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Temozolomide-associated Hypermutation in Gliomas

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Abstract

Low-grade gliomas cause considerable morbidity and most will recur after initial therapy. At recurrence, low-grade gliomas can undergo transformation to high-grade gliomas (grade III or grade IV), which are associated with worse prognosis. Temozolomide (TMZ) provides survival benefit in patients with glioblastomas (GBMs) but its value in patients with low-grade gliomas is less clear. A subset of TMZ-treated, IDH-mutant, low-grade astrocytomas recur as more malignant tumors with thousands of *de novo*, coding mutations bearing a signature of TMZ-induced hypermutation. Preliminary studies raise the hypothesis that TMZ-induced hypermutation may contribute to malignant transformation, although with highly variable latency. On the other hand, hypermutated gliomas have radically altered genomes that present new opportunities for therapeutic intervention. In light of these findings and the immunotherapy clinical trials they inspired, how do patients and providers approach the risks and benefits of TMZ therapy? This review discusses what is known about the mechanism and consequences of TMZ-induced hypermutation and outstanding questions regarding its clinical significance.

Introduction

Disruption of DNA repair pathways increases mutagenesis and genomic instability, thereby promoting cancer progression and resistance to therapies.[1,2] IDH-mutant, low-grade astrocytomas treated with TMZ can recur as more malignant tumors with DNA mismatch repair (MMR) defects and a hypermutator phenotype,[3-5] as observed in TMZ-treated GBM patients.[4-8] TMZ-induced hypermutation is defined by a dramatic increase in the mutation rate and a TMZ-associated mutational signature in post-treatment recurrences. In order to present the current knowledge of TMZ-induced hypermutation in gliomas, this review first provides relevant background on the new molecular classification of gliomas, TMZ clinical trial results, and mechanisms of TMZ action in relation to DNA repair and the genesis of hypermutated clones. The review focuses on TMZ-induced hypermutation in IDH-mutant gliomas. At the conclusion of the review, we discuss immune checkpoint inhibitors as a new experimental therapy for the hypermutated glioma patient population.

The 2016 World Health Organization (WHO) Molecular Classification of Gliomas

Objective classification of gliomas is critical for understanding which gliomas may undergo TMZ-associated hypermutation, and how this event might influence therapeutic response and survival. In 2016, the guidelines for the WHO classification of gliomas were revised from the 2007 guidelines to incorporate molecular parameters.[9] By molecular classification, grade II and grade III astrocytic tumors are further stratified into IDH-mutant (most of grade II and grade III) and IDH-wildtype groups. The majority of grade II and grade III astrocytic tumors fall into the IDH-mutant group and show characteristic mutation of *TP53*, and loss of ATRX staining. The diagnostic criteria for grade II or III oligodendrogliomas include both IDH-mutation status and combined whole

chromosome arm losses of 1p and 19q (1p/19q co-deletion). In addition, the diagnoses of oligoastrocytoma and anaplastic oligoastrocytoma are now strongly discouraged and to be used only when diagnostic molecular testing is not possible or in the very rare instance of a dual genotype oligoastrocytoma.[9]

IDH mutation status and 1p/19q co-deletion have prognostic value for gliomas. The Cancer Genome Atlas (TCGA) network performed genome-wide analyses of 293 low-grade gliomas from adults.[10] From these data, three molecular subgroups of WHO grade II and grade III gliomas emerged which correlated with clinical outcomes, recurrence, and survival: (i) IDH-mutant gliomas with associated 1p/19q co-deletion, (ii) IDH-mutant gliomas without 1p/19q co-deletion, and (iii) IDH-wildtype tumors without 1p/19q co-deletion. The median survival for patients with mutant IDH plus 1p/19q co-deletion, mutant IDH without 1p/19q co-deletion and IDH-wildtype gliomas without 1p/19q co-deletion are 8, 6.3 and 1.7 years, respectively.[10] Given their longer overall survival, the mutagenic consequences of TMZ are particularly relevant in these patient subgroups.

Grade IV GBMs are further stratified into IDH-wildtype (90%) or IDH mutant (10%) molecular subtypes. IDH-wildtype GBMs are also known as primary GBMs that arise *de novo*. IDH-mutant GBMs, or secondary GBMs, may arise from a low-grade diffuse astrocytoma or anaplastic astrocytoma.[11,12,] IDH-mutant GBMs have high rates of *ATRX* mutations (~80%), which are less common in IDH-wildtype GBMs (7%); rather, up to 83% of primary GBMs have frequent *TERT* promoter mutations.[13-15] Patients with IDH-wildtype GBMs have a median overall survival of 15 months, whereas patients with IDH-mutant GBMs have a median survival of 31 months.[16] TMZ, given concurrently with radiation post-operatively and adjuvantly (the Stupp regimen), increases overall survival in GBMs and is therefore the standard of care.[17]

Malignant Transformation of Low-Grade Gliomas

As a group, low-grade gliomas are histologically and biologically heterogeneous and are associated with marked diversity in survival times. Although some patients may survive for more than a decade, most tumors recur. At recurrence, a tumor can undergo malignant transformation to a high-grade glioma (grade III or grade IV), which are associated with worse prognosis.[18] On MRI, malignant transformation correlates with the development of focal contrast enhancement which is often used as a radiographic surrogate of malignant transformation in clinical practice.[19]

The interval between initial presentation and malignant transformation is highly variable, with an incidence ranging from 17 to 73% across several clinical studies and median interval after initial resection ranging from 2.1 to 10.1 years.[18] Known risk factors for malignant transformation of an initial low-grade glioma include larger preoperative tumor volume, higher tumor growth rate, and decreased extent of surgical resection.[20,21] Genetic alterations associated with malignant transformation in IDH-mutant gliomas include the acquisition at recurrence of genetic alterations in the RB and Akt-mTOR pathways, activation of the MYC and RTK-RAS-PI3K pathways, upregulation of the FOXM1- and E2F2-mediated cell cycle transitions, and epigenetic silencing of developmental transcription factors.[3,22] In a study of 204 patients with grade 2 gliomas treated prospectively on North Central Cancer Treatment Group (NCCTG) clinical trials,

malignant transformation occurred in 70% of low-grade astrocytomas compared to 45% in oligodendrogliomas.[23] Of note, this study was performed prior to molecular subtyping of gliomas and thus the proportion of malignant transformation in the corresponding molecularly defined groups may differ.

The Role of Chemotherapy in the Management of Gliomas

The role of TMZ treatment in patients with high-grade gliomas is well established. In general, the treatment paradigm for malignant gliomas is maximal safe surgical resection followed by adjuvant chemoradiation. The landmark EORTC-NCIC study published by Stupp *et al.* established that post-operative radiotherapy with concurrent and adjuvant TMZ improved survival in patients with GBM compared to adjuvant radiotherapy alone, and a subsequent study demonstrated improved survival with adjuvant TMZ even for elderly patients receiving adjuvant hypofractionated radiotherapy.[24,25] Studies in anaplastic oligodendrogliomas have demonstrated a benefit with the adjuvant procarbazine, lomustine (CCNU), and vincristine chemotherapy regimen, (PCV) while an ongoing study in patients with 1p/19q intact anaplastic tumors (CATNON) recently reported a survival improvement in patients receiving adjuvant TMZ.[26-28] RTOG 9813, a phase III study of radiotherapy with TMZ versus nitrosourea in adults with anaplastic astrocytoma, showed no significant difference in survival between the two treatment arms, but TMZ was better tolerated; 1p/19q status was not reported, but *IDH1* mutation was found to be prognostic for overall survival.[29] An ongoing study (CODEL) is directly comparing radiation plus concurrent and adjuvant TMZ versus radiation plus adjuvant PCV in patients with 1p/19q co-deleted, high-risk, grade II (with high risk defined as either age ≥40 and any surgical therapy or age <40 and subtotal resection or biopsy) and grade III gliomas.[30] Thus, TMZ in combination with adjuvant radiotherapy constitutes standard of care for all WHO grade IV and 1p/19q intact WHO grade III gliomas and soon the efficacy of TMZ will be known for 1p/19q co-deleted WHO grade III gliomas.

The clinical management of low-grade gliomas remains controversial, but is evolving rapidly. After resection, treatment options include observation, adjuvant radiation alone, chemotherapy alone, or radiation plus chemotherapy. Patients with low-grade glioma are typically young and have longer expected survival compared to patients with high-grade gliomas. Thus, treatment-related toxicities and quality of life are important clinical considerations. Early studies demonstrated that adjuvant radiotherapy improved progression-free survival compared to observation for patients with LGG, but that salvage radiotherapy at the time of progression yielded equivalent overall survival.[31] These results led many to advocate for initial observation after surgical resection in an effort to delay adjuvant radiotherapy and associated risk of long-term neurocognitive decline. More recent work has focused on tailoring adjuvant treatment based on individual risk factors, with the RTOG 9802 study demonstrating improved PFS and OS with the addition of adjuvant PCV to radiotherapy versus radiotherapy alone for patients with high-risk LGG, defined as <40 years with subtotal resection/biopsy or ≥40 years with any degree of resection.[32] Although IDH-mutation status was assessed post-hoc in a subset of patients, 1p/19q status was not reported, so the differences between IDH-mutated subtypes is unknown. Whether TMZ can be substituted for PCV chemotherapy

remains unproven. Furthermore, it is not clear how TMZ and PCV compare in the induction of hypermutation. In addition to RTOG 9813, RTOG 0424, a phase II study of radiation with concurrent and adjuvant TMZ, demonstrated promising results; many practitioners have extrapolated positive results with TMZ in malignant gliomas to the low-grade setting, given the favorable toxicity profile of TMZ.[29,33] The results of the CODEL study should determine whether TMZ is an appropriate substitute for PCV.

While RTOG 9802 clearly established a role for adjuvant chemotherapy in patients with LGGs, the study did not address the underlying controversy regarding the timing of radiation and chemotherapy. Appropriately selected patients could be considered for adjuvant chemotherapy alone, with combination chemoradiotherapy reserved for the time of progression, but it remains to be seen if this strategy yields survival results comparable to those observed in RTOG 9802. A recently published phase II study examined the utility of adjuvant TMZ alone in patients with high-risk LGG, and found that TMZ yielded a high rate of radiographic stability and meaningfully delayed the receipt of radiotherapy, with most patients on the study not having received radiation with a median follow-up of almost six years.[34] Long-term survival on the study was comparable to patients enrolled on RTOG 9802 receiving adjuvant radiation alone, but patients on the study were not salvaged with combined chemoradiotherapy, as would currently be considered standard of care. In an unplanned subgroup analysis, the study demonstrated particularly favorable results for patients with 1p/19q co-deletion and limited residual disease after surgical resection, suggesting that a selected subgroup of patients may be appropriate candidates for adjuvant TMZ alone. It is also important to note that malignant transformation was observed in 33 of 55 patients on the study that underwent surgical resection for progression after therapy, similar to previously observed rates of malignant transformation.[20,31,35] Thus, the appropriate role for adjuvant therapies in the management of LGG remains controversial. Future adjuvant treatment decisions for low-grade gliomas are likely to be based on an individual patient's risk profile, incorporating clinical, molecular, and radiographic features, and assuming biomarkers can be identified, an assessment of the risk of hypermutation.

The Cytotoxic Mechanism of TMZ

The mechanism of TMZ induced cytotoxicity and mutagenicity is well studied. TMZ is a lipophilic, monofunctional alkylating agent and an imidazotetrazine derivative of the alkylating agent dacarbazine. TMZ is administered orally and well-tolerated, with main toxicities of mild nausea, vomiting, and dose limiting myelosuppression.[36] Unlike dacarbazine, which requires metabolic activation, TMZ is a prodrug that is absorbed intact at acidic pH, allowing oral administration, but rapidly decomposes at pH >7 to form monomethyl triazene 5-(3-methyltriazen-1-yl)- imidazole-4-carboxamide (MTIC). MTIC further reacts with water to form 5-aminoimidazole-4-carboxamide (AIC) and the methyldiazonium cation. The methyldiazonium cation then preferentially adds methyl groups to DNA at N^7 positions of guanine in guanine rich regions (N^7-MeG), N^3 adenine (N^3-MeA) and O^6 guanine residues (O^6-meG).[37,38]

Although N^7-MeG is the major DNA adduct induced by TMZ, the cytotoxicity and mutagenicity is primarily attributed to the O^6-meG lesion.[38] O^6-meG lesions are directly

repaired in cells by the suicide enzyme, O^6-methylguanine DNA methyltransferase (MGMT), which removes the methyl adduct in a one-step alkyl transfer reaction that transfers the alkyl group from the oxygen in the DNA to the sulfur of a cysteine residue in the catalytic pocket of MGMT, thereby restoring guanine and inactivating MGMT. A single MGMT molecule can repair only one alkyl adduct, therefore the repair of O^6-meG adducts is dependent on the number of MGMT molecules per cell and on the rate of MGMT regeneration.[39]

Unrepaired O^6-meG DNA adducts are cytotoxic. The methylation of the O^6 position of guanine changes the normal hydrogen bonding of guanine with cytosine resulting in mispairing of guanine with thymine instead during DNA replication.[38] The DNA MMR machinery recognizes the mispairing of guanine with thymine and repairs the daughter strand, but leaves behind the O^6-meG in the template strand. This leads to repeated attempts by the MMR pathway to repair the same mismatched base in a process called futile cycling. Futile cycling results in DNA double-strand breaks and cell death.[40] Thus, cytotoxicity from TMZ is dependent on an intact MMR pathway and low levels of MGMT.

Indeed, in GBM patients, epigenetic silencing of the *MGMT* gene by promoter methylation is associated with longer overall survival when patients were treated with alkylating chemotherapy (either carmustine or TMZ) and radiation.[41,42,] A comprehensive characterization by The Cancer Genome Atlas (TCGA) consortium of more than 500 GBMs showed that the *MGMT* promoter methylation status distinguished TMZ responders from nonresponders in the classical subtype of GBM.[43] Hegi *et al.* showed that methylation of *MGMT* is associated with longer survival in patients with GBM who receive TMZ.[44] In agreement with these results, high MGMT protein staining in GBM patient samples is associated with TMZ resistance.[45] In the phase III EORTC-NCIC by Stupp *et al*., the addition of concomitant and adjuvant TMZ to adjuvant radiotherapy improved survival in GBM patients compared to adjuvant radiotherapy alone; *MGMT* promoter methylation was the strongest predictor for outcome and benefit from TMZ.[24] Yet, even for gliomas with unmethylated *MGMT* promoters, for which TMZ is less effective, clinical data showed a slight trend towards survival benefit with adjuvant TMZ. As a result, TMZ is often also given to patients with high-grade glioma with unmethylated *MGMT*.[44]

Mechanism of TMZ-induced Hypermutation

Alkylating agents, such as TMZ and procarbazine, are also mutagenic, due to the O^6-meG adducts they induce.[38,46] TMZ, like other alkylating agents, is a known carcinogen, with case reports linking TMZ use to the development of acute myelogenous leukemia and acute lymphoblastic leukemia in humans.[47,48] The stoichiometric limitation of MGMT-mediated repair of O^6-meG adducts renders MMR pathway status a critical determinant of whether tumor cells die or exhibit resistance in response to alkylating agents. In the context of cellular MGMT deficiency, futile cycling by intact MMR causes cell death, whereas loss of MMR function can lead to the mispairing of guanine with thymine.[46] Resistance to TMZ can arise in cells deficient in MMR, as unrepaired DNA damage can no longer lead to cell death in this context.[49,50]

In a radiographic study of untreated low-grade gliomas before and after TMZ, tumor growth was slower among untreated 1p/19q co-deleted tumors compared to untreated non-co-deleted tumors (3.4 vs 5.9 mm/year). Tumor growth was also slower among untreated tumors that do not overexpress p53, measured by immunohistochemistry, compared to untreated tumors overexpressing p53 (4.2 vs 6.3 mm/year).[51] After the administration of TMZ, the growth of both astrocytoma and oligodendroglioma subtypes were slowed. However, the duration of the effect was shorter in patients whose tumors overexpressed p53 (an immunohistochemical result that often but not always reflects *TP53* mutation)[52] and did not harbor 1p/19q co-deletion, suggesting a more accelerated acquisition of TMZ resistance in astrocytomas versus oligodendrogliomas and a potential relationship between *TP53* mutation and glioma cell response to TMZ.[51]

In the absence of MGMT-mediated repair and intact MMR, cells may incur a large number of G:C>A:T transitions throughout the genome upon DNA replication, leading to TMZ resistance and a "hypermutator phenotype" in recurrent tumors.[6,7] Spontaneous deamination of 5-methylcytosine to uracil can also cause G:C>A:T transitions, though at much lower rates and mainly at CpG dinucleotides.[53] Therefore, hypermutation is defined not only by the abundance of mutations, but also by a strong signature of G:C>A:T transitions at non-CpG sites.

The first report of hypermutation induced by alkylating agents in human GBMs was a targeted sequencing study of 518 protein kinases across 9 patients.[5] Consistent with a lack of MGMT-mediated repair, two patients with TMZ-treated, recurrent tumors with inactivating mutations in the MMR pathway gene *MSH6* had an increase in G:C>A:T transitions at non-CpG dinucleotides. The TCGA consortium also found 7 hypermutated recurrences from patients treated with TMZ or CCNU, which chloroethylates DNA. All hypermutated recurrences showed *MGMT* promoter methylation and higher proportions of non-CpG site G:C>A:T transitions.[4] MMR mutations in patients with *MGMT* promoter methylation were exclusively G:C>A:T transitions at non-CpG sites, suggesting MMR inactivation and hypermutation was a result of *MGMT* promoter methylation and chemotherapy-induced mutagenesis rather than spontaneous deamination. Felsberg *et al.* looked at changes in the *MGMT* promoter methylation status and the expression of MGMT and the MMR proteins, MLH1, MSH2, MSH6 and PMS2, in pairs of primary and recurrent GBMs of 80 patients who were treated with radiation and TMZ. Reduced expression of MMR proteins, but not changes in *MGMT* promoter methylation, was characteristic of GBMs recurring after standard of care treatment.[54] Hypermutation was not assessed in this study however. A recent comprehensive analysis of hypermutation across >81,000 adult and pediatric tumors describes a mutation signature based on mutations in a specific nucleotide context that identifies hypermutated brain tumors with alkylating-associated mutations.[55]

TMZ-induced Hypermutation and Malignant Transformation of Low-grade Astrocytomas

Johnson *et al.* performed a comparison of the mutational profile of 23 IDH-mutant, low-grade astrocytomas at initial diagnosis versus at tumor recurrence to determine the extent to which mutations in the initial tumors differ from their subsequent

recurrent tumors and how treatment with TMZ affects the mutational profile of recurrent tumors.[3] Exome sequencing was performed on primary tumor tissues and the patient matched tumor recurrences. Among the ten TMZ-treated low-grade astrocytomas, six harbored TMZ-induced hypermutation, composed of thousands of coding mutations which were not seen in the initial tumor prior to TMZ therapy (31.9 to 90.9 mutations per Mb). The vast majority of the new mutations were G:C>A:T transitions, the signature of TMZ-induced mutagenesis.[3,5] All six hypermutated tumors underwent malignant transformation to GBM and had acquired new somatic mutations in MMR genes as well as increased methylation of the *MGMT* promoter region.[56] Furthermore, TMZ-induced mutations consistently affected genes in the RB and Akt-mTOR signaling pathways, which are frequently deregulated in GBMs.[3,4] This preliminary study and others[22] led to the hypothesis that TMZ-induced hypermutation could drive malignant transformation in low-grade astrocytomas.

These studies proposed an evolutionary path for the TMZ-resistant tumor clones that repopulate hypermutated recurrences (Figure 1).[3,56] A TMZ-associated mutation (G:C>A:T transition unique to the post-TMZ recurrence) in at least one MMR gene was observed in five of six hypermutated recurrences.[3] In two cases, pre-existing heterozygous deletions encompassing *MGMT*, or an MMR gene, were followed by TMZ-associated mutations in one of the genes of interest. Hypermutated recurrences showed more than one thousand TMZ-related, clonal mutations in coding regions and had significantly higher average *MGMT* promoter methylation levels compared to TMZ-treated non-hypermutated recurrences, or compared to the untreated initial tumors. These studies suggest that during TMZ treatment, a selective advantage may exist for a minority of cells with higher *MGMT* promoter methylation once MMR activity is abrogated by TMZ-induced mutation. In this theoretical context, each resistant tumor cell has a mostly unique repertoire of mutations confering a distinct selective advantage, depending on which genes and pathways are altered. Indeed, alterations of PI3K signaling, the RB pathway, and cell cycle gene expression serve as one explanation for the dominant clones in many hypermutated recurrences.[3,57] The increased mutational load from TMZ includes new driver mutations that are linked to malignant transformation of IDH-mutant astrocytomas, though additional cases are needed to verify these trends.[3]

Outstanding Questions on Hypermutated Gliomas

These studies raise several clinically important questions. First, what is the true risk of TMZ-induced malignant transformation among IDH-mutant, low-grade gliomas treated with TMZ? Knowing the risk of TMZ-induced hypermutation is essential for patients and clinicians to accurately assess the risks and benefits of TMZ treatment. This may be difficult to quantify, however, as re-operations are reserved for selected patients, and thus hypermutation status cannot be determined directly in an unbiased cohort. Detection of tumor-associated mutations in cell-free DNA from blood or cerebral spinal fluid may represent a minimally-invasive technique that can address this bias.[58]

Are there patient or tumor factors that are predictive for risk of hypermutation? It is important to determine why hypermutation occurred in some, but not all astrocytomas treated with TMZ. Knowledge of markers of susceptibility to TMZ-induced hypermutation

could be key to strategies to prevent or delay malignant transformation in low-grade glioma patients treated with TMZ.

Is there a threshold dose and/or duration of TMZ exposure that increases the risk of TMZ-associated hypermutation? The time between the initiation of TMZ treatment and radiographic progression of a hypermutated tumor ranged from 12 to 90 months, and the duration of TMZ treatment also varied.[3] Clinical or biological factors underlying this wide range of latencies, from creation of the hypermutated cells during treatment to a clinically significant clonal expansion, have not been defined. It is important to identify a TMZ regimen that maximizes treatment benefit while minimizing the risk of TMZ-induced hypermutation and hematologic toxicities.

Do TMZ-induced hypermutated GBMs arising from initially low-grade, IDH-mutant gliomas have different prognoses compared to their non-hypermutated GBM counterparts? Hypermutation has been associated with improved prognosis in other cancer sites. Patients with MMR-deficient hypermutated sporadic colorectal cancers have improved outcomes over patients with non-hypermutated cancers of the same stage, although hypermutation in these cases are not treatment-related.[59] Hypermutated uterine serous carcinomas with somatic mutations in MMR genes and *POLE* exhibit a significantly better prognosis when compared to the non-hypermutated uterine serous carcinomas patients, although the reasons are unclear.[60] Wang *et al.* examined longitudinal genomic and transcriptomic data from 114 GBM patients and showed that 17 out of 100 GBM patients treated with TMZ recurred with hypermutated tumors. The median survival time of patients with hypermutated primary GBM with wildtype *IDH1* was 24 months, versus 18 months in other patients with GBM having wildtype *IDH1*, although it is unclear whether this difference was statistically significant.[61] Kim *et al.* identified 5 hypermutated GBMs from a cohort of tumor samples from 21 patients with pairs of primary and first recurrent GBMs.[8] Three of the patients with hypermutation were treated with TMZ and radiation, all five had *MGMT* promoter methylation and mutations in MMR genes and one was a secondary GBM with mutated *IDH1*; it is not clear if the three patients treated with TMZ had TMZ-induced mutations. The five patients with hypermutated GBMs survived for 35, 64, 107, 191, and 245 days after their second surgeries at recurrence compared to a previously reported median survival of 7.8 months after surgery upon first recurrence.[8,62] Although this data suggests that hypermutated GBMs may have worse clinical outcomes, conclusions cannot be drawn from the small sample size.

Are TMZ-induced hypermutated gliomas more sensitive to radiation or other chemotherapies? A study of germline ultra-hypermutated cancers with biallelic MMR deficiency and with loss of polymerase proofreading function suggested that there is an upper limit of 10,000 to 20,000 exonic mutations, above which further mutational burden may be incompatible with cell survival.[63] With the onslaught of additional DNA damage in the setting of a high mutational burden, hypermutated cancers may have increased sensitivity to DNA-damaging agents.[64] In addition, treatment of MGMT-deficient GBMs with TMZ introduces a strong selective pressure to lose MMR function. Radiation can induce a variety of DNA damage lesions, including DNA double-strand breaks, single strand breaks and oxidative base damage. However, the relationship between MMR deficiency and radiosensitivity is not straightforward; MMR proficiency has been

associated with radiosensitivity following low dose-rate IR whereas loss of MMR was associated with radiosensitivity following acute high dose-rate IR.[65]

In addition to its canonical role in the repair of base-base mismatches and insertion and deletion loops during replication, MMR proteins have non-canonical functions in the repair of oxidatively damaged DNA lesions and in modulating homologous recombination repair of DNA double-strand breaks, which may be therapeutically exploited.[66,67] Hewish *et al*. screened a pair of isogenic MLH1-deficient and MLH1-proficient cancer cell lines with a library of clinically used drugs and found that cytarabine was selectively toxic to MLH1- and MSH2-deficient tumor cells due to increased levels of cellular oxidative stress, suggesting that MMR-deficient cancers may be more sensitive to cytarabine-based chemotherapy regimens.[68] Martin *et al.* showed that inhibition of the base excision repair protein DNA polymerase POLB was synthetically lethal with MSH2 deficiency and inhibition of mitochondrial DNA polymerase POLG was synthetically lethal with MLH1 deficiency due to accumulation of the toxic and mutagenic oxidative DNA lesions 8-oxoG, providing a rationale for the development of POLB and POLG inhibitors.[69] In a small molecule screen to identify drugs that are selectively lethal to cells lacking functional MSH2, methotrexate was identified as being highly selective for cells with MSH2 deficiency, which was attributed to the persistence of 8-oxoG lesions in the MSH2-deficient cells.[70] Dietlan *et al.* performed a functional screen which identified a druggable synthetic lethal interaction between MSH3 deficiency and the DNA-dependent protein kinase catalytic subunit, an essential component of the machinery for nonhomolous end-joining repair of DNA double-strand breaks.[71] Aquilina *et al.* showed that methylation tolerant cell lines that were generated after multiple exposures to the alkylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) were more sensitive to CCNU compared to the parental cell line.[72] In addition, methylation tolerant cell lines generated after multiple exposures to the alkylating agent *N*-methyl-*N*-nitrosourea (MNU) that were also MMR deficient were more sensitive to CCNU than isogenic parental cell lines with functional MMR.[72] Sensitivity to CCNU and tolerance to MNU (via loss of MMR) were both detectable only when the MGMT repair protein was inactivated by the MGMT inhibitor, O^6-benzylguanine. The study did not identify the lesion induced by CCNU that is the substrate for MMR, but it may involve an interstrand DNA cross-link, which is the cytotoxic lesion formed by CCNU.[73] A more recent preclinical study by Strizelberger *et al.* showed similar findings in GBM cells; TMZ resistance mediated by MMR deficiency in MGMT-methylated GBM cells was accompanied by increased sensitivity to CCNU and to combined CCNU and TMZ.[74] Since MMR proficiency is inversely related to CCNU toxicity, hypermutated glioma cells may be sensitive to CCNU, although this remains to be determined. Interestingly, the combination of radiotherapy, CCNU, and TMZ has shown promising long-term survival data in patients with newly diagnosed glioblastoma, especially in the patients with MGMT methylated tumors.[75] Overall, exploiting the loss of the noncanonical functions of MMR may yield new therapeutic strategies for patients with hypermutated cancers.

Immune Checkpoint Inhibitors for Hypermutated Tumors

Immune checkpoint inhibition is an attractive therapeutic avenue that exploits the mutational burden and clonal mutational architecture of hypermutated tumors. There has been considerable enthusiasm regarding the success of immune checkpoint inhibitors in treating recurrences from a variety of cancer types. The most pronounced responses have been among tumors known to have high mutational burdens,[76] such as subsets of non-small cell lung cancers,[77-81] malignant melanomas,[82,83] renal cell carcinomas,[84] and MMR-deficient tumors.[85] Observations from a phase II clinical trial of immune checkpoint inhibitors among colorectal cancers and MMR-deficient extracranial malignancies support these hypotheses. Response rates of 40% were observed for MMR-deficient colorectal cancers, compared with 0% with MMR-intact colorectal cancers. Interestingly, all patients with somatic MMR deficits had objective responses to checkpoint inhibition, compared with 27% of patients with germline MMR deficits.[85] On May 23, 2017, the PD-1 inhibitor pembrolizumab was approved for the treatment of adult and pediatric patients with unresectable or metastatic, microsatellite instability-high or MMR-deficient solid tumors regardless of tumor site or histology. Importantly, this is the first time that a drug has received FDA approval based on a biomarker, agnostic of cancer site.[86]

Even among purportedly immunogenic tumors, not all patients have durable responses to checkpoint inhibition, and mixed responses are often observed. Biomarkers of response to immune checkpoint inhibitors have included PD-L1 staining and lymphocyte infiltration. However, the predictive power of these markers are variable across histologies and complicated by inconsistencies in immunohistochemistry technique.[87,88] More recently, neoantigen burden,[89] and tumor clonal architecture[90] have been proposed as novel genomic biomarkers for efficacious checkpoint inhibition. These studies have used whole exome sequencing data to predict potential non-self protein epitopes that are likely to bind and be presented on tumor cells by MHC-1. These findings suggest that checkpoint inhibition may stochastically amplify T-cell clones with specificity for a small number of tumor neoantigens. For tumors with highly branched evolution, T-cell specificity to some but not all tumor cells may result in partial responses to treatment. Thus, the extent of intratumoral heterogeneity of hypermutated glioma could be a critical factor in response prediction.[90]

Several immune-based therapies, including peptide vaccines, dendritic cell vaccines, and engineered T cell therapy are under active investigation for gliomas. Secondary GBMs with alkylator-induced hypermutation may elicit a robust immunogenic response. Given the high mutational burden and the clonal mutational architecture of TMZ-induced hypermutated GBMs, immune checkpoint therapies may be a promising therapy for GBMs with TMZ-associated hypermutation. Furthermore, these tumors are highly enriched for mutations in the Akt-mTOR pathway,[3] resulting in post-transcriptional increase in PD-L1 membrane presentation, an immune escape mechanism in these tumors.[91]

The potential for immune checkpoint inhibitors in hypermutated GBMs are highlighted by two case reports. A case report of two siblings with hypermutated GBMs arising from germline biallelic MMR deficits demonstrated clinically significant responses to the PD-1 inhibitor nivolumab.[92] This was the first report demonstrating durable responses of recurrent GBM to immune checkpoint inhibition. Another case report showed clinical response to the PD-1 inhibitor, pembrolizumab (MK-3475), in a patient

with a germline *POLE* mutation and GBM with PNET features who developed metastatic disease to the spine after receiving chemoradiation and maintenance TMZ.[93] Genomic analyses on the tissue from the pretreatment frontotemporal GBM and two spinal metastases showed that all samples were hypermutated and between 2,040 and 3,254 neoantigenic mutations were identified per sample. Immunohistochemical analyses performed on the resected spinal metastases before and three weeks after treatment with pembrolizumab showed cytolytic lymphocyte infiltration into the tumors post-pembrolizumab.[93] These reports provide support for the utility of immune checkpoint inhibitors in hypermutated gliomas and suggest that routine genomic testing for hypermutation may be useful for predicting increased sensitivity for immune checkpoint inhibitors. However, response to immune checkpoint inhibitors in gliomas with TMZ-induced hypermutation may differ compared to hypermutated gliomas arising from germline or somatic mutations in MMR genes or in *POLE* or *POLD.*

Immune checkpoint inhibitors are now being evaluated for newly diagnosed GBM (CHECKMATE-498,NCT02617589, and CHECKMATE-548, NCT02667587) and for recurrent GBM (CHECKMATE-143, NCT02017717). Of note, secondary GBMs are excluded from the CHECKMATE-498 and -548 trials. Based on Kim *et al.*, a low risk for TMZ-induced hypermutation in *IDH1*-wildtype primary GBMs under the Stupp regimen might be expected, though the requirement for a second surgery results in a biased cohort.[94] A recent study looked at tumor mutational burden, PD-1/PD-L1 expression and DNA MMR defects in 327 glioma patient samples and showed that these potential biomarkers of response to immune checkpoint inhibitors occur infrequently in gliomas; a high tumor mutation load was associated with loss of MLH1, MSH2, MSH6 and PMS2 proteins, but was only found in 3.5% of GBM patients.[95] These results underscores the importance of patient selection in clinical trials of immune checkpoint inhibitors in gliomas. Currently, pembrolizumab is being investigated in patients with recurrent malignant gliomas with a hypermutator phenotype (NCT02658279). “Hypermutator phenotype” in this study is defined as tumors with at least 30 mutations (non-synonymous, somatic, point or indel mutations) detected by the MSK-IMPACT or comparable next generation sequencing performed in a CLIA environment, or if tumors have a mutation in a MMR gene or in genes known to be associated with hypermutator phenotypes or microsatellite instability, including but not limited to *MLH1, MSH2, MSH6, PMS2, POLE* and *POLD*. This trial will help address the question of whether hypermutated gliomas (grades II-IV) are vulnerable to immune checkpoint inhibitor therapies.

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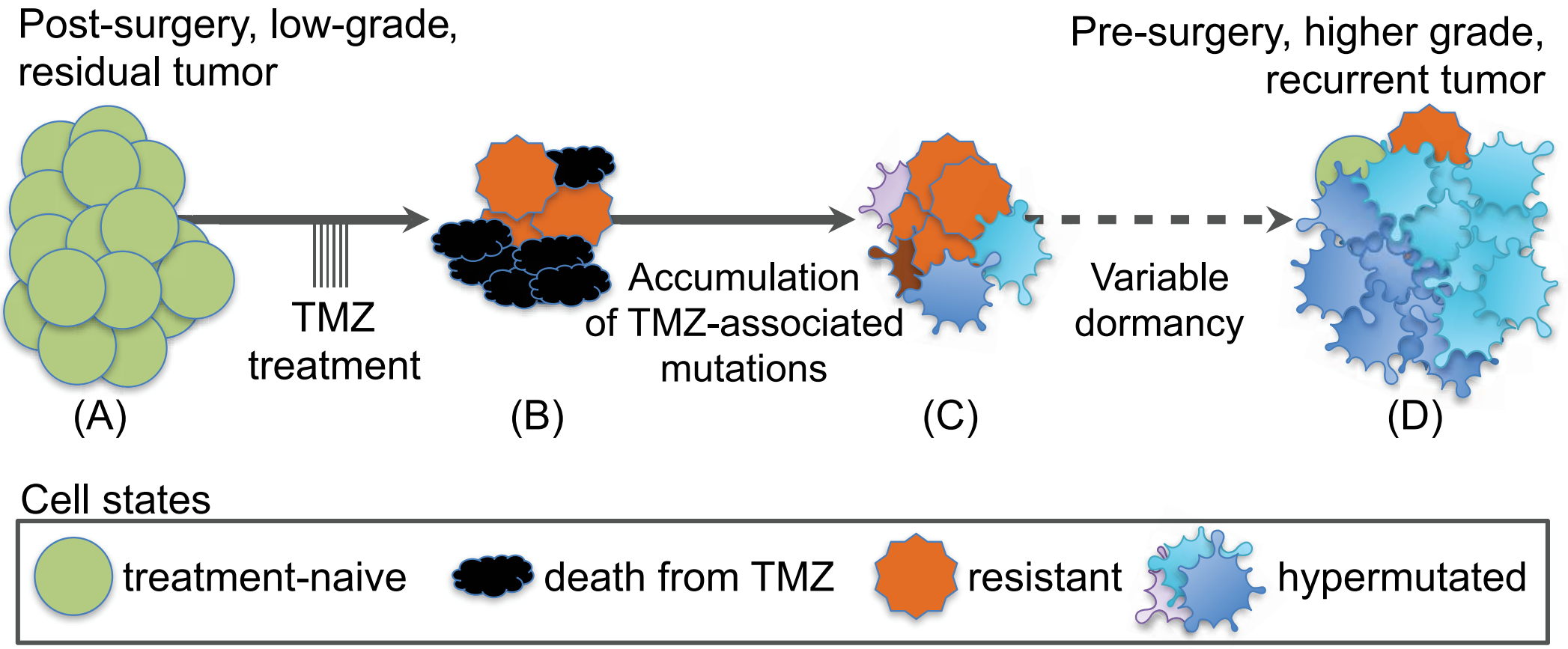
Fig. 1 Model of TMZ-associated malignant transformation. An initial low grade glioma (A) is resected and residual disease is treated with TMZ causing tumor cell death (B). If DNA repair capacity is low and TMZ-associated mutations occur within key amino acids of mismatch repair (MMR) genes, the loss of MMR function may render cells resistant to TMZ. Resistant cells can acquire high numbers of *de novo* TMZ-associated mutations, including thousands in coding regions, resulting in hypermutation (C). After widely varying periods of dormancy, clonal expansions of hypermutated cells drive formation of higher grade tumor recurrences. Multiple unique hypermutated tumor clones may expand concurrently, as depicted by the different colored groups of hypermutated cells (D).

Figure


Post-surgery, low-grade, residual tumor
Pre-surgery, higher grade, recurrent tumor
TMZ treatment
Accumulation of TMZ-associated mutations
Variable dormancy
(A)
(B)
(C)
(D)
Cell states
treatment-naive
death from TMZ
resistant
hypermutated