Cancer Treatment Reviews 82 (2020) 101935

Contents lists available at ScienceDirect

Cancer Treatment Reviews

journal homepage: www.elsevier.com/locate/ctrv

Anti-Tumour Treatment

Biomarker-guided implementation of the old drug temozolomide as a novel treatment option for patients with metastatic colorectal cancer

Filippo Pietrantonio[a,b,*], Giovanni Randon[b], Dario Romagnoli[c], Samantha Di Donato[d], Matteo Benelli[c], Filippo de Braud[a,b]

[a] Oncology and Hemato-oncology Department, University of Milan, via Festa del Perdono, 7, 20122 Milan, Italy
[b] Department of Medical Oncology, Fondazione IRCCS Istituto Nazionale dei Tumori, via Giacomo Venezian 1, 20133 Milan, Italy
[c] Bioinformatics Unit, Oncology Department, Nuovo Ospedale-Santo Stefano, Via Suor Niccolina Infermiera 20, 59100 Prato, Italy
[d] Medical Oncology Department, Nuovo Ospedale-Santo Stefano, Via Suor Niccolina Infermiera 20, 59100 Prato, Italy

ARTICLE INFO

Keywords:
Temozolomide
Metastatic colorectal cancer
MGMT, Mismatch repair, Base excision repair
Immune checkpoint inhibitors

ABSTRACT

Temozolomide is an oral alkylating agent used for treating several cancers including glioblastoma and melanoma. Promising, albeit limited, activity and efficacy of temozolomide have been reported in pretreated patients with metastatic colorectal cancer bearing *MGMT* promoter methylation. MGMT silencing and proficiency of the mismatch repair system were considered the major predictive biomarkers of sensitivity to temozolomide. Refinement of established biomarkers and integration with those related to alteration in specific DNA-damage response pathways such as base excision repair are promising strategies for selecting metastatic colorectal patients to this old drug with several potential novel applications. Then, mounting preclinical and clinical observations have linked acquired resistance to temozolomide to emergence of alterations in the mismatch repair system. Whilst accounting for tumor cells capability of escaping apoptosis when exposed to temozolomide, inactivation of key mismatch-repair proteins will ultimately lead to increasing tumor mutational burden. This drug-induced mismatch deficient-like phenotype is being exploited in proof-of-concept trials combining temozolomide and immune checkpoint inhibitors in metastatic colorectal cancer.

MGMT promoter methylation as predictive biomarker of treatment with alkylating agents in metastatic colorectal cancer

In patients with metastatic colorectal cancer (mCRC), the occurrence of chemo-refractory disease and the failure of guidelines-recommended standard treatments pose a major challenge, at least when an adequate performance status would allow the potential administration of further treatment lines. In the era of personalized medicine, tumor molecular profiling may lead to the identification of therapeutic targets that may represent predictive biomarkers for pharmacological intervention. The DNA repair gene O6-methylguanine-DNA methyltransferase (*MGMT*) is responsible for the elimination of alkyl groups from the O6-position of guanine (Fig. 1). MGMT inactivation due to gene promoter hypermethylation may be involved in the early steps of colorectal tumorigenesis accounting for an increase of G-to-A point mutations of cancer-associated genes (e.g. in *KRAS* and *TP53*)[1]. *MGMT* promoter hypermethylation is observed in up to 40% of colorectal cancers and might confer enhanced sensitivity to alkylating agents such as methyltriazen-imidazole-carboximide prodrugs dacarbazine and temozolomide, as previously shown for patients with glioblastoma and advanced melanoma - for whom temozolomide represents a standard option [2–4]. Following the initial case report of two patients with refractory mCRC and tumor loss of MGMT who achieved an exceptional response to single-agent temozolomide [5], several phase II non-randomized studies were started with the aim of assessing the activity of dacarbazine and temozolomide after failure of the standard treatment options. In the initial trial with dacarbazine, responses according to RECIST criteria were observed only in 2 out of 68 patients, although all responders had tumors bearing *MGMT* promoter methylation [6]. Therefore, even if the trial failed to meet its primary endpoint of overall response rate (ORR), the biological rationale linking *MGMT* silencing with clinical benefit was considered strong enough to perform the subsequent temozolomide monotherapy trials in patients with mCRC who were molecularly selected for the presence of *MGMT* methylation [7–11]. In all these trials, methylation-specific PCR (MSP) was adopted as the assay for qualitatively detecting the presence or absence of

* Corresponding author at: Oncology and Hemato-oncology Department, University of Milan, via Festa del Perdono, 7, 20122 Milan, Italy and Department of Medical Oncology, Fondazione IRCCS Istituto Nazionale dei Tumori, via Giacomo Venezian 1, 20133 Milan, Italy.
E-mail address: filippo.pietrantonio@istitutotumori.mi.it (F. Pietrantonio).

https://doi.org/10.1016/j.ctrv.2019.101935
Received 5 October 2019; Received in revised form 21 November 2019; Accepted 22 November 2019

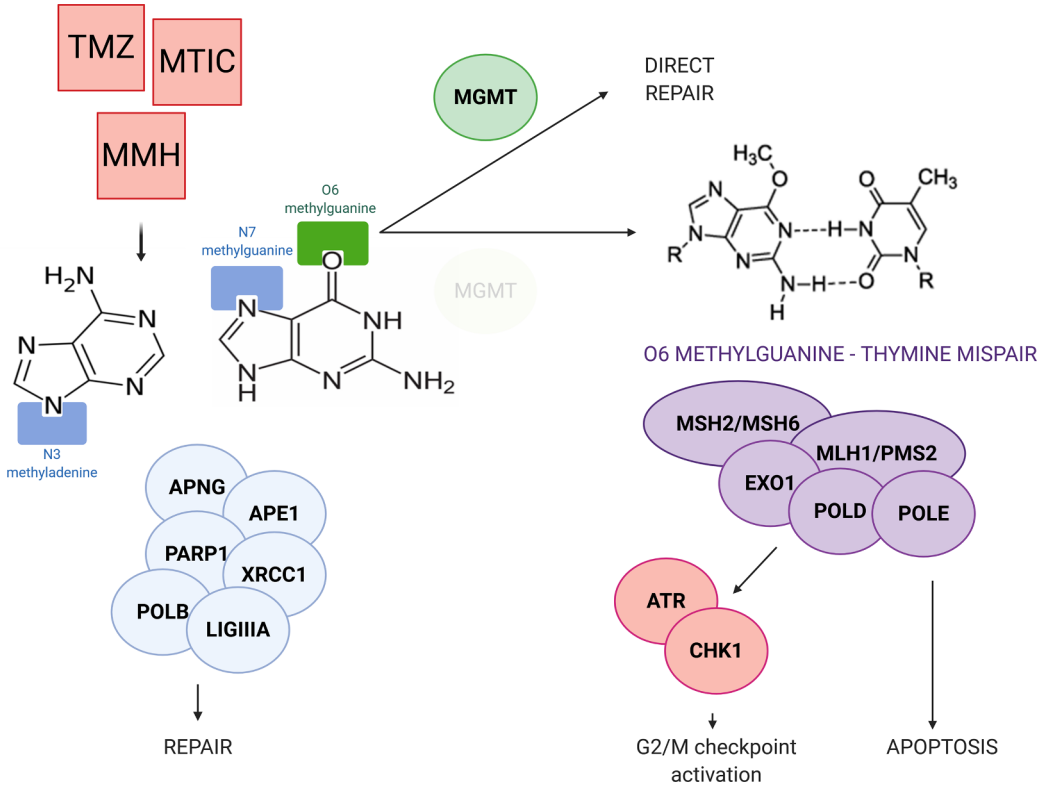

Fig. 1. DNA repair pathways involved in processing major temozolomide-induced alkylations. Methyl group in O6 atom of guanine is directly removed by MGMT; in contexts of MGMT deficiency O6 methylguanine is mispaired with thymine and processed by the mismatch repair system. Persistent activation of mismatch repair (MMR) in face of O6-methylguanine and thymine mispairs is implicated in apoptotic cell death. Reliance upon functional MMR for inducing apoptosis after alkylation-induced DNA damage is explained by different models. In the futile cycle model, an apoptotic response may be triggered by insurmountable DNA damage after continued MMR processing of the O6methylguanine-thymine mispairs. According to the direct signaling model, ATR is activated by the MuTSα complex, thus initiating the G2/M cell-cycle checkpoint pathway. Methyl groups in N3 position of adenine and N7 position of guanine are processed by base excision repair proteins for repair. **Abbreviations.** MTIC: methyl-triazen-imidazole-carboxamide. MMH: monomethylhydrazine. TMZ: temozolomide.

Table 1
Phase II trials on alkylating agents dacarbazine and temozolomide in metastatic colorectal cancer.

	Schedule	*MGMT* methylation assessment assay	N	ORR N (%)	DCR N (%)	mPFS (months)	mOS (months)	Non hematological toxicity (% G3-G4)	Hematological toxicity (% G3-G4)	Ref.
Amatu 2013[a]	DTIC 250 mg/sqm IV d1-d4, q21	MSP	26	2 (8)	11 (44)	–	–	14	10	[6]
Hochauser 2013[b]	TMZ 150 mg/sqm PO 7 d on/7 d off, q28	MSP	37	1 (3)	16 (43)	–	–	–	–	[7]
Pietrantonio 2014	TMZ 150 mg/sqm PO d1-d5, q28	MSP	32	4 (12)	10 (31)	1.8	8.4	0	3	[8]
Pietrantonio 2016	TMZ 75 mg/sqm PO d1-21, q28	MSP; IHC (exploratory)	32	5 (16)	7 (21)	2.3	6.7	9	0	[9]
Amatu 2016	TMZ 200 mg/sqm PO d1-5, q28	MSP; MB (exploratory)	29	1 (3)	14 (48)	2.6	6.2	24	41	[10]
Calegari 2017	TMZ 200 mg/sqmPO d1-5, q28	MSP	41	4 (10)	13 (31)	1.9	5.1	43	21	[11]

Abbreviations. DTIC: dacarbazine. IHC: immunohistochemistry. IV: intravenous. MB: methyl-BEAMing. mOS: median overall survival. mPFS: median progression-free survival. MSP: methylation-specific PCR. ORR: overall response rate. PO: per-os. TMZ: temozolomide.

[a] Here reported data about patients with *MGMT* promoter methylated tumors.

[b] Here reported data about patients with metastatic colorectal cancer included in the trial.

MGMT methylation, as previously validated for patients with glioblastoma [12]. The summary of all 6 monocenter trials is shown in Table 1. The mean ORR to single agent temozolomide in *MGMT* methylated refractory mCRC patients selected by MSP is about 9%. Based on the modest activity of temozolomide in these heavily pretreated patient populations, the median progression-free survival (PFS)

and overall survival (OS) results were overall poor. However, these results clearly suggested that the molecular selection for temozolomide-based treatment based on MSP is necessary but not sufficient and, from a precision medicine point of view, temozolomide cannot represent a new bullet for the treatment of all patients with *MGMT* methylated mCRC, but only for a relatively small subgroup of them.

Currently, regorafenib and trifluridine/tipiracil represent two approved standard treatments used from the third line setting and beyond in patients with molecularly unselected mCRC. The approval of the two drugs was based on the positive results of several phase III placebo-controlled trials, which led to demonstrate a median OS gain of 1.4 and 1.8 months for regorafenib and trifluridine/tipiracil, respectively, in Western studies [13,14], and 1.8 and 0.7 months, respectively, in Asian ones [15,16]. The OS benefit achieved by these drugs in trial patients was therefore quite limited, albeit statistically significant, whereas their costs and toxicity burden are particularly relevant in the clinical practice, especially if considering the extremely palliative goal of treatments used in the third line setting and beyond. Finally, the ORR according to RECIST was negligible - only about 1% - in trials with both regorafenib and trifluridine/tipiracil, therefore not allowing to potentially use such agents in the clinical practice in order to reduce the tumor burden and potentially improve the health status and quality of life of patients with poor performance status. Even if the use of temozolomide is not supported by level I evidences and phase III trials, such agent may offer several potential advantages compared to the available standard treatment options: 1) significantly lower cost; 2) manageable safety profile; 3) the chance to achieve tumor response; 4) the chance to investigate DNA damage response (DDR)-related biomarkers that may be potentially implemented during the design of clinical trials, whereas no biomarkers have been so far considered as sufficiently promising to predict the benefit from both regorafenib and trifluridine/tipiracil. Besides being a candidate “targeted” chemotherapy agent, temozolomide shares with regorafenib and trifluridine/tipiracil the advantage of oral administration and is safe when used both at the standard schedule (150 mg/sqm days 1–5 q28) and a dose-dense one (75 mg/sqm days 1–21 q28). The only dose-limiting toxicity of temozolomide is myelosuppression, and its safety profile may be favorable when indirectly compared to regorafenib and even the relatively well tolerated agent trifluridine/tipiracil. However, it should be pointed out that several promising strategies are emerging as breakthrough treatments for specific molecular subgroups of patients with mCRC, such as BRAF +/- MEK inhibitors added to anti-EGFR agents for patients with BRAF V600E mutated [17], immunotherapy for MSI-high [18], dual HER2 blockade for HER2-positive [19], entrectinib or larotrectinib for *ALK*, *ROS1* or *NTRK1-3* fusion positive [20,21], and RET selective inhibitors such as BLU-667 and LOXO-292 for *RET* fusion positive disease [22]. However, except for BRAF V600E mutations (observed in about 8% of all patients with mCRC [23], the individual frequency of all the above-mentioned predictive biomarkers is below 1–4% in the metastatic setting. Therefore, most patients with mCRC are not currently eligible for immunotherapy or novel targeted strategies, highlighting the urgent need of exploring treatment options that may be effective in pretreated patients, particularly those with *RAS* mutated disease.

Improving the efficacy of temozolomide in patients with metastatic colorectal cancer: combination strategies

An improvement of the efficacy of temozolomide may be expected from its use in earlier treatment lines and its combination with other agents with known activity in patients with mCRC. Considering this rationale, we ran an open-label, phase II non-randomized study on the combination of temozolomide plus irinotecan (TEMIRI), which showed promising and higher than expected activity [24]. Key inclusion criteria were: failure of at least two prior treatment lines including at least one prior irinotecan-based regimen, irinotecan-sensitive disease based on presence of an irinotecan-free interval (time from irinotecan last dose and disease progression) of 3 months or greater, presence of tumor *MGMT* methylation assessed by MSP and mismatch repair (MMR) proficiency. TEMIRI regimen achieved an ORR of 24%, a median PFS and OS of 4.4 and 13.8 months, respectively, in patients that had been carefully selected by combined clinical and molecular criteria, despite 68% of them had received at least three previous treatment lines. Retained sensitivity to irinotecan might have contributed to the high activity of the combination, as reported also in the setting of anti-EGFR re-treatments [25]. However, given the limited activity of both temozolomide and irinotecan as single-agents in pretreated patients [26], our results suggested that the two drugs may have synergistic rather than additive effects. Such synergistic effect may be partially independent from MGMT and MMR status [27], since O6 methylated guanine is expected to induce a topoisomerase 1 cleavage complex and poison topoisomerase 1 enzyme [28], ultimately leading to DNA replication-mediated double strand breaks [29].

Additionally, the combination of fluoropyrimidines and temozolomide relies on in-vitro evidence of schedule-dependent synergism between 5-fluorouracil (5FU) and temozolomide [30]. Depletion of deoxythymidine triphosphate by the capecitabine metabolite fluorodeoxyuridine monophosphate would leave unmatched O6 methylguanine resulting in DNA double-strand breaks in S-phase. Moreover, thymidylate synthase inhibition by capecitabine results in deoxynucleoside triphosphate imbalance and mutagenicity due to 5FU:-Guanine (G) mismatched nucleotides; 5FU:G mismatched nucleotides are substrates for MMR network and their processing leads to apoptosis; a similar DNA damage response is evoked upon temozolomide induced O6 methylguanine-thymine mispairs [31]. Based on these preclinical data and reinforcing the use of temozolomide as a combination treatment in earlier lines, we recently reported the results of a multicenter, open-label, randomized phase II study aimed at investigating the efficacy of capecitabine and temozolomide (CAPTEM) regimen versus FOLFIRI after failure of a prior first-line oxaliplatin-based treatment in patients with *MGMT* methylated and *RAS* mutated mCRC [32]. This trial enrolled 86 patients that were randomized on 1:1 basis to receive second-line treatment with either intravenous biweekly FOLFIRI regimen (irinotecan, leucovorin and fluorouracil at standard doses) or fully oral four-weekly CAPTEM regimen (temozolomide for 5 days at 75 mg/sqm bid on days 10–14 and capecitabine 750 mg/sqm bid on days 1–14 of a 28-day cycle) until disease progression or unacceptable toxicity. Even if the trial failed to meet its primary endpoint of PFS superiority of CAPTEM over FOLFIRI, activity and efficacy outcomes of the two regimens were super-imposable (11.6% overall response rate and 3.5 months median PFS in both arms, 9.5 versus 10.6 months in CAPTEM and FOLFIRI arm, respectively). Moreover, the fully oral CAPTEM combination was associated with a better safety profile and quality of life, potentially leading to improved treatment compliance. When putting these results in the context of the current treatment landscape, we acknowledge that several effective combinations of chemotherapy plus biological agents are recommended in the second-line. Therefore, since our second-line randomized trial was negative and failed to show superiority of CAPTEM compared to standard FOLFIRI, the most reasonable setting for further investigating the role of temozolomide may be from the third setting and beyond, ideally based on the simultaneous implementation of predictive biomarkers during the trial design.

Refining the selection of patients for temozolomide treatment thanks to microsatellite instability testing and comprehensive MGMT assessment

MMR proteins are involved in processing and repair of single nucleotide misincorporations, as well as small insertion/deletion (indel) loops. MSH2-MSH6 (MutSα) heterodimer recognizes single mismatches and small indels, whereas MSH2-MSH3 (MutSβ) the larger indels. Then MLH1/PMS2 (MutLα) complex is recruited thus allowing recruitment

of the exonuclease EXO1, DNA polymerases POLE/POLD and DNA ligases for excision of error-containing strand, DNA re-synthesis and ligation [33]. Reliance upon functional MMR for inducing apoptosis after alkylation-induced DNA damage is explained by different models, including the futile cycle model and a direct signaling model [34,35]. Namely MMR proficiency has been linked to sensitivity to several DNA damaging agents, including temozolomide [36] and MMR deficient cells display intrinsic resistance to several alkylating agents [37]. Therefore, even if MSI-high status is found only in 4% of patients with metastatic disease [38], it is reasonable to exclude such patients from temozolomide-based treatment, even if concomitant *MGMT* hypermethylation is detected.

In metastatic melanoma the role of MGMT as a biomarker remains still unclear. A small retrospective study has linked *MGMT* promoter methylation to higher activity of single-agent temozolomide as first-line therapy (ORR 62% vs 15% in unmethylated patients [39]). However, these results were not recalled in similar retrospective studies regarding both standard-schedule dacarbazine or temozolomide [40,41] or dose-dense temozolomide [42]. *MGMT* promoter methylation assessed by the qualitative MSP is a predictive biomarker in patients with glioblastoma [12,43]. Even if *MGMT* promoter methylation may also represent a favorable prognostic factor [44], its prognostic role in CRC is controversial and the few available studies reported conflicting results, mainly in early stage disease [45]. Moreover, the prognostic relevance could be masked by its significant association with other negative prognostic biomarkers such as *KRAS* mutations. The pivotal phase II trial of dacarbazine in pretreated patients clearly suggested the potential clinical usefulness of patients selection based on the presence of *MGMT* methylation [6], since only patients with *MGMT* methylation achieved a RECIST response. Nevertheless, the activity of dacarbazine was low even when focusing on this molecular subgroup (2 responders out of 26 patients, accounting for an ORR of 8%). Although subsequent phase II trials of temozolomide restricted the enrollment to patients with MSP-detected *MGMT* methylation (Table 1), the poor specificity of such assay (highlighted by the dramatically high fraction of non-responders) has been recalled in all the five monotherapy trials, as well as in the combination trials investigating TEMIRI and CAPTEM regimens [24,32]. The low positive predictive values of MSP for the identification of patients with RECIST response are summarized in Supplementary Table S1.

Several issues account for the lack of accuracy in patients' selection when adopting MSP as the unique assay for assessing MGMT status. Firstly, even if high concordance (90%) has been reported between *MGMT* promoter methylation in primary and metastatic tissues [6], a lower concordance (27%) has been reported between archival tumor tissue and baseline samples obtained immediately before temozolomide administration [10]. Decline of the quantitative methylation rate from archival and fresh tissues might be related to prior exposure to several chemotherapeutic agents such as 5-FU [46] and has been reported in patients with glioblastoma at the time of disease recurrence [47]. Also, long-term storage of archival tissue might negatively affect the accuracy of the assays used for assessing MGMT status [48]. Second, MSP might not catch real MGMT status as outlined by consistent disagreement of gene promoter methylation and protein expression across several tumors [49].

Several MGMT-centered assays have been investigated with the aim of refining the selection of patients with mCRC for temozolomide-based treatment. Post-hoc analyses of phase II trials have shown that qualitative MSP may be outperformed by digital PCR quantification of *MGMT* promoter methylation % (i.e. Methyl-BEAMing [MB] assay, with MGMT quantitative methylation of 63% identified as the optimal cut-off for predicting treatment response) [50–52]. Although increasing specificity, up to 50% of patients with MB-high tumors do not achieve tumor response to temozolomide monotherapy, whereas a small subset of patients with MB-low (less than 10%) still experienced a tumor shrinkage [52]. Interestingly, immunohistochemistry (IHC) was investigated as a fast, low-cost and widespread assay for assessing tumor expression of MGMT protein. In glioblastoma, IHC is not validated due to several limitations linked to operator sensitivity, lack of prognostic relevance, poor concordance with methylation assays and low tumor specificity due to MGMT staining in non-neoplastic cells [53]. In the setting of mCRC, IHC has been retrospectively evaluated as an exploratory biomarker in non-randomized trials with temozolomide monotherapy and temozolomide-based combinations (Supplementary Table S1). Such analyses highlighted a suboptimal concordance between MGMT protein expression (assessed by IHC) and *MGMT* quantitative methylation (measured by MB) [51], suggesting that different techniques should not be used interchangeably. Despite such limitations, IHC has consistently shown a negative predictive value of 100%, as none of the tumors with retained MGMT staining achieved a RECIST response. To date, the highest accuracy (87%) for predicting RECIST response arose from a comprehensive MGMT assessment thanks to the use of multiple assays (i.e. MB, mass-spectrometry and RNAseq), thus outperforming individual assays such as MB or proteomic analysis by mass-spectrometry [52]. Although the best prediction may be likely dependent on quantitative/semiquantitative methylation assays (MB or pyrosequencing) combined with protein expression with IHC, several techniques are far from clinical-stage spread and their prospective validation is needed.

The potential predictive role of IHC was investigated thanks to the tumor tissue samples centrally collected during the screening procedures of the CAPTEM trial [32]. Notably, MGMT-centered assays had been previously investigated only in patients receiving temozolomide and in absence of a control group, thus not allowing to discriminate between their potential predictive role or just the prognostic one. In the randomized phase II CAPTEM trial a significant difference was reported in disease control rate according to treatment arm between patients with immunohistochemically MGMT positive and negative tumors (interaction test $P = 0.028$). Similarly, we reported a non-significant difference in outcomes such as ORR, PFS and OS between CAPTEM and FOLFIRI according to MGMT protein expression status, even if the lack of statistically significant interaction test may have been related to the small sample size. Despite identification of IHC as a promising biomarker for clinical benefit to temozolomide-based regimens, ORR to the CAPTEM regimen was still only 16% even in patients with MGMT IHC negative tumors, suggesting that MGMT-centered extensive assessment is still not sufficient to accurately identify the subgroup of patients with the chance of treatment benefit. With this regard, to investigate whether colorectal cancers with *MGMT* methylation and low-to-absent MGMT expression may represent a distinct molecular subtype with specific features, we performed here an in-silico analysis of The Cancer Genome Atlas [54]. Analysis of DNA-methylation data from TCGA's colorectal cancer samples (TCGA-COAD and TCGA-READ) showed the presence of *MGMT* promoter hyper-methylation in 32% of cases (Fig. 2A and Supplementary Material). Interestingly, *MGMT* itself was the only gene resulting as differently expressed between patients with *MGMT* hyper-methylated versus unmethylated status, in line with the observation that *MGMT* methylation may occur early in colorectal tumorigenesis. As expected, *MGMT* under-expression was observed in patients with promoter hypermethylation (Wilcoxon $P < 10^{-3}$, Fig. 2B). Residual expression of *MGMT* accounted for the majority of cases, with only 27% of cases showing low-to-absent gene expression. Patients with hyper-methylation and low-to-absent gene expression level of *MGMT* (Meth/Low) showed similar clinical and molecular characteristics than those with residual gene-expression (Meth/Normal) or unaltered methylation status (Unmeth), including sex, age, frequently altered genes and MSI status (Fig. 2C). Interestingly, Meth/Low cases were more prevalent in the right side than Meth/Normal and

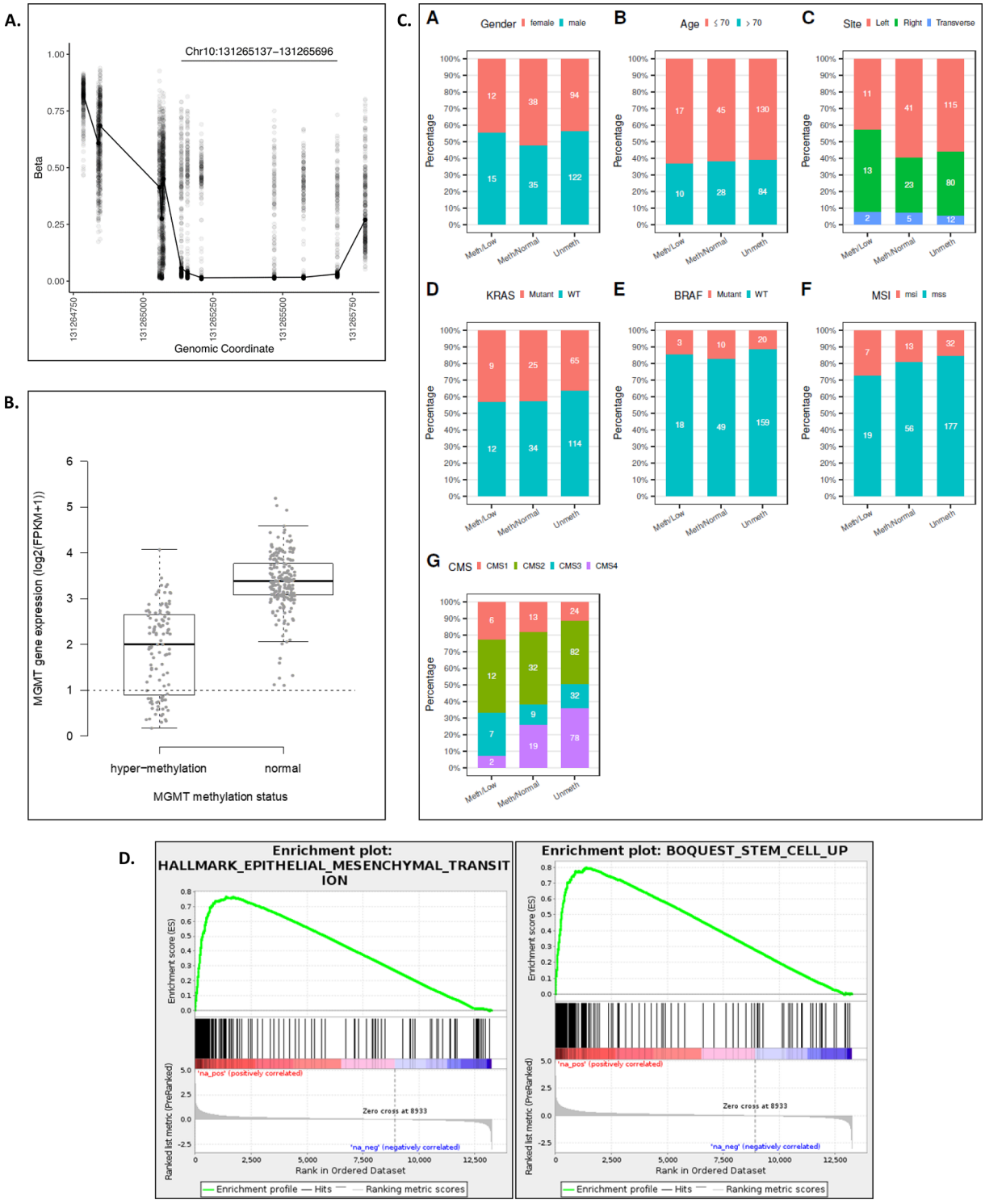

Fig. 2. (A) DNA-methylation level across TCGA CRC samples for CpG sites mapping in the genomic region around *MGMT* promoter. The horizontal segment refers to the 6 CpG sites considered to define the methylation status of *MGMT*. (B) Box plots of the distribution of MGMT gene expression level in samples showing hypermethylated and normal status of *MGMT*. (C) Bar plots showing the distribution of Hyper/Low, Hyper/Normal, Normal/Normal TCGA CRC patients stratified by (A) Gender, (B) Age, (C) Side, (D) *KRAS* mutant, (E) *BRAF* mutant, (F) Microsatellite status status and (G) CMS. (D) Examples from results of GSEA analysis in hyper/low versus hyper/normal CRC TCGA samples, including HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION (left, FDR = 0, Enrichment Score = 0.76) and BOQUEST_STEM_CELL_UP (right, FDR = 0, Enrichment Score = 0.80) gene lists.

Unmeth. Compared to remaining cases, Meth/Low patients' subgroup showed different distribution of consensus molecular subtype (CMS) classes, with a reduction of CMS4 and increase of CMS3 and CMS1 classes. In line with this data, in-silico functional analysis of Meth/low versus Meth/normal patients demonstrates significant depletion of stem-like and epithelial-mesenchymal transition terms (Fig. 2D). Collectively these data show that *MGMT* promoter methylation assessed by means of qualitative assays (i.e. dichotomized as present or absent) is clearly insufficient for selecting patients with higher chance of response to temozolomide. Supporting this, MGMT assessment by means of

second-level assays (IHC, mass spectrometry, RNAseq and methyl-BEAMing) may refine the predictive accuracy for response. As shown by our in-silico original data reported in this review, low/absent MGMT expression was restricted to a small subset of samples with methylated *MGMT* promoter. From a practical point of view, the available evidence in colorectal cancer suggests that any residual MGMT expression or a low level of *MGMT* methylation percentage is associated with lack of activity from temozolomide. However, there is still a long way to optimal molecular selection of patients, that may be also limited by the potential intra- and/or inter-lesion heterogeneity of MGMT status as reported for glioblastoma [55].

Future perspectives on biomarkers: base excision repair status

Temozolomide causes mainly methylation of guanine on the N7 and O6 atoms, and adenine on the N3 atom [56]. While MGMT accounts for direct repair of O6-methyl adducts of guanine, alkylated bases other than O6 methyl-guanine are processed by the base excision repair (BER) network (Fig. 1). BER is a multistep pathway starting from recognition and removal of small DNA lesions by specific DNA glycosylases (alkylpurine DNA-N glycosylase [APNG] is responsible for sensing and removing temozolomide induced alkylated bases). The resulting abasic site is recognized by an apurinic/apyrimidinic endonuclease (APE1) providing excision of damaged nucleotide. DNA single strand break is stabilized by PARP1 and XRCC1 until gap filling synthesis and ligation occur by means of a DNA polymerase (such as POLB) and a DNA ligase [57]. Crucially, cells display higher sensitivity to alkylating agents when BER is dysregulated leaving unrepaired cytotoxic intermediates. This concept is regarded as BER imbalance [58] and might arise from both deficiency of BER key enzymes (e.g. POLB) or overexpression of the BER initiating enzyme APNG coupled with POLB deficiency [59]. Despite BER is responsible for repairing the majority of temozolomide-induced DNA alkylations, few clinical studies have investigated with conflicting results if specific BER alterations might be involved in the susceptibility or resistance to temozolomide. In a small trial of temozolomide followed by fotemustine in patients with metastatic melanoma, lower mRNA levels of *APNG*, *PARP1* and *XRCC1* were associated with improved PFS [60]. Then, in a post-hoc analysis of the trial investigating the role temozolomide as concurrent and adjuvant to radiation therapy in patients with newly-diagnosed glioblastoma APNG expression assessed by IHC failed to show any significant association with OS according to treatment regimen (radiotherapy alone or radiotherapy plus temozolomide) in patients with *MGMT* promoter methylation [61].

Moving from a strong preclinical rationale [62–64], many efforts [65–71] (Table 2) have been made to increase the tumor sensitivity to temozolomide thanks to the pharmacological effect of agents such as PARP1/2 inhibitors or methoxyamine (that inhibits BER by binding to apurinic/apirymidinc sites and blocking APE1 nuclease activity). Results of phase I/II trials have been overall disappointing. Regarding mCRC, the combination of the PARP inhibitor veliparib with temozolomide was tested by a single-arm phase II trial carried out in MGMT unselected chemo-refractory patients, including those with MMR deficiency [66]. Even if this trial formally met the primary endpoint of disease-control rate after 2 cycles of temozolomide and veliparib (24%), both median PFS and OS (1.8 and 6.6 months, respectively) were quite disappointing. Moreover, the ORR was only 4% for patients with MMR proficiency treated with 150 mg/sqm of temozolomide and veliparib, and no responses were seen in patients treated with 200 mg/sqm temozolomide plus veliparib. Regarding upstream BER inhibition with the APE1 inhibitor methoxyamine, its combination with temozolomide was investigated by a single-arm phase II trial in patients with chemorefractory mCRC, who were again not selected for MGMT status [71]. The ORR was only 6% (1 out of 16 patients) and occurred in a patient with *MGMT* promoter methylation.

The reasons for unsuccessful results of the combination trials with temozolomide plus BER pathway inhibitors may be primarily referred to the absence of proper molecular selection. In fact, the combination of PARP inhibitors or methoxyamine with temozolomide should be assessed in the patient subgroup with *MGMT* hypermethylated and MMR proficient tumors. Moreover, in context of intrinsic BER imbalance, PARP inhibition could paradoxically impair temozolomide-induced cytotoxicity [72]. Consensual upregulation of key genes involved in BER is observed only in a minority of cases of CRC patients [73]. In conclusion, further studies should address clinical-stage assays for evaluating BER status and therefore potentially predicting the expected benefit from the use of BER inhibitors as sensitizers to the DNA damage induced by temozolomide.

The use of temozolomide as a “priming” therapy for sensitizing microsatellite stable colorectal cancer to immune checkpoint inhibitors

Mutational signatures across human cancers might be traced back to enzymatic modifications of DNA, defective DNA repair pathways or mutagen exposure; among the latter, temozolomide is able to induce a specific signature characterized by C > T transitions found in samples of patients with melanoma and glioblastoma previously exposed to this drug [74]. Notably, C > T transitions are frequently found in MSI-high cancers characterized by small insertions and deletions at mono/polynucleotide repeats [75]. In glioblastoma, the inactivation of MMR-related genes has been reported at the time disease recurrence after initial temozolomide-based treatment [76–80] (Supplementary Table S2). Overall, the prevalence of glioblastoma relapses with hypermutated phenotype due to MMR inactivation after temozolomide treatment is about 10–20% [81]. As MMR deficient cells are resistant to temozolomide, the emergence of alterations in MMR genes could be regarded as an adaptive mechanism of resistance to temozolomide. Since both temozolomide and carboplatin/cisplatin share dependency on intact MMR for inducing apoptosis [36,82], the emergence of a MMR deficiency-miming phenotype has been reported in ovarian cancer tumors after exposure to platinum-based chemotherapy [83]. More recently, we were able to confirm the hypermutator effects of temozolomide also in colorectal cancer preclinical models and in patients with mCRC selected for baseline microsatellite stable (MSS) status, low tumor mutational burden (TMB) and *MGMT* promoter hypermethylation.

From a clinical point of view, patients displaying initial benefit from temozolomide-based treatment were endowed with a deep change of tumor mutational landscape at time of progression. Specifically, 2 out of 5 patients with acquired resistance to temozolomide underwent tumor rebiopsy and the progressing lesions were characterized by the acquisition of high TMB (> 60 mutations/Mb) and pathogenic mutations in key MMR genes (MSH6 p.T1219I and MSH6 p.T1219I/MSH2 p.782I/MSH6 p.G1224E, respectively), while the loss of MGMT expression was retained also after disease progression. Conversely, patients with non-hypermutated progressing lesions (TMB < 10 mutations/Mb) had increased MGMT expression compared to pre-treatment samples, suggesting that the selection and expansion of tumor subclones with MGMT expression may have played a crucial role in determining secondary resistance to temozolomide [84]. Similar findings were reported for another patient treated with the temozolomide plus irinotecan regimen [24]: post-progression tumor tissue displayed high TMB (68 mutations/Mb) in contrast to the pre-treatment sample (4 mutations/Mb). Despite increasing mutational load no somatic mutations in MMR repair were found with MSS status retained, and BRCA2 E2198* emerged likely as a passenger mutation. Acquisition of high tumor mutational burden independently from MMR following temozolomide might be related to alterations in additional DNA repair

Table 2
Trials investigating safety and efficacy of base excision repair inhibitors and temozolomide in solid tumors.

BER target	Phase	Molecular selection	N	Drug	Setting	Schedule	ORR (%)	Exploratory biomarkers	Ref.
PARP	I	–	33	rucaparib	Solid tumors	**TMZ** 100 mg/sqm qd PO d1-5 q28 **Rucaparib**[a] 12 mg/sqm qd PO	9	–	[65]
PARP	II	–	75	veliparib	mCRC	**TMZ** 150 or 200 mg/sqm qd PO d1-5 q28 **Veliparib** 40 mg bid PO d1-7 q28	4 0[b]	PTEN, MGMT	[66]
PARP	II	–	104	veliparib	SCLC	**TMZ** 200 mg/sqm qd PO d1-5 q28 **Veliparib/placebo** 40 mg bid PO d1-7 q28	39[c]	PARP1 IHC, SLFN11 IHC, MGMT (PCR) CTCs	[67]
PARP	II	–	346	veliparib	Metastatic melanoma	**TMZ** 150 to 200 mg/sqm qd PO d1-5 q28 **Veliparib/placebo** 20 or 40 mg bid PO d1-7 q28	10.3 8.7 7[d]	P16 IHC, ERCC1 IHC	[68]
APE1	II	–	19	TRC102	Relapsed GBM	**TMZ** 150 mg/sqm qd PO d1-5 q28 **Methoxyamine** 150 mg qd PO d1-5 q28	0	APNG, MGMT (PCR)	[69]
APE1	I	–	52	TRC102	Solid tumors and lymphomas	**TMZ** 150 mg/sqm qd PO d1-5 q28 **Methoxyamine** 125 mg qd PO d1-5 q28[e]	7.6	γH2Ax p, NBS1, RAD51	[70]
APE1	II	–	16	TRC102	mCRC	**TMZ** 150 mg/sqm qd PO d1-5 q28 **Methoxyamine** 125 mg qd PO d1-5 q28	6	MGMT (IHC)	[71]

Abbreviations. BER: base-excision repair. GBM: glioblastoma multiforme. mCRC: metastatic colorectal cancer. ORR: overall response rate. PO: per-os. SCLC: small-cell lung cancer. TMZ: temozolomide.

[a] Recommended dose.

[b] ORR is referred to patients with mismatch-repair proficient tumors treated with standard-dose temozolomide or high-dose temozolomide. In the cohort of patients with mismatch-repair deficient tumors (N = 5) no objective responses were observed.

[c] (vs 14% in the temozolomide/placebo group)

[d] Referred to veliparib 20/40/placebo respectively.

[e] Recommended dose

pathways responsible for repairing alkylated adducts. BER-initiating enzyme APNG might be involved in temozolomide resistance due to increase in repair of N3 and N7 adducts in clinical setting [61]. Increased expression of APNG has been linked to genomic instability due to increase in spontaneous mutations, especially frameshift ones [85,86]. Overall, these data suggest that acquired resistance to temozolomide might develop independently from the induction of MGMT expression and may include MMR inactivation and/or acquisition of a mutator phenotype (Fig. 3).

In patients with mCRC, clinical benefit from immune checkpoint inhibitors (ICIs) is strictly restricted to MSI-high subgroup [18]. In pretreated patients, both nivolumab +/- ipilimumab [87,88] and pembrolizumab [18,89] led to rapid and durable responses. In first-line setting, an unprecedented ORR of 60% was achieved by the combination of low-dose ipilimumab and nivolumab [90]. Despite such encouraging results, it must be pointed out that MSI-high mCRCs represent only 4% of all cases, and therefore most of remaining patients (i.e. those with MSS status) are currently excluded from immunotherapy approaches. Sensitizing MSS cancers to immunotherapy is a major challenge since several strategies have failed so far, including combination of PD-1/PD-L1 blockade with anti-CTLA-4 monoclonal antibodies [91], MEK inhibitors [92] and post-induction "lightened" combination with 5-FU/LV and the anti-angiogenic agent bevacizumab [93]. However, promising data were reported for the combination of nivolumab and regorafenib thanks to the potential suppression of pro-tumorigenic tumor-associated macrophages induced by regorafenib [94]. We hypothesized that temozolomide could reshape the mutational landscape of *MGMT* silenced mCRC and could be used as a "priming" treatment able to sensitize MSS cancers to sequential or concomitant ICIs. Three proof-of-concept phase II trials are currently ongoing (Table 3). Different trial design will address unanswered questions regarding the role of temozolomide in potentially inducing the MSI-high like phenotype. Firstly, MSI-high like status is expected only in a patients' subgroup following temozolomide treatment and, therefore, only those patients who acquire a high TMB might benefit from ICIs. ARETHUSA and NCT03879811 trials will test the feasibility

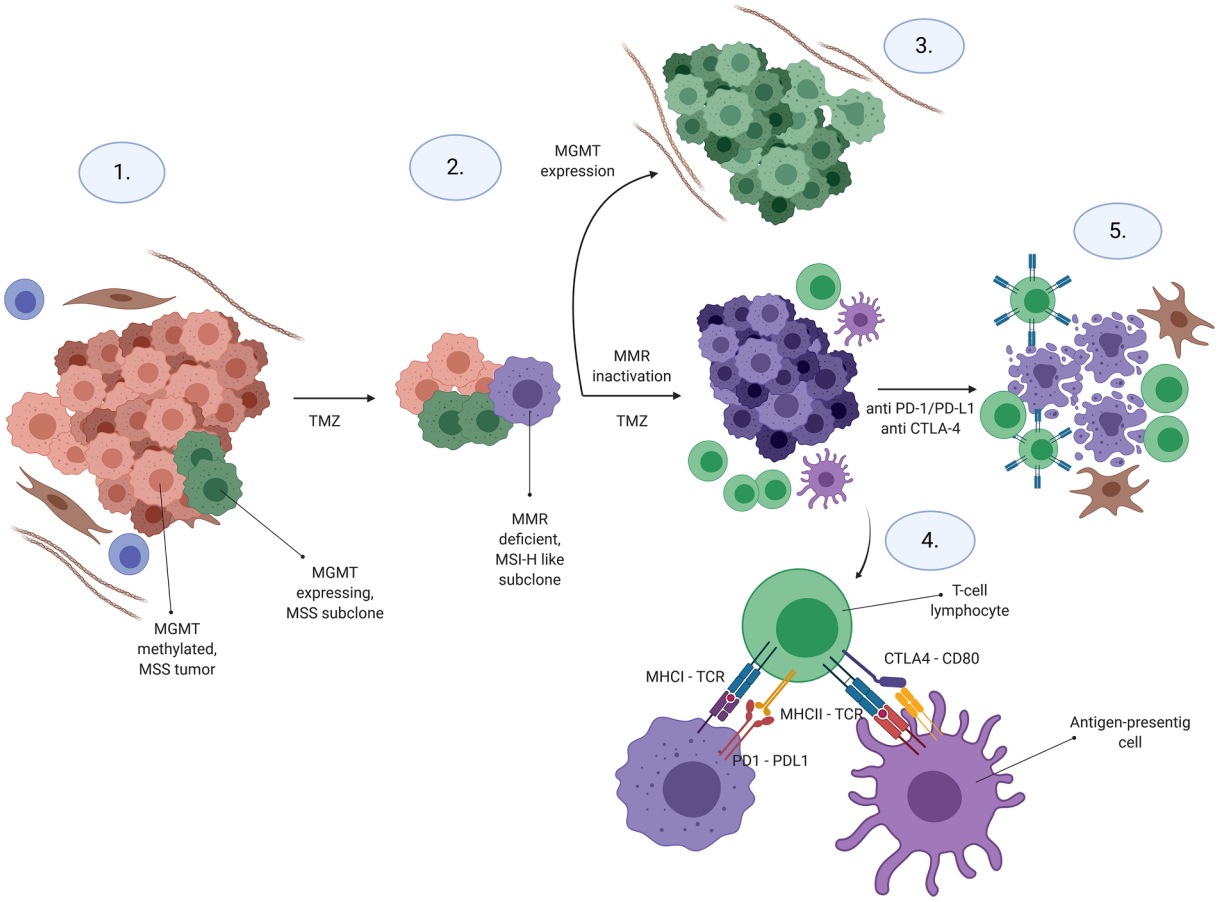

Fig. 3. Putative mechanism of temozolomide induced hypermutation in metastatic colorectal cancer. Initially MGMT deficient, microsatellite stable metastatic colorectal cancer is treated with temozolomide (1) with response and lesion shrinkage (2). Acquired resistance to temozolomide might develop due to MGMT re-expression/selection of MGMT expressing subclones (3) of due to inactivation of key genes involved in the mismatch repair and acquisition of a high tumor mutational burden (4). This hypermutant temozolomide-resistant tumor might elicit antitumor response hampered by expression of immune checkpoint proteins as observed in initially MSI-high tumors. Treatment with immune checkpoint inhibitors is expected to boost antitumor immunity (5). **Abbreviations.** MMR: mismatch repair. MSI-H: microsatellite instable-high. MSS: microsatellite stable. TMZ: temozolomide.

of this approach by means of a tumor re-biopsy following disease progression on temozolomide or after a pre-planned number of cycles (1 or 3, respectively). Then, only patients with demonstrated high TMB will be sequentially treated with an anti-PD-1 agent. While these trials are correctly excluding from immunotherapy those patients with retained non-hypermutated status, some warnings exist regarding: 1) rapid performance status deterioration of heavily pretreated patients following several courses of temozolomide; 2) lack of priming phase of naïve T cells at central level by highly immunogenic neoantigens, that may be preferentially stimulated by anti-CTLA-4 compared to anti-PD-1 agents; 3) lack of prevention of the potential occurrence of temozolomide-induced prolonged immune-suppression, that may be again preferentially stimulated by anti-CTLA-4 agents [95]; 4) the induction of mutations and MMR gene alterations in single cells may be cumulative and time-dependent, so that short-term temozolomide treatment may be not sufficient to boost the efficacy of immunotherapy. Moving from these considerations, we designed the MAYA trial (NCT03832621). Patients will be initially treated with temozolomide (priming phase) and, if no progressive disease is observed after 2 temozolomide courses, low dose ipilimumab plus nivolumab will be administered along with temozolomide. Patients progressing at first radiological restaging are considered *bona-fide* as primary resistant and therefore likely to bearing innate resistance mechanism other than MMR. Those who gained clinical benefit from temozolomide will be treated with ICI combo regardless of TMB status as acquired resistance invariably develops within 6 months after temozolomide as outlined by phase II trials of temozolomide as single agent.

Translational analyses of all the three trials will hopefully help to better understand the clinical relevance, dynamics and potential subclonality of temozolomide-induced hypermutation that might hamper its activity as a priming therapy for immune checkpoint inhibitors. Clonality and antigenicity of neoepitopes rather than just tumor mutational burden are emerging biomarkers for response to immune checkpoint inhibitors [96]. Frameshift neo-epitopes generated in MSI-high tumors are likely to elicit effective anti-tumor immune response in ICIs treated tumors [97]. Anyway, high tumor mutational burden might represent a clinical-stage biomarker of response to ICIs irrespective of the underlying DDR-related alteration as shown in both MSI-high [98] or MSS mCRC tumors such as those harboring *POLE* mutations [99].

Specific strategies and their biological insights could be transferred to other settings such as maintenance treatment following first-line therapy in mCRC or other diseases showing *MGMT* methylation. A biomarker-guided approach could be explored for tumors with MGMT deficiency and MSS/MMR proficiency status and limited benefit from ICIs, such as triple negative breast cancer. This agnostic approach would provide in many cases two effective treatments sequentially: a tailored chemotherapeutic therapy and immunotherapy at onset of resistance

Table 3
Ongoing phase II trials investigating immune checkpoint inhibitors in mCRC patients following TMZ induction therapy.

Phase	MMR status	MGMT status	*RAS/BRAF* status	Line of treatment	Design and Schedule		Primary endpoint	Ref.
					Phase 1 Priming	Phase 2 Treatment		
II	MSS (PCR)	MGMT promoter methylation (pyrosequencing) MGMT low expression (IHC)	–	At least 2 prior therapies including oxaliplatin and irinotecan-based regimens	TMZ 150 mg/sqm d1-5 q28 x2 cycles. If CR/PR/SD phase 2 follows	TMZ 150 mg/sqm d 1-5 q28 Ipilimumab 1 mg/Kg q56 Nivolumab 480 mg d1 q28	8-month PFS rate	NCT03832621 "MAYA"
II	MSS (IHC)	MGMT promoter methylation (MethylBEAMing) MGMT low expression (IHC)	*RAS* mutated	–	TMZ 150–200 mg/sqm d1-5 q28 until PD. Rebiopsy If TMB > 20 mut/Mb phase 2 follows	Pembrolizumab 200 mg d1 q21 up to 24 months	ORR	NCT03519412 "ARETHUSA"
II	MSS (IHC or PCR)	MGMT promoter methylation (PCR)	–	At least 2 prior therapies including oxaliplatin and irinotecan-based regimens	TMZ 150–200 mg/sqmd 1-5 q28 1–3 cycles[a]	Nivolumab 480 mg d1 q28	ORR	NCT03879811

Abbreviations. CR: complete response. IHC: immunohistochemistry. MMR: mismatch repair. ORR: overall response rate. PD: progressive disease. PFS: progression-free survival. PR: partial response. SD: stable disease.f TMZ: temozolomide.

[a] Initially 3 patients will be treated with TMZ, after 1 cycle rebiopsy will be performed. If TMB will be found increased in at least 1/3 patients accrual proceeds with the same schedule. Alternatively, 6 patients will be treated with TMZ and rebiopsed after completing 3 cycles. If TMB will be found increased in 1/6 patients, 12 more patients will be treated at the same schedule.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ctrv.2019.101935.

References

[1] Esteller M, Toyota M, Sanchez-Cespedes M, Capella G, Peinado MA, Watkins DN, 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;60:2368–71.
[2] Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005;352:987–96.
[3] Yang AS, Chapman PB. The history and future of chemotherapy for melanoma. Hematol Oncol Clin North Am 2009;23(583–97):x.
[4] Middleton MR, Grob JJ, Aaronson N, Fierlbeck G, Tilgen W, Seiter S, et al. Randomized phase III study of temozolomide versus dacarbazine in the treatment of patients with advanced metastatic malignant melanoma. J Clin Oncol 2000;18:158–66.
[5] Shacham-Shmueli E, Beny A, Geva R, Blachar A, Figer A, Aderka D. Response to temozolomide in patients with metastatic colorectal cancer with loss of MGMT expression: a new approach in the era of personalized medicine? J Clin Oncol 2011;29:e262–5.
[6] Amatu A, Sartore-Bianchi A, Moutinho C, Belotti A, Bencardino K, Chirico G, 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;19:2265–72.
[7] Hochhauser D, Glynne-Jones R, Potter V, Grávalos C, Doyle TJ, Pathiraja K, 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;12:809–18.
[8] Pietrantonio F, Perrone F, de Braud F, Castano A, Maggi C, Bossi I, et al. Activity of temozolomide in patients with advanced chemorefractory colorectal cancer and MGMT promoter methylation. Ann Oncol 2014;25:404–8.
[9] Pietrantonio F, de Braud F, Milione M, Maggi C, Iacovelli R, Dotti KF, et al. Dose-dense temozolomide in patients with MGMT-silenced chemorefractory colorectal cancer. Target Oncol 2016;11:337–43.
[10] Amatu A, Barault L, Moutinho C, Cassingena A, Bencardino K, Ghezzi S, et al. Tumor MGMT promoter hypermethylation changes over time limit temozolomide efficacy in a phase II trial for metastatic colorectal cancer. Ann Oncol 2016;27:1062–7.
[11] Calegari MA, Inno A, Monterisi S, Orlandi A, Santini D, Basso M, et al. A phase 2 study of temozolomide in pretreated metastatic colorectal cancer with MGMT promoter methylation. Br J Cancer 2017;116:1279–86.
[12] Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 2005;352:997–1003.
[13] Grothey A, Van Cutsem E, Sobrero A, Siena S, Falcone A, Ychou M, et al. Regorafenib monotherapy for previously treated metastatic colorectal cancer (CORRECT): an international, multicentre, randomised, placebo-controlled, phase 3 trial. Lancet 2013;381:303–12.
[14] Mayer RJ, Van Cutsem E, Falcone A, Yoshino T, Garcia-Carbonero R, Mizunuma N, et al. Randomized trial of TAS-102 for refractory metastatic colorectal cancer. N Engl J Med 2015;372:1909–19.
[15] Li J, Qin S, Xu R, Yau TC, Ma B, Pan H, et al. Regorafenib plus best supportive care versus placebo plus best supportive care in Asian patients with previously treated metastatic colorectal cancer (CONCUR): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol 2015;16:619–29.
[16] Xu J, Kim TW, Shen L, Sriuranpong V, Pan H, Xu R, et al. Results of a randomized, double-blind, placebo-controlled, phase III trial of trifluridine/tipiracil (TAS-102) Monotherapy in Asian Patients with previously treated metastatic colorectal cancer: the TERRA study. J Clin Oncol 2018;36:350–8.
[17] Cutsem EV, Huijberts S, Grothey A, Yaeger R, Cuyle P-J, Elez E, et al. Binimetinib, encorafenib, and cetuximab triplet therapy for patients with BRAF V600E–mutant metastatic colorectal cancer: safety lead-in results from the phase III BEACON colorectal cancer study. J Clin Oncol 2019;37:1460–9.
[18] Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, et al. PD-1 blockade in tumors with mismatch-repair deficiency. N Engl J Med 2015;372:2509–20.
[19] Sartore-Bianchi A, Trusolino L, Martino C, Bencardino K, Lonardi S, Bergamo F, et al. Dual-targeted therapy with trastuzumab and lapatinib in treatment-refractory, KRAS codon 12/13 wild-type, HER2-positive metastatic colorectal cancer (HERACLES): a proof-of-concept, multicentre, open-label, phase 2 trial. Lancet Oncol 2016;17:738–46.
[20] Drilon A, Siena S, Ou SI, Patel M, Ahn MJ, Lee J, et al. Safety and antitumor activity of the multitargeted pan-TRK, ROS1, and ALK inhibitor entrectinib: combined results from two phase I trials (ALKA-372-001 and STARTRK-1). Cancer Discov

2017;7:400–9.
[21] Drilon A, Laetsch TW, Kummar S, DuBois SG, Lassen UN, Demetri GD, et al. Efficacy of larotrectinib in TRK fusion-positive cancers in adults and children. N Engl J Med 2018;378:731–9.
[22] Subbiah V, Gainor JF, Rahal R, Brubaker JD, Kim JL, Maynard M, et al. Precision targeted therapy with BLU-667 for. Cancer Discov 2018;8:836–49.
[23] Orlandi A, Calegari A, Inno A, Berenato R, Caporale M, Niger M, et al. BRAF in metastatic colorectal cancer: the future starts now. Pharmacogenomics 2015;16:2069–81.
[24] Morano F, Corallo S, Niger M, Barault L, Milione M, Berenato R, et al. Temozolomide and irinotecan (TEMIRI regimen) as salvage treatment of irinotecan-sensitive advanced colorectal cancer patients bearing MGMT methylation. Ann Oncol 2018;29:1800–6.
[25] Shapiro JD, Thavaneswaran S, Underhill CR, Robledo KP, Karapetis CS, Day FL, et al. Cetuximab alone or with irinotecan for resistant KRAS-, NRAS-, BRAF- and PIK3CA-wild-type metastatic colorectal cancer: the AGITG randomized phase II ICECREAM study. Clin Colorectal Cancer. 2018;17:313–9.
[26] Sobrero AF, Maurel J, Fehrenbacher L, Scheithauer W, Abubakr YA, Lutz MP, et al. EPIC: phase III trial of cetuximab plus irinotecan after fluoropyrimidine and oxaliplatin failure in patients with metastatic colorectal cancer. J Clin Oncol 2008;26:2311–9.
[27] Houghton PJ, Stewart CF, Cheshire PJ, Richmond LB, Kirstein MN, Poquette CA, et al. Antitumor activity of temozolomide combined with irinotecan is partly independent of O6-methylguanine-DNA methyltransferase and mismatch repair phenotypes in xenograft models. Clin Cancer Res 2000;6:4110–8.
[28] Pourquier P, Waltman JL, Urasaki Y, Loktionova NA, Pegg AE, Nitiss JL, et al. Topoisomerase I-mediated cytotoxicity of N-methyl-N'-nitro-N-nitrosoguanidine: trapping of topoisomerase I by the O6-methylguanine. Cancer Res 2001;61:53–8.
[29] Pommier Y. Topoisomerase I inhibitors: camptothecins and beyond. Nat Rev Cancer 2006;6:789–802.
[30] Fine RL, Gulati AP, Krantz BA, Moss RA, Schreibman S, Tsushima DA, et al. Capecitabine and temozolomide (CAPTEM) for metastatic, well-differentiated neuroendocrine cancers: the Pancreas Center at Columbia University experience. Cancer Chemother Pharmacol 2013;71:663–70.
[31] Li LS, Morales JC, Veigl M, Sedwick D, Greer S, Meyers M, et al. DNA mismatch repair (MMR)-dependent 5-fluorouracil cytotoxicity and the potential for new therapeutic targets. Br J Pharmacol 2009;158:679–92.
[32] Pietrantonio F, Lobefaro R, Antista M, Lonardi S, Raimondi A, Morano F, et al. Capecitabine and temozolomide versus FOLFIRI in *RAS* mutated, *MGMT* methylated metastatic colorectal cancer. Clin Cancer Res 2019. https://doi.org/10.1158/1078-0432.CCR-19-3024. In press.
[33] Gupta D, Heinen CD. The mismatch repair-dependent DNA damage response: mechanisms and implications. DNA Repair (Amst). 2019;78:60–9.
[34] Quiros S, Roos WP, Kaina B. Processing of O6-methylguanine into DNA double-strand breaks requires two rounds of replication whereas apoptosis is also induced in subsequent cell cycles. Cell Cycle 2010;9:168–78.
[35] Soll JM, Sobol RW, Mosammaparast N. Regulation of DNA alkylation damage repair: lessons and therapeutic opportunities. Trends Biochem Sci 2017;42:206–18.
[36] Fink D, Aebi S, Howell SB. The role of DNA mismatch repair in drug resistance. Clin Cancer Res 1998;4:1–6.
[37] de Wind N, Dekker M, Berns A, Radman M, te Riele H. Inactivation of the mouse Msh2 gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. Cell 1995;82:321–30.
[38] Fabrizio DA, George TJ, Dunne RF, Frampton G, Sun J, Gowen K, et al. Beyond microsatellite testing: assessment of tumor mutational burden identifies subsets of colorectal cancer who may respond to immune checkpoint inhibition. J Gastrointest Oncol 2018;9:610–7.
[39] Tuominen R, Jewell R, van den Oord JJ, Wolter P, Stierner U, Lindholm C, et al. MGMT promoter methylation is associated with temozolomide response and prolonged progression-free survival in disseminated cutaneous melanoma. Int J Cancer 2015;136:2844–53.
[40] Busch C, Geisler J, Lillehaug JR, Lønning PE. MGMT expression levels predict disease stabilisation, progression-free and overall survival in patients with advanced melanomas treated with DTIC. Eur J Cancer 2010;46:2127–33.
[41] Hassel JC, Sucker A, Edler L, Kurzen H, Moll I, Stresemann C, et al. MGMT gene promoter methylation correlates with tolerance of temozolomide treatment in melanoma but not with clinical outcome. Br J Cancer 2010;103:820–6.
[42] Rietschel P, Wolchok JD, Krown S, Gerst S, Jungbluth AA, Busam K, et al. Phase II study of extended-dose temozolomide in patients with melanoma. J Clin Oncol 2008;26:2299–304.
[43] Weller M, Felsberg J, Hartmann C, Berger H, Steinbach JP, Schramm J, et al. Molecular predictors of progression-free and overall survival in patients with newly diagnosed glioblastoma: a prospective translational study of the German Glioma Network. J Clin Oncol 2009;27:5743–50.
[44] Rivera AL, Pelloski CE, Gilbert MR, Colman H, De La Cruz C, Sulman EP, et al. MGMT promoter methylation is predictive of response to radiotherapy and prognostic in the absence of adjuvant alkylating chemotherapy for glioblastoma. Neuro Oncol 2010;12:116–21.
[45] Inno A, Fanetti G, Di Bartolomeo M, Gori S, Maggi C, Cirillo M, et al. Role of MGMT as biomarker in colorectal cancer. World J Clin Cases 2014;2:835–9.
[46] Murakami J, Lee YJ, Kokeguchi S, Tsujigiwa H, Asaumi J, Nagatsuka H, et al. Depletion of O6-methylguanine-DNA methyltransferase by O6-benzylguanine enhances 5-FU cytotoxicity in colon and oral cancer cell lines. Oncol Rep 2007;17:1461–7.
[47] Brandes AA, Franceschi E, Tosoni A, Bartolini S, Bacci A, Agati R, et al. O(6)-methylguanine DNA-methyltransferase methylation status can change between first surgery for newly diagnosed glioblastoma and second surgery for recurrence: clinical implications. Neuro Oncol 2010;12:283–8.
[48] Kalmár A, Péterfia B, Hollósi P, Wichmann B, Bodor A, Patai Á, et al. Bisulfite-based DNA methylation analysis from recent and archived formalin-fixed, paraffin embedded colorectal tissue samples. Pathol Oncol Res 2015;21:1149–56.
[49] Brell M, Ibáñez J, Tortosa A. O6-Methylguanine-DNA methyltransferase protein expression by immunohistochemistry in brain and non-brain systemic tumours: systematic review and meta-analysis of correlation with methylation-specific polymerase chain reaction. BMC Cancer 2011;11:35.
[50] Barault L, Amatu A, Bleeker FE, Moutinho C, Falcomatà C, Fiano V, 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;26:1994–9.
[51] Sartore-Bianchi A, Pietrantonio F, Amatu A, Milione M, Cassingena A, Ghezzi S, et al. Digital PCR assessment of MGMT promoter methylation coupled with reduced protein expression optimises prediction of response to alkylating agents in metastatic colorectal cancer patients. Eur J Cancer 2017;71:43–50.
[52] Schwartz S, Szeto C, Tian Y, Cecchi F, Corallo S, Calegari MA, et al. Refining the selection of patients with metastatic colorectal cancer for treatment with temozolomide using proteomic analysis of O6-methylguanine-DNA-methyltransferase. Eur J Cancer 2019;107:164–74.
[53] Weller M, Stupp R, Reifenberger G, Brandes AA, van den Bent MJ, Wick W, et al. MGMT promoter methylation in malignant gliomas: ready for personalized medicine? Nat Rev Neurol 2010;6:39–51.
[54] Network CGA. Comprehensive molecular characterization of human colon and rectal cancer. Nature 2012;487:330–7.
[55] Parker NR, Hudson AL, Khong P, Parkinson JF, Dwight T, Ikin RJ, et al. Intratumoral heterogeneity identified at the epigenetic, genetic and transcriptional level in glioblastoma. Sci Rep 2016;6:22477.
[56] Tentori L, Graziani G. Pharmacological strategies to increase the antitumor activity of methylating agents. Curr Med Chem 2002;9:1285–301.
[57] Kim YJ, Wilson DM. Overview of base excision repair biochemistry. Curr Mol Pharmacol. 2012;5:3–13.
[58] Fu D, Calvo JA, Samson LD. Balancing repair and tolerance of DNA damage caused by alkylating agents. Nat Rev Cancer 2012;12:104–20.
[59] Trivedi RN, Wang XH, Jelezcova E, Goellner EM, Tang JB, Sobol RW. Human methyl purine DNA glycosylase and DNA polymerase beta expression collectively predict sensitivity to temozolomide. Mol Pharmacol 2008;74:505–16.
[60] Guida M, Tommasi S, Strippoli S, Natalicchio MI, De Summa S, Pinto R, et al. The search for a melanoma-tailored chemotherapy in the new era of personalized therapy: a phase II study of chemo-modulating temozolomide followed by fotemustine and a cooperative study of GOIM (Gruppo Oncologico Italia Meridionale). BMC Cancer 2018;18:552.
[61] Agnihotri S, Gajadhar AS, Ternamian C, Gorlia T, Diefes KL, Mischel PS, et al. Alkylpurine-DNA-N-glycosylase confers resistance to temozolomide in xenograft models of glioblastoma multiforme and is associated with poor survival in patients. J Clin Invest 2012;122:253–66.
[62] Liu L, Nakatsuru Y, Gerson SL. Base excision repair as a therapeutic target in colon cancer. Clin Cancer Res 2002;8:2985–91.
[63] Fishel ML, He Y, Smith ML, Kelley MR. Manipulation of base excision repair to sensitize ovarian cancer cells to alkylating agent temozolomide. Clin Cancer Res 2007;13:260–7.
[64] Liu L, Taverna P, Whitacre CM, Chatterjee S, Gerson SL. Pharmacologic disruption of base excision repair sensitizes mismatch repair-deficient and -proficient colon cancer cells to methylating agents. Clin Cancer Res 1999;5:2908–17.
[65] Plummer R, Jones C, Middleton M, Wilson R, Evans J, Olsen A, et al. Phase I study of the poly(ADP-ribose) polymerase inhibitor, AG014699, in combination with temozolomide in patients with advanced solid tumors. Clin Cancer Res 2008;14:7917–23.
[66] Pishvaian MJ, Slack RS, Jiang W, He AR, Hwang JJ, Hankin A, et al. A phase 2 study of the PARP inhibitor veliparib plus temozolomide in patients with heavily pretreated metastatic colorectal cancer. Cancer 2018;124:2337–46.
[67] Pietanza MC, Waqar SN, Krug LM, Dowlati A, Hann CL, Chiappori A, et al. Randomized, double-blind, phase II study of temozolomide in combination with either Veliparib or Placebo in patients with relapsed-sensitive or refractory small-cell lung cancer. J Clin Oncol 2018;36:2386–94.
[68] Middleton MR, Friedlander P, Hamid O, Daud A, Plummer R, Falotico N, et al. Randomized phase II study evaluating veliparib (ABT-888) with temozolomide in patients with metastatic melanoma. Ann Oncol 2015;26:2173–9.
[69] Ahluwalia M, Drappatz J, Ye X, Walbert T, Holdhoff M, Lesser G, et al. ACTR-18. Phase II trial of temozolomide and TRC 102, base excision repair inhibitor, in bevacizumab NAÏVE glioblastoma at first recurrence. Neuro-Oncol 2018;20:vi15-vi.
[70] Meehan RS, Coyne GHOS, Kummar S, Collins JM, Anderson L, Zlott J, et al. A phase I trial of TRC102 (methoxyamine HCl) with temozolomide (TMZ) in patients with solid tumors and lymphomas. J Clin Oncol 2017;35:2518-.
[71] Coyne GO, Bonilla CM, Zlott J, Takebe N, Meehan R, Juwara L, et al. Abstract LB-293: a phase II trial of TRC102 (methoxyamine HCl) in combination with temozolomide in patients with relapsed metastatic colorectal carcinoma. Cancer Res 2019;79:LB-293-LB-.
[72] Tang JB, Goellner EM, Wang XH, Trivedi RN, St Croix CM, Jelezcova E, et al. Bioenergetic metabolites regulate base excision repair-dependent cell death in response to DNA damage. Mol Cancer Res 2010;8:67–79.
[73] Leguisamo NM, Gloria HC, Kalil AN, Martins TV, Azambuja DB, Meira LB, et al. Base excision repair imbalance in colorectal cancer has prognostic value and modulates response to chemotherapy. Oncotarget 2017;8:54199–214.
[74] Alexandrov LB, Stratton MR. Mutational signatures: the patterns of somatic

mutations hidden in cancer genomes. Curr Opin Genet Dev 2014;24:52–60.
[75] Ma J, Setton J, Lee NY, Riaz N, Powell SN. The therapeutic significance of mutational signatures from DNA repair deficiency in cancer. Nat Commun 2018;9:3292.
[76] Wang J, Cazzato E, Ladewig E, Frattini V, Rosenbloom DI, Zairis S, et al. Clonal evolution of glioblastoma under therapy. Nat Genet 2016;48:768–76.
[77] Cahill DP, Levine KK, Betensky RA, Codd PJ, Romany CA, Reavie LB, et al. Loss of the mismatch repair protein MSH6 in human glioblastomas is associated with tumor progression during temozolomide treatment. Clin Cancer Res 2007;13:2038–45.
[78] Shinsato Y, Furukawa T, Yunoue S, Yonezawa H, Minami K, Nishizawa Y, et al. Reduction of MLH1 and PMS2 confers temozolomide resistance and is associated with recurrence of glioblastoma. Oncotarget 2013;4:2261–70.
[79] Sun Q, Pei C, Li Q, Dong T, Dong Y, Xing W, et al. Up-regulation of MSH6 is associated with temozolomide resistance in human glioblastoma. Biochem Biophys Res Commun 2018;496:1040–6.
[80] Indraccolo S, Lombardi G, Fassan M, Pasqualini L, Giunco S, Marcato R, et al. Genetic, epigenetic, and immunologic profiling of MMR-deficient relapsed glioblastoma. Clin Cancer Res 2019;25:1828–37.
[81] Daniel P, Sabri S, Chaddad A, Meehan B, Jean-Claude B, Rak J, et al. Temozolomide induced hypermutation in glioma: evolutionary mechanisms and therapeutic opportunities. Front Oncol 2019;9:41.
[82] Fink D, Nebel S, Norris PS, Baergen RN, Wilczynski SP, Costa MJ, et al. Enrichment for DNA mismatch repair-deficient cells during treatment with cisplatin. Int J Cancer 1998;77:741–6.
[83] Samimi G, Fink D, Varki NM, Husain A, Hoskins WJ, Alberts DS, et al. Analysis of MLH1 and MSH2 expression in ovarian cancer before and after platinum drug-based chemotherapy. Clin Cancer Res 2000;6:1415–21.
[84] Germano G, Lamba S, Rospo G, Barault L, Magrì A, Maione F, et al. Inactivation of DNA repair triggers neoantigen generation and impairs tumour growth. Nature 2017;552:116–20.
[85] Eyler DE, Burnham KA, Wilson TE, O'Brien PJ. Mechanisms of glycosylase induced genomic instability. PLoS ONE 2017;12:e0174041.
[86] Klapacz J, Lingaraju GM, Guo HH, Shah D, Moar-Shoshani A, Loeb LA, et al. Frameshift mutagenesis and microsatellite instability induced by human alkyladenine DNA glycosylase. Mol Cell 2010;37:843–53.
[87] Overman MJ, McDermott R, Leach JL, Lonardi S, Lenz HJ, Morse MA, et al. Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): an open-label, multicentre, phase 2 study. Lancet Oncol 2017;18:1182–91.
[88] Overman MJ, Lonardi S, Wong KYM, Lenz HJ, Gelsomino F, Aglietta M, et al. Durable clinical benefit with Nivolumab plus Ipilimumab in DNA mismatch repair-deficient/microsatellite instability-high metastatic colorectal Cancer. J Clin Oncol 2018;36:773–9.
[89] Le DT, Kavan P, Kim TW, Burge ME, Cutsem EV, Hara H, et al. KEYNOTE- 164: pembrolizumab for patients with advanced microsatellite instability high (MSI-H) colorectal cancer. J Clin Oncol 2018;36:3514-.
[90] Lenz H-JJ, Van Cutsem E, Limon ML, Wong KY, Hendlisz A, Aglietta M, et al. LBA18_PRDurable clinical benefit with nivolumab (NIVO) plus low-dose ipilimumab (IPI) as first-line therapy in microsatellite instability-high/mismatch repair deficient (MSI-H/dMMR) metastatic colorectal cancer (mCRC). Ann Oncol 2018;29.
[91] Chen EX, Jonker DJ, Kennecke HF, Berry SR, Couture F, Ahmad CE, et al. CCTG CO.26 trial: A phase II randomized study of durvalumab (D) plus tremelimumab (T) and best supportive care (BSC) versus BSC alone in patients (pts) with advanced refractory colorectal carcinoma (rCRC). J Clin Oncol 2019;37:481-.
[92] Eng C, Kim TW, Bendell J, Argilés G, Tebbutt NC, Di Bartolomeo M, et al. Atezolizumab with or without cobimetinib versus regorafenib in previously treated metastatic colorectal cancer (IMblaze370): a multicentre, open-label, phase 3, randomised, controlled trial. Lancet Oncol 2019;20:849–61.
[93] Grothey A, Tabernero J, Arnold D, De Gramont A, Ducreux MP, O'Dwyer PJ, et al. LBA19Fluoropyrimidine (FP) + bevacizumab (BEV) + atezolizumab vs FP/BEV in BRAFwt metastatic colorectal cancer (mCRC): Findings from Cohort 2 of MODUL – a multicentre, randomized trial of biomarker-driven maintenance treatment following first-line induction therapy. Ann Oncol 2018;29.
[94] Fukuoka S, Hara H, Takahashi N, Kojima T, Kawazoe A, Asayama M, et al. Regorafenib plus nivolumab in patients with advanced gastric (GC) or colorectal cancer (CRC): An open-label, dose-finding, and dose-expansion phase 1b trial (REGONIVO, EPOC1603). J Clin Oncol 2019;37:2522-.
[95] Patel SP, Kim DW, Bassett RL, Cain S, Washington E, Hwu WJ, et al. A phase II study of ipilimumab plus temozolomide in patients with metastatic melanoma. Cancer Immunol Immunother 2017;66:1359–66.
[96] McGranahan N, Swanton C. Neoantigen quality, not quantity. Sci Transl Med 2019;11:eaax7918.
[97] Dudley JC, Lin MT, Le DT, Eshleman JR. Microsatellite Instability as a Biomarker for PD-1 Blockade. Clin Cancer Res 2016;22:813–20.
[98] Schrock AB, Ouyang C, Sandhu J, Sokol E, Jin D, Ross JS, et al. Tumor mutational burden is predictive of response to immune checkpoint inhibitors in MSI-high metastatic colorectal cancer. Ann Oncol 2019;30:1096–103.
[99] Silberman R, Steiner DF, Lo AA, Gomez A, Zehnder JL, Chu G, et al. Complete and prolonged response to immune checkpoint blockade in POLE-mutated colorectal cancer. JCO Precision Oncol 2019:1–5.