided colon	18 (44)
Left-sided colon	20 (49)
Rectum	3 (7)
No. of metastatic sites	
1 metastatic site	18 (44)
≥2 metastatic sites	23 (56)
Sites of metastases	
Liver	32 (78)
Lung	24 (58)
Peritoneum	6 (15)
No. of previous treatments	
Median (range)	3 (2–5)
Objective best response rate (RECIST)	
PR	9 (22)
SD	6 (15)
PD	26 (63)

ECOG, Eastern Cooperative Oncology Group; F, female; M, male; No., number; PR, partial response; RECIST, Response Evaluation Criteria in Solid Tumours; SD, stable disease; PD, progressive disease; TMZ, temozolomide.

3.2. MGMT status

All 41 archived samples were evaluable by MS and IHC; 35 were analysed by MB, and 39 were of sufficient quality for MGMT assessment by RNA-seq (Fig. 1). Of patients assessed by MS-based proteomics, 18 (44%) had 'low' tumour expression of MGMT protein (<200 amol/μg of tumour protein) and were therefore considered likely to respond to TMZ. The remainder (n = 23) were 'high' protein expressors prone to TMZ resistance. As expected in this population of patients enriched for the study of exceptional responders, low MGMT protein expression was relatively frequent (44%); by comparison, the prevalence of low MGMT expression among all samples of CRC analysed in the authors' clinical laboratory during the past year (n = 104) was 14% (Table 2). Among MGMT 'low' subgroup, no significant association with specific clinicopathological features was observed when comparing responders versus non-responders to TMZ (data not shown).

MGMT promoter methylation above the previously validated 63% cut-off was observed in 12 (34%) patients. In the 35 tumours analysed by MB and MS, the agreement rate between methods was 77%; $P = 0.004$. Using the experimental cut-point for mRNA expression fit to the data (≤3.5 log2[TPM+1]), low mRNA expression was observed in the majority of samples (n = 23; 59%; Table 2). In the 39 patients analysed for MS and RNA-seq, the agreement rate was 77%; $P = 0.0008$.

IHC for MGMT was scored as negative in four (10%) samples, weakly positive in eight (20%) and intense positive in the remaining 29 (70%). Even if IHC and MC analyses results showed a significant correlation ($P = 0.0003$ by chi-square test), seven patients classified as MGMT negative by means of MS had intense MGMT expression by means of IHC.

3.3. Response and survival

Quantitative proteomics retrospectively identified nine of nine RECIST-defined responders to TMZ; all nine

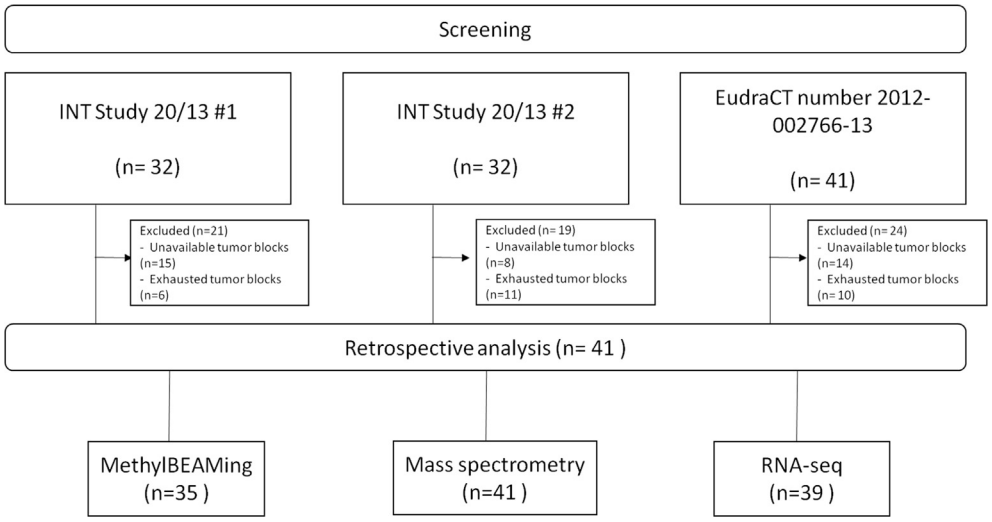

Fig. 1. Consort diagram of the translational study. Archived formalin-fixed paraffin-embedded tissue sections were obtained from patients who had received temozolomide in one of three Phase II clinical trials. Forty-one samples were evaluable by proteomics, 35 were analysed by digital MB and 39 were analysed by RNA-seq.

Table 2
Overall response rate (ORR) of TMZ-treated patients by MGMT status as assessed by three methods: mass spectrometry–based proteomics, methyl-BEAMing and RNA-seq.

Assessment method/status	n (%)	ORR	*P**
MGMT protein (N = 41)			
<200 amol/μg	18 (44)	50%	0.0001
≥200 amol/μg	23 (56)	0%	
MGMT hypermethylation (N = 35)			
>63%	12 (34)	50%	0.011
≤63%	23 (66)	9%	
MGMT RNA-seq (N = 39)			
≤3.5 log2[TPM+1]	23 (59)	35%	0.115
>3.5 log2[TPM+1]	16 (41)	6%	

MGMT, O6-methylguanine-DNA-methyltransferase; N, number; ORR, overall response rate; TPM, transcripts per million.
*Two-tailed Fisher's exact test.

responders had low MGMT protein levels by MS. Other nine patients with low MGMT protein expression did not have RECIST-defined response on TMZ (ORR of low MGMT protein: 50%). None of the patients with high MGMT protein responded to TMZ (ORR of high MGMT protein: 0%; $P = 0.0001$; Table 2; Fig. 2A).

Positive MGMT methylation status by MB retrospectively identified six of eight responders to TMZ; other six patients with positive MGMT status by MB were non-responders (ORR of *MGMT* hypermethylation: 50%). Two patients with negative methylation status responded to TMZ (ORR: 9%; $P = 0.011$; Table 2; Fig. 2B). Patients with low mRNA-expressing tumours by RNA-seq had a non-significantly higher ORR than higher mRNA expressors (35% vs 6%; $P = 0.115$; Table 2; Fig. 2C).

In survival analyses, patients with low MGMT protein levels (<200 amol/μg) had longer median PFS (mPFS) than patients with high MGMT levels (3.7 vs 1.8 months; $P = 0.014$; Fig. 3A). MGMT levels remained a statistically significant predictor of PFS when paired with other prognostic factors in Cox proportional hazards models; no other variable tested was more explanatory than MGMT protein level (Table 3). Differences in OS by MGMT protein level were similar to PFS differences but did not reach statistical significance (8.7 vs 7.4 months, hazard ratio [HR] = 0.55, $P = 0.077$; Fig. 3B). There were no statistically significant differences in PFS or OS among patients stratified by MB or RNA-seq (Fig. 3C–F).

3.4. Gene set analysis

No statistically significant differential expression (*t*-test) was observed in the 19,270 genes analysed between responders and non-responders to TMZ, after correcting for false discovery rate (using Benjamini-Hochberg adjustment). Even when focusing analysis on genes likely related to TMZ processing (i.e. the DNA damage repair pathway), none was significantly differentially expressed (q-values range 0.85–0.94).

Similarly, no genes were found to be significantly differentially expressed between MS-defined MGMT subgroups nor between RNA-seq–defined MGMT subgroups.

In lieu of statistically significant differences, the 50 most differentially expressed genes between subgroups were analysed for gene set enrichment (Supplementary Table 1). Within response- and MS-defined subgroups, there was no significant association with any process related to drug response or DNA damage response. Within the gene sets most differentially expressed between MGMT high and low by RNA-seq, the top gene sets enriched were related to drug metabolism and chemical carcinogenesis (Supplementary Table 2).

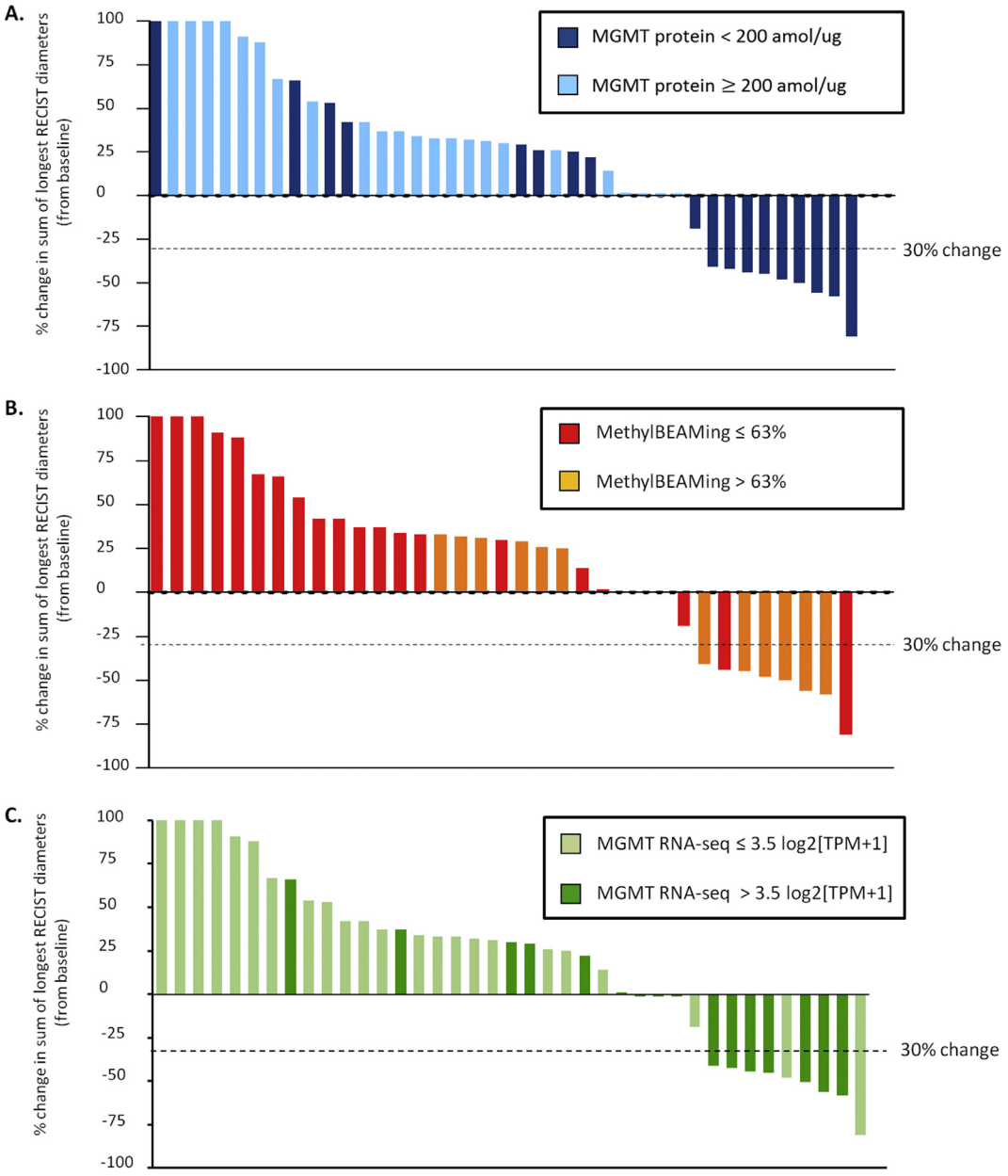

Fig. 2. Percent change in sum of the longest RECIST diameters (from baseline) among temozolomide-treated patients by (n = 41) (A) MGMT protein status, (n = 35) (B) *MGMT* promoter hypermethylation status and (n = 39) (C) by MGMT RNA level. RECIST, Response Evaluation Criteria in Solid Tumours; MGMT, O6-methylguanine-DNA-methyltransferase.

While no genes were significantly differentially expressed between pre-defined subgroups, nine genes were found to be significantly correlated with MGMT mRNA expression values (Supplementary Table 3). However, these nine genes were not significantly associated with any process involving DNA damage response.

No significant mRNA correlations were observed with MGMT protein levels in MS.

3.5. Predictive modelling

Predictive models of TMZ response (ORR) were built using each of the MGMT assays (MS, MB and RNA-seq) and for combinations. These models were run using their established cut-offs as well as raw, continuous MGMT values with various experimental cut-offs. The predefined cut-offs in MS protein and RNA-seq expression values were defined *a priori* by observing the limit of detection of the assay and by identifying the midpoint in a clearly bimodal distribution in TCGA COADREAD MGMT expression data, respectively. Ten datasets and 14 classification algorithms combined into 140 different modelling strategies, evaluating the predictive performance of these strategies in unseen samples required building an additional 2772 unique predictive submodels. The best modelling strategy included all three MGMT assessment methods (MB, MS and RNA-seq) with their

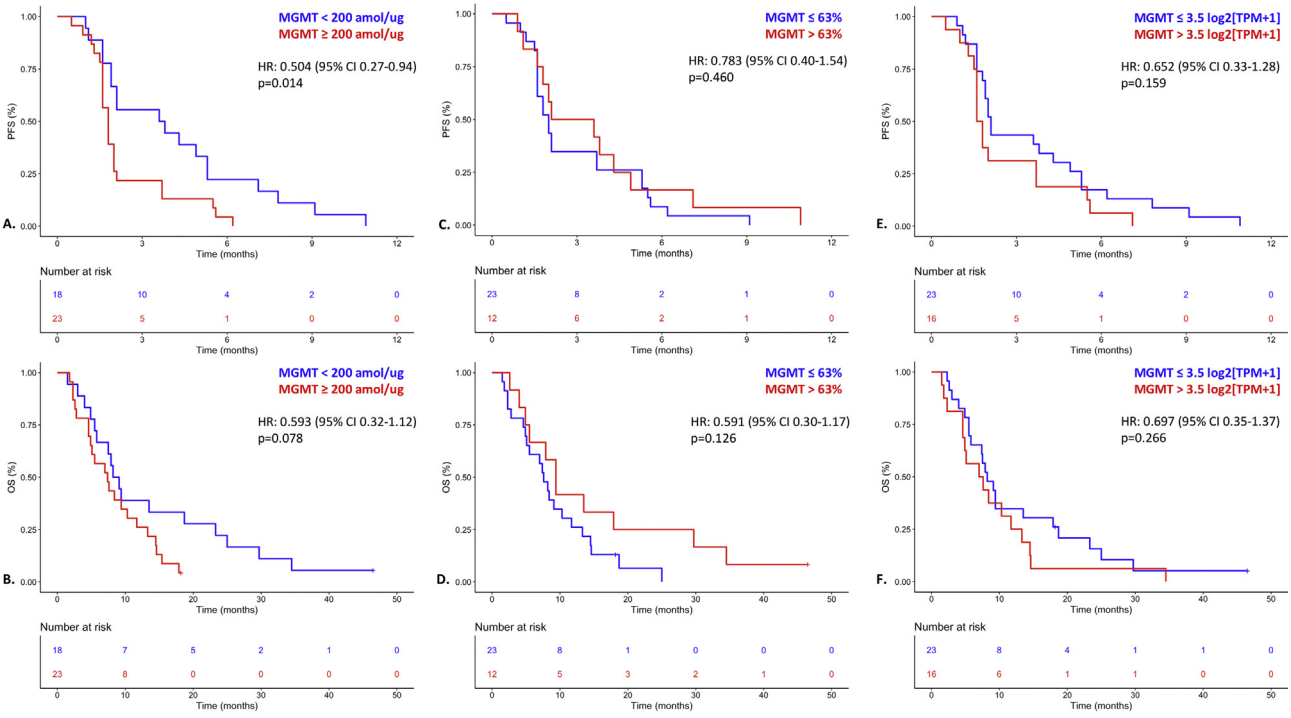

Fig. 3. PFS (A) and OS (B) of temozolomide-treated patients with metastatic colorectal cancer, by MGMT protein expression level, PFS (C) and OS (D) stratified by MGMT methylation status and PFS (E) and OS (F), by MGMT mRNA level (RNA-seq). CI, confidence interval; HR, hazard ratio; MGMT, O6-methylguanine-DNA-methyltransferase; OS, overall survival; PFS, progression-free survival.

Table 3
Analysing potential confounders to MGMT association with PFS.

Features		Confounder		MGMT	
		HR (95% CI)	*P* value	HR (95% CI)	*P* value
BRAF mutation	Yes vs no	2.26 (0.78–6.58)	0.137	0.44 (0.22–0.86)	0.018
RAS mutation	Yes vs no	0.66 (0.35–1.25)	0.203	0.44 (0.23–0.88)	0.020
Gender	M vs F	1.27 (0.66–2.44)	0.424	0.46 (0.23–0.93)	0.032
ECOG	1–2 vs 0	1.39 (0.85–2.27)	0.202	0.48 (0.24–0.95)	0.038
Number of previous treatment	4 vs 2[a]	0.97 (0.70–1.34)	0.910	0.40 (0.19–0.83)	0.014
LDH baseline level (U/L)	521 vs 244[a]	1.07 (1.01–1.12)[b]	0.017	0.35 (0.17–0.72)	0.005
Number of metastatic sites	≥2 vs 1	1.57 (0.96–2.56)	0.083	0.36 (0.17–0.74)	0.004
Neutrophil/lymphocyte at baseline	5.2 vs 2.7[a]	1.04 (0.97–1.11)	0.149	0.44 (0.22–0.88)	0.046
Peritoneal disease	Yes vs no	1.58 (0.73–3.40)	0.219	0.83 (0.20–0.84)	0.012
Primary tumour location	Right vs left	1.36 (0.68–2.74)	0.381	0.45 (0.23–0.92)	0.026
Site of the archived tissue	INT 20/13 #1-#2 vs EudraCT 2012-002766-13	0.81 (0.42–1.56)	0.631	0.41 (0.21–0.83)	0.019
Age (years)	73 vs 59[a]	1.01 (0.73–1.38)[c]	0.855	0.43 (0.22–0.86)	0.022

CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; HR, hazard ratio; LDH, lactate dehydrogenase; MGMT, O6-methylguanine-DNA-methyltransferase.
The *P* values are associated with the explanatory coefficient for each potential confounder in the presence of MS MGMT status in a bivariate Cox-proportional hazard model for PFS. Age, number of previous treatments, neutrophil/lymphocyte at baseline and LDH were explored as continuous variables. Age is defined as the years elapsed between birth and date at histological diagnosis. The table is sorted in order of the clinical likelihood of each variable to be a confounder.
[a] The reported values are the third and first quartiles of the variable distribution.
[b] HR per 50 U/L increase in LDH.
[c] HR per 10 years increase in age.

established cut-offs. This model was 87% accurate in predicting TMZ response in unseen samples and performed better than that using MGMT protein quantity alone (80% accurate; Fig. 4, Supplementary Table 4). For each of the three assessment methods, experimental MGMT cut-offs (optimised using leave-out-pair cross-validation) did not perform better than the predefined cut-offs (Fig. 5, Supplementary Table 5).

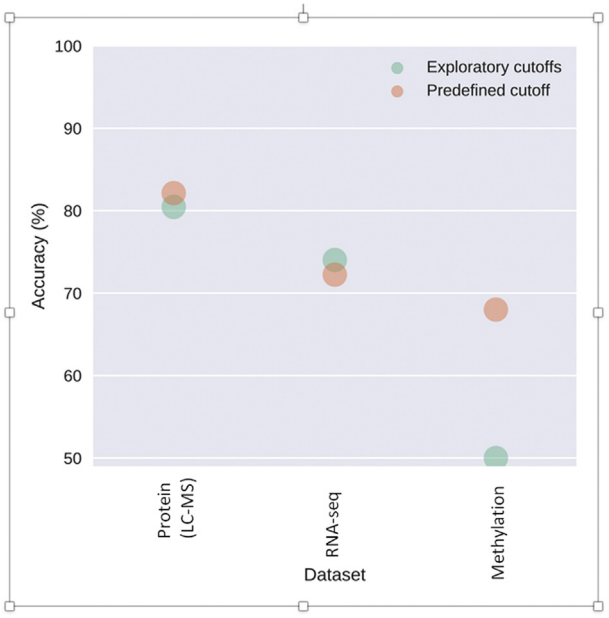

Fig. 4. Average accuracy of predictive models per leave-pair-out cross-validation. Two classification strategies were employed: predefined cut-offs and exploratory cut-offs determined as optimum in a training set. Predefined and exploratory cut-offs were assessed in the exact same training and testing sets for direct comparison.

4. Discussion

In this *post-hoc* pooled analysis of three phase II trials in refractory mCRC patients receiving TMZ, a proteomic test for MGMT protein had a 100% sensitivity and a 50% specificity when using clinical response as the gold standard. Although the sample size was too small to reach definitive conclusions, the proteomic test seemed to outperform both digital MB and RNA-seq in predicting response to TMZ. MGMT protein expression below a predefined threshold was significantly associated with longer mPFS, independently from other prognostic variables. Regarding MB and RNA-seq tests, no significant association with survival could be demonstrated since a comparable survival of subgroups could not be confirmed or excluded, possibly for the limited study power. Patients with high MGMT protein expression had similar PFS to that reported for mCRC patients in clinical trials of TMZ. Therefore, the disappointing results of such trials may reflect the limited ability of standard *MGMT* assessment methods (e.g. MSP) to select the optimal candidates for TMZ.

In order to develop a hypothetical MGMT assay with maximal accuracy in identifying responders to TMZ (ORR), a predictive model was built using three different MGMT assays and their combinations. A

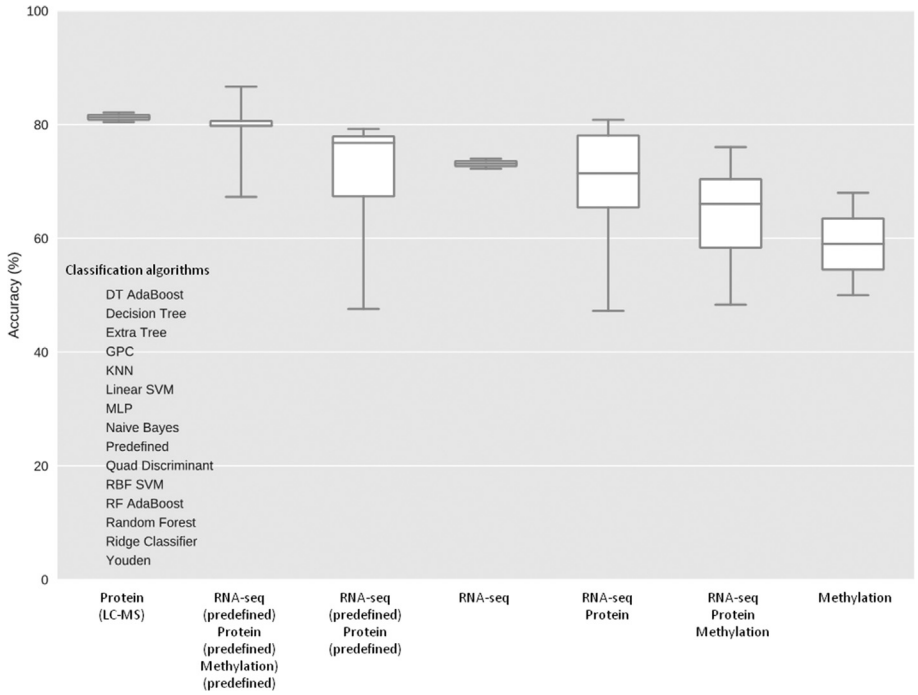

Fig. 5. Average predictive accuracy in unseen samples for 58 predictive modelling strategies by MGMT assessment method group. Groups are ordered left-to-right by average accuracy. Groups labelled 'predefined' are discretised by their predefined cut-offs prior to predictive modelling. Each point represents a different predictive modelling strategy (i.e. combination of MGMT assessment method group and classification algorithm). Univariate datasets were analysed using only Youden analysis and predefined cut-offs. Multivariate datasets were used as input for all other classification algorithms shown. Although prediction strategies that use all three MGMT assessment methods outperformed the univariate proteomic cut-off, the accuracy in the proteomic data is the most robust (lowest data dispersion) in this small cohort. MGMT, O6-methylguanine-DNA-methyltransferase.

combination of all three MGMT assessment methods was 87% accurate versus 80% accuracy using the proteomic test alone. Modelling confirmed that the thresholds for MGMT expression and MGMT methylation used to stratify patients in this study were more robust than other exploratory thresholds. These results point to the potential clinical value of MGMT protein quantitation, either alone or in combination with other methods.

MS technique is also valuable for two reasons: first it seems to outperform IHC since the specificity of IHC may not be sufficient to categorise MGMT negativity. Second, MS may allow the selective detection of the active form of MGMT protein. In fact, the alkylated inactive form of MGMT is rapidly cleared by ubiquitin-mediated proteasomal degradation following conformational changes.

Concerning the discrepancies between RNA and MS, most of the discordant cases demonstrated a silencing at the RNA level, while protein was found at high level. This could be explained by a slow turnover of the protein in absence of DNA damage. In fact, in absence of DNA alkylation, the cells might switch off the transcription of MGMT, which will not affect the protein level already available. Additional process of transcription regulation might also be involved, such as deregulation of UBR1, a protein ligase E3 proved to affect MGMT transcription level [26].

The importance of identifying potential responders to TMZ is emphasised by recently published findings of impairment of DNA mismatch repair or hypermutated status after the emergence of acquired resistance to TMZ in mCRC patients, thus becoming potentially eligible for immune checkpoint inhibitors [27]. Studies in microsatellite stable mCRC are investigating the optimal duration of TMZ therapy prior to tumour mutational burden (TMB) testing, as well as 'priming' treatment with TMZ followed, at the time of TMB high associated disease progression by sequential PD-1 blockade (ARETHUSA trial; clinicaltrials.gov identifier NCT03519412) or short-term induction treatment with TMZ followed in the absence of disease progression by its combination with CTLA-4 plus PD-1 blockade (MAYA trial). In parallel, our recent work suggested a potential synergy between TMZ and other active agents commonly used in mCRC, such as irinotecan (TEMIRI regimen), with novel translational data regarding molecular selection at both gene and protein levels [28].

Methylation-mediated silencing of MGMT has been reported in 38% of mCRC [7], and the frequency of low MGMT protein expression in our study is similarly encouraging. Of 104 samples of CRC analysed in our clinical laboratory during the past year, 15 (14%) underexpressed MGMT protein thus likely to respond to TMZ, and this percentage is similar to response rates to alkylating agents in refractory mCRC [8–13].

In this study, about a half of patients with low MGMT expression failed to respond to TMZ, suggesting a role for other factors such as DNA damage repair, cell cycle and immune profile. Indeed, other transcriptional and post-transcriptional processes might be involved in *MGMT* expression in CRC [29,30]. An analysis of 70 genes with known involvement in DNA damage repair and immune-mediated response failed to find differential gene expression in MGMT subgroups. Future studies may identify genetic signatures that could further refine predictions of response to TMZ.

Furthermore, our study has potential relevance not only for mCRC patients since alkylating agents are part of the treatment management of several tumours, such as sarcoma, triple negative breast cancer and mainly GBM, and evidence has been collected regarding the predictive role of MGMT methylation for tumour response [5,31,32]. In the setting of GBM, where TMZ is one of the cornerstones of treatment, MGMT status is generally assessed through MSP promoter methylation analysis, but this method showed to be unable to accurately select patients responsive to the treatment [33]. Detection of MGMT mRNA is a potential alternative to guide patients' selection since it could identify cases with MGMT deregulation induced by mechanisms independent from MGMT methylation. It was evidenced that in cases with discordant results, RNA levels displayed a higher correlation with response to TMZ: patients with low methylated tumours showing low RNA levels had an improved outcome, whereas patients with methylated MGMT but high RNA levels had a worse prognosis than those with reduced RNA expression [34].

This study has some clear limitations. The absence of a control group treated without TMZ leaves open the possibility that the investigated biomarkers may be prognostic rather than predictive. Moreover, there is evidence that MGMT status may change during the course of disease [8], limiting the reliability of data from tumour tissue obtained at the time of diagnosis. Finally, the absence of prospective validation of our results limits their current use outside a research setting. Of note, a phase II trial (NCT02414009) led by our group is currently enrolling patients with *MGMT*-methylated – as assessed by MSP – and *RAS*-mutated mCRC, who failed a previous oxaliplatin-based treatment, randomly allocated to either second-line FOLFIRI regimen or capecitabine plus TMZ (CAPTEM regimen). This trial has almost concluded its target enrolment and will give us the chance to validate the potential predictive utility of our MGMT-centred panel of biomarkers.

Despite these limitations, the results of this preliminary study support the ability of a proteomic MGMT assay to refine the selection of TMZ responders and suggest that quantitated MGMT protein may be a useful biomarker in clinical settings.

Conflict of interest statement

S.S. is a former employee of NantOmics. C.S., F.C., Y.T., S.B. and T.H. are employees of NantOmics. F.P. has received consultant/advisory board fees from Roche, Amgen, Eli Lilly, Sanofi, Merck-Serono and Bayer. M.D.B. has received consultant/advisory board fees from Eli Lilly. F.D.B. has received consultant/advisory board fees from Roche, Novartis, Amgen and Celgene. The remaining authors declare no conflict of interest.

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Availability of data and material

Data supporting the results of this article are available from the authors upon request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2018.11.016.

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