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Restoration of Temozolomide Sensitivity by Poly(ADP-Ribose) Polymerase inhibitors in Mismatch Repair Deficient Glioblastoma is Independent of Base Excision Repair

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Abstract

Purpose: Emergence of mismatch repair (MMR) deficiency is a frequent mechanism of acquired resistance to the alkylating chemotherapeutic temozolomide (TMZ) in gliomas. Poly(ADP-ribose) polymerase inhibitors (PARPi) have been shown to potentiate TMZ cytotoxicity in several cancer types, including gliomas. We tested whether PARP inhibition could re-sensitize MSH6-null MMR-deficient gliomas to TMZ, and assessed the role of the base excision repair (BER) DNA damage repair pathway in PARPi-mediated effects.

Methods: Isogenic pairs of MSH6 wild-type and MSH6-inactivated human glioblastoma (GBM) cells (including both *IDH1/2* wild-type and *IDH1* mutant), as well as MSH6-null cells derived from a patient with recurrent GBM were treated with TMZ, the PARPi veliparib or olaparib, and combination thereof. Efficacy of PARPi combined with TMZ was assessed *in vivo*. We used genetic and pharmacological approaches to dissect the contribution of BER.

Results: While having no detectable effect in MSH6 wild-type GBMs, PARPi selectively restored TMZ sensitivity in MSH6-deficient GBM cells. This genotype-specific restoration of activity translated *in vivo*, where combination treatment of veliparib and TMZ showed potent suppression of tumor growth of MSH6-inactivated orthotopic xenografts, compared with TMZ monotherapy. Unlike PARPi, genetic and pharmacological blockage of BER pathway did not re-sensitize MSH6-inactivated GBM cells to TMZ. Similarly, CRISPR PARP1 knockout did not re-sensitize MSH6-inactivated GBM cells to TMZ.

Conclusions: PARPi restoration of TMZ chemosensitivity in MSH6-inactivated glioma represents a promising strategy to overcome acquired chemoresistance caused by MMR

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deficiency. Mechanistically, this PARPi-mediated synthetic phenotype was independent of BER blockage and was not recapitulated by loss of PARP1.

Keywords

Glioma; temozolomide; PARP inhibitor; Mismatch Repair; Base excision repair

Introduction

Mismatch repair (MMR) is one of the critical pathways responsible for repair of base-base mismatches arising during DNA replication or otherwise caused by DNA damage. MMR deficiency resulting from inactivating MMR gene mutations not only predisposes cells to tumorigenesis, but also affects a tumor cell's response to DNA damaging agents. MMR deficiency causes drug resistance to alkylating agents that mediate the formation of O6 methylguanine-containing mismatches(1). Mechanistically, the recognition of alkylator-induced O6meG:T mismatches by the multiprotein complex MutSα (comprised of the heterodimer of MSH2 and MSH6) is thought to result in repeated replacement of the mispaired thymidine, rendering a futile cycle of replication/repair, and ultimately programmed cell death(2,3). In the absence of a functional MMR response, O^6-meG:T is not targeted for this "futile repair" cycle, and the cells avoid programmed cell death and survive, albeit with the accumulation of a massive number of DNA mis-pairs, resulting in a characteristic cytidine-to-thymidine "hypermutator" phenotype(4).

Temozolomide (TMZ) is the most commonly used alkylator for gliomas, with significant clinical activity in both lower-grade tumors carrying isocitrate dehydrogenase 1 (*IDH1*) mutations(5,6), as well as primary *IDH1*-wild-type glioblastoma (GBM) exhibiting methylation of the O6-methyguanine DNA methyltransferase (*MGMT*) promoter(7,8). Unfortunately, prolonged treatment with TMZ is often followed by the development of acquired resistance to TMZ, contributing to malignant progression, tumor recurrence and mortality. Inactivation of mismatch repair (MMR) genes, i.e., *MSH2*, *MSH6*, *MLH1* and *PMS2*, has been identified in both IDH mutant(9,10) and IDH wild-type(11,12) recurrent malignant gliomas that have been previously treated, whereas these alterations are extremely rare in primary tumors(13,14). Studies have identified MSH6 inactivation as a key molecular mechanism of acquired TMZ resistance in glioma cells(15), and strong association between TMZ treatment and the development of MMR deficiency in patient tumors(16). Furthermore, MMR alterations after TMZ treatment of low-grade gliomas are considered a driver for malignant progression to higher grade and a post-TMZ hypermutator phenotype(14).

To potentiate TMZ cytotoxicity, combination therapies that modulate DNA repair pathways have been evaluated as a potential strategy to overcome TMZ resistance. TMZ induces not only Ome6G but also large numbers of N3-methyladenine and N7-methylguanine adducts that are rapidly processed by the base excision repair (BER) DNA repair pathway. N3-methyladenine and N7-methylguanine lesions activate the nuclear enzyme poly (ADP-ribose) polymerases (PARPs) which synthesize poly(ADP-ribose) chains (PARylation) and facilitate the recruitment of XRCC1, pol-beta and DNA ligase to the DNA strand break sites,

thus playing an critical role in the initiation of BER machinery. PARPi combinations have been shown to increase TMZ sensitivity in the treatment of various cancer cell types(17–20), and are under active investigation in IDH wild-type(21,22) and IDH mutant(23,24) gliomas. Because of the documented role of PARP in single-strand break repair and BER, PARP inhibitor (PARPi) mediated potentiation of TMZ sensitivity has been speculated to be due to its inhibition of BER, nevertheless direct evidence is lacking.

Here, we show that PARPis restore TMZ sensitivity in MSH6-inactivated, MMR-deficient TMZ resistant gliomas (both IDH wild-type and IDH mutant) *in vitro* and *in vivo*. Notably, this restoration effect is selectively observed in MSH6-inactivated cells, representing a genotype-specific synthetic phenotype. Furthermore, PARPi mediated restoration of TMZ sensitivity is independent of BER, as we show that genetic and pharmacological blockage of BER pathway enzymes and PARP1 knock-out fail to recapitulate this phenotype.

Materials and Methods

Cell and Compounds

Human glioblastoma cell line LN229 was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and was authenticated in 2017 by comparison of STR profile to the ATCC public dataset. Gli36 was provided by Dr. Khalid Shah, Boston, MA. Normal Human Astrocyte (NHA) was purchased from ScienCell. LN229 and NHA were maintained in Delbecco's modified Eagle medium (DMEM) with 4.5g/L glucose, L-glutamine and sodium pyruvate supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin/Amphotericin. Patient-derived glioma neurosphere lines (MGG4, MGG123, MGG152) were established from patient tumors and cultured in serum-free neural stem cell medium as described previously.(25–27) Temozolomide and Methoxamine were purchased from Sigma. Veliparib was from APE-BIO, Olaparib was from Selleckchem and APE inhibitor was from EMD MILLIPORE.

Cell viability assay

Cells were seeded in 96-well plates at 1,000–2,000 cells per well. After overnight incubation, compounds were serially diluted and added to wells. Cell viability was evaluated on day 6 by Cell Titer Glo (Promega) according to the manufacturer's instruction.

Western blot analysis

Cells were lysed in radioimmunoprecipitation (RIPA) buffer (Boston Bioproducts) with a cocktail of protease and phosphatase inhibitors (Roche). Protein (10–15 ug) was separated by 4–20% SDS-PAGE and transferred to polyvinylidene difluoride membranes by electroblotting. After blocking with 5% non-fat dry milk in TBS-T (20 mM Tris [pH,7.5] 150mM NaCl, 0.1% Tween20) for 1–2 hours at room temperature, membranes were incubated with primary antibody at 4°C overnight. Membranes were washed in TBS-T and incubated with appropriate peroxidase conjugated secondary antibodies for 1 hour at room temperature. Signals were visualized using the enhanced chemiluminescense (ECL) kit (Amersham Bioscience). Primary antibodies used were: MSH6 (#5425), XRCC1 (#2735) (Cell Signal Technology), PARP1 (sc-8007)(Santa Cruz) and β-actin (A1978)(Sigma).

MSH6-shRNA, XRCC1-shRNA and PARP1-knockout cell lines generation

Lentivirus vector plasmids containing shRNA sequences for MSH6 (TRCN0000286578 shMSH6no.1) and XRCC1 (TRCN0000007913 no.1, TRCN0000011211 no.2), and non-targeting shRNA sequence (SHC002 shNS) were obtained from Sigma. shMSH6no.2, a second shRNA for MSH6, was from Dharmacon (V3LHS 318784). For *PARP1* knockout, lentivirus vector plasmids containing SpCas9 (pLentiCRISPR v2) with/without sgRNA sequences for *PARP1* (No.1:TTCTAGTCGCCCATGTTTGA, No.2: CCCCTTGCACGTACTTCTGT) were obtained from GenScript. To generate lentiviral particles, 293T cells were transfected with a lentiviral plasmid, packaging plasmid (pCMV-dR8.2), and envelope plasmid (pCMV-VSV-G) with FuGene (Promega). Cells were infected with lentivirus in the presence of polybrene (8 μg/ml) for 8 hours. Three days later, cells were selected with puromycin (0.6 μg/ml for LN229, 0.2 μg/ml for Gli36, MGG4 and MGG152) for 3–4 days before use. Knockdown and knockout were confirmed by western blot.

Animal Study

Five million LN229-shNS cells and LN229-MSH6sh1 cells were implanted subcutaneously into the flank of 7-week-old female nude mice (NCI). When the maximum diameter of established tumors reached 5 mm, mice were randomized and treated with PBS (n=6), TMZ (n=6, 50mg/kg i.p.), Veliparib (n=6, 50mg/kg oral gavage), TMZ plus Veliparib (n=6) 5 consecutive days per week for 2 cycles with one week interval before the second treatment cycle. TMZ was dissolved in DMSO, diluted with PBS and injected intraperitoneally. Veliparib was dissolved in DMSO, diluted with 10% 2-hydroxyl-propul-β-dextrine/PBS and administrated orally. Tumor diameters were measured twice a week using a digital caliper. Tumor volumes were calculated using the formula: tumor volume (mm^3) = tumor length × tumor $width^2$ /2. Orthotopic model of MSH6-deficient glioblastoma was established by intracerebral implantation of 5×10^5 LN229-MSH6sh1 cells in 7-week-old female nude mice (NCI) as previously described.(26) Seven days later, mice were randomized and treated with PBS (n=5), TMZ (n=5), TMZ plus Veliparib (n=5) at the same doses and schedules as the flank model. Animals were euthanized when significant deterioration of neurological or general conditions was noted. All animal procedures were approved by Institutional Animal Care and Use Committee at Massachusetts General Hospital.

Statistical Methods

The Prism (GraphPad) software package was used for statistical analysis. Comparisons between 2 groups were done with Student *t*-test (unpaired). Differences of tumor volumes were analyzed by non-parametric Mann-Whitney test. Survival analysis was performed using the Kaplan-Meier method, and the log-rank test (two-sided) was used to compare survival differences between treatment arms. *P* values less than 0.05 were considered statistically significant.

RESULTS

PARP inhibitors restored TMZ sensitivity in MSH6-inactivated glioblastoma cell lines and patient-derived MSH6 deficient glioblastoma cells.

We used lentivirus vectors expressing MSH6 shRNA or control (non-targeting, NS) shRNA to establish an isogenic model system of MSH6 inactivation using GBM cell lines (LN229 and Gli36) and patient-derived GBM sphere lines, (MGG4, primary *IDH1* wild-type GBM; and MGG152, recurrent, *IDH1* mutant GBM)(28)(Figure 1A–C, Supplementary Fig.1D). As expected, these shMSH6 cell lines showed TMZ resistance across a wide range of TMZ doses (Supplementary Fig. 1A–D). MSH6 knockdown did not alter sensitivity to PARPi monotherapy in LN229 (Figure 1D). Combination treatment with TMZ and PARPi, at PARPi doses only marginally (<20%) cytotoxic as monotherapy, greatly restored TMZ sensitivity across shMSH6 GBM cell lines (Figure 1A–C, Supplementary Fig.1E). Importantly, PARPis had no, or only marginal, additional effects on TMZ-induced reduction of cell viability in MSH6 proficient control (shNS) GBM cells (Figure 1A–C, Supplementary Fig.1E). Testing of a second independent shRNA sequence targeting MSH6 in LN229 and Gli36 showed induction of TMZ resistance and similar restoration of TMZ sensitivity by PARPis, suggesting on-target effects of the two shRNAs (Supplementary Fig. 1F–I). The second shRNA for MSH6 also confirmed that MSH6 silencing did not meaningfully alter response to PARPis (Supplementary Fig. 1J). Next, we tested PARPis in a patient-derived GBM neurosphere line (MGG123) that was derived from a GBM that recurred after TMZ treatment. We confirmed that MGG123 had loss of MSH6 and exhibited high resistance to TMZ (Figure 1E). In accord with the results with MSH6 knockdown GBM cells, PARPi treatment restored response to TMZ in MSH6 deficient MGG123 even when PARPi monotherapy had a marginal effect (Figure 1F). The combination of TMZ and veliparib had only modest toxicity for proliferating human normal astrocytes compared with TMZ monotherapy (Figure 1G).

Veliparib combination with TMZ inhibits tumor growth of MSH6-deficient glioblastoma compared with TMZ monotherapy

To assess the effects of veliparib combination with TMZ on MSH6-deficient GBM *in vivo*, we treated nude mice bearing LN229-shNS and LN229-shMSH6 flank tumors with vehicle, veliparib alone, TMZ alone, or TMZ combination with veliparib. Veliparib alone had no effect on tumor growth compared to vehicle in both LN229-shNS and LN229-shMSH6 tumor models, consistent with the results obtained *in vitro*. Both TMZ and combination showed potent inhibition of LN229-shNS tumor growth – adding veliparib to TMZ provided no significant therapeutic benefit to TMZ alone (Figure 2A). In the LN229-shMSH6 tumor model, however, the combination treatment of veliparib with TMZ significantly inhibited tumor outgrowth better than TMZ monotherapy. (*P=0.019, day32 TMZ vs TMZ + veliparib, student t-test), (Figure 2A). We further tested the effect of veliparib combination with TMZ in mice harboring intracerebral LN229 MSH6-knockdown GBM xenografts. We omitted the group of veliparib monotherapy given the lack of efficacy in flank tumor models. One week after intracerebral implantation, animals started receiving TMZ alone or TMZ combined with veliparib treatment. Veliparib combination with TMZ resulted in an extension of animal survival (P=0.02; long lank test), compared with TMZ alone that did not

extend survival in this model (Figure 2B). During the treatment, animals receiving the regimens involving TMZ lost 10% body weight, however they recovered promptly after completion of treatment. Combining veliparib with TMZ did not confer additional toxicity as the body weights of TMZ and combination groups were comparable (Figure 2C).

PARP inhibitor restoration of TMZ sensitivity is independent of BER signaling blockage.

PARPis including veliparib potentiate TMZ-induced cytotoxicity in diverse tumor types(17–20). A proposed mechanism of this PARPi effect is mediated via inhibition of DNA single-strand break (SSB) repair via interfering with BER pathway (Figure 3A). The BER inhibitors Methoxyamine (MeOX) and APE inhibitor (APEi) have been reported to potentiate the cytotoxicity of alkylating agents including TMZ by disrupting BER through an accumulation of mutagenic and toxic apurinic/apyrimidinic (AP) sites (APEi) or preventing AP endodeoxyribonuclease cleavage (MeOX)(29–32)(Figure 3A). Therefore, we tested APEi and MeOX to determine whether these BER pathway inhibitors could recapitulate the observed PARPi mediated re-sensitization to TMZ in LN229-shMSH6 and Gli36-shMSH6 cells. APEi alone was less cytotoxic to MSH6 silenced Gli36 cells as compared with control cells (Figure 3D). To our surprise, unlike PARPi, neither of these BER repair-modulating drugs re-sensitized MSH6 knockdown cells to TMZ, even at concentrations that displayed signs of toxicity as a single agent (Figure 3B–E).

The scaffold protein XRCC1 plays a major role in coordinating BER by interacting with polymerase beta, PARP and DNA ligase III at sites of DNA damage (Figure 3A). XRCC1-deficient mouse embryonic fibroblasts have been shown to exhibit hyper-sensitivity to alkylating agents (33). We evaluated whether shRNA knockdown of XRCC1 altered TMZ sensitivity in our isogenic GBM cells with and without MSH6 knockdown (Figure 3F). XRCC1 knockdown did not alter the TMZ resistant phenotype of shMSH6 GBM cells and this was consistent with two independent shRNA constructs (Figure 3G,H, Supplementary Fig. 2A, B). With intact MSH6, XRCC1 silencing rendered GBM cells slightly but significantly less responsive to TMZ (Figure 3G,H, Supplementary Fig. 2A, B), unlike what was reported with Xrcc1−/− mouse embryonic fibroblasts (33,34). XRCC1 knockdown did not significantly alter response to PARPi in MSH6 wild-type and knockdown GBM cells. (Figure 3GH, Supplementary Fig. 2C). Notably however, both veliparib and olaparib retained the ability to restore TMZ sensitivity in GBM cells that had dual knockdown of MSH6 and XRCC1 (Figure 3G,H); we did not observe additional therapeutic benefit by silencing XRCC1. These results indicate that PARPi restoration of TMZ sensitivity in MSH6 inactivated GBM cells was not due to its blockage of BER signaling.

PARP inhibitor restoration of TMZ sensitivity is independent of PARP1

The biological effects mediated by PARPis are distinct from PARP knockdown, and PARPis often confer greater cytotoxicity than PARP1 knockdown when combined with TMZ (35,36). PARPis physically impair the dissociation of PARP from DNA, which is required for the proper progress of repair process, and this “PARP trapping” at DNA damage sites has been shown, in certain contexts, to be more cytotoxic than persistent SSBs in the absence of PARP(37). PARPi have varying degrees of “PARP trapping” (olaparib greater than veliparib), and the possible involvement of this mechanism motivated us to test whether

PARP1 knockdown restores TMZ sensitivity in MSH6-deficient cells as was observed with PARPis.

We used CRISPR-Cas9 lentivirus to inactivate PARP1 in our isogenic LN229 cells with and without MSH6 inactivation (Figure 4A). Two independent sgRNAs for PARP1 consistently rendered shMSH6 GBM cells less responsive to PARPis, reflecting loss of their main target (Figure 4B). Strikingly, PARP1 knockout did not alter responsiveness to TMZ in both MSH6 intact and MSH6 deficient GBM cells (Figure 4C, D). Furthermore, analogous to what was observed with BER inhibitors, both veliparib and olaparib re-sensitized GBM cells with double silencing of MSH6/PARP1 to TMZ (Figure 4E, F). These results show that PARP1 knockout does not recapitulate the PARPi-mediated effect of TMZ re-sensitization in MSH6 deficient GBM cells.

DISCUSSION

Multiple National Cancer Institute (NCI) consortia sponsored trials are investigating the potential efficacy of PARPi in combination with TMZ in both newly-diagnosed (NCT02152982, NCT00770471) and recurrent (NCT01026493, NCT01390571) GBM (38). Notably, preclinical studies have shown that gliomas can have upfront variability to PARPi when combined with TMZ, with TMZ-sensitive MGMT promoter methylated GBMs noted to be responders(21,22,39). It is important to understand mechanisms underlying the potentiation effect by PARPi and to identify molecularly defined subsets of gliomas that likely benefit from this combination treatment.

We here show that PARPi restores TMZ cytotoxicity selectively in GBM cells characterized by MMR-mediated TMZ resistance, both *in vitro* and *in vivo*. MSH6 knockdown did not alter sensitivity to PARPi, and PARPi monotherapy itself had little effect in either MMR deficient or wild-type cells. Therefore, enhanced cytotoxicity was not the result of a traditional synthetic lethal interaction, as described previously for PARP inhibition and homologous recombination repair deficiency resulting from BRCA gene alterations(40) or IDH mutations(23,24). Our findings are consistent with reports showing that PARPi potentiation of TMZ cytotoxicity is limited to tumors that are intrinsically resistant to TMZ or have acquired resistance(41,42). Our studies conclusively demonstrate, in experiments using isogenic cell models across a large panel of GBM lines, and in an orthotopic model, that MMR deficiency arising in gliomas from MSH6 inactivation is a unique molecular alteration predicting PARPi restoration of TMZ chemosensitivity. Importantly, relatively low concentrations of PARPi were sufficient to re-sensitize MMR-deficient glioma cells to TMZ, contrasting to negligible combination effect seen in MMR-proficient control cells at the same PARPi concentrations. Together with significant *in vivo* efficacy and safety in our orthotopic mouse model, our results suggest a wide therapeutic window that could be clinically meaningful for dosing regimens that otherwise circumvent toxicity issues reported in early veliparib combination trials (43).

From a mechanistic standpoint, it is well-established that the majority of TMZ-induced adducts are N3-methyladenine and N7-methylguanine which are rapidly processed by BER machinery, whereas O6meG adducts, despite accounting for only 5% of total lesions, are

responsible for TMZ cytotoxicity. In the combination treatment of PARPi with TMZ, PARPi has been postulated to impair BER by inhibiting PARP-mediated PARylation and cause cytotoxicity through increasing N3-methyladenine and N7-methylguanine-induced damages (44,45). In accord with this working hypothesis, alterations to BER enzymes result in cell death by an accumulation of toxic and mutagenic BER intermediates after treatment with alkylating agents(46). Disruption of BER via APE inhibition and XRCC1 inactivation might be expected to therefore lead to the restoration or potentiation of TMZ sensitivity. However, our results show that BER perturbation by APE inhibition and XRCC1 inactivation does not recapitulate the effects of PARPis in MMR deficient, TMZ-resistant glioma cells. Furthermore, PARPi restoration of TMZ responsiveness was not affected by XRCC1 genetic inactivation, and there was no benefit in combining PARPi and XRCC1 depletion, consistent with the combination effects of PARPi 4-AN and TMZ seen in Xrcc1−/− mouse embryonic fibroblasts(34). Together, these data strongly suggest that PARPi-mediated re-sensitization to TMZ in MMR-deficient glioma cells is independent of BER pathway inactivation.

PARPi exert anti-cancer effects via not only catalytic inhibition of PARPs but also trapping PARPs on sites of DNA damage, and these diverse PARPi functions explain why PARPi and PARP1 knockout often have different biological effects. Indeed, we observed a striking discrepancy between the phenotypes of PARPi when compared to genetic PARP1 knockout as only the former not the latter sensitized MSH6 deficient GBM cells to TMZ. We found that PARPi (particularly olaparib) combination therapy with TMZ for MMR deficient glioma does not appear to depend on the expression levels of PARP1, as a mediator of PARP-trapping damage signaling, further suggesting that PARP1 trapping may not play a major role in this phenotype. Notably, veliparib, which has a lesser trapping ability than olaparib, was still capable of restoring sensitivity to TMZ in the absence of PARP1. The known targets of veliparib and olaparib include PARP1 and 2 (47,48), but these inhibitors could cross-target other PARP enzymes, as has been reported for the ability of olaparib to inhibit PARP3 (49). Thus, while catalytic inhibition of PARP1 alone is not sufficient, catalytic inhibition and/or selective trapping of other PARP family members is necessary for producing chemo-sensitization of MMR-deficient glioma cells. Although the functions of many of seventeen known PARP family members have not been characterized, PARP2, 3 and 10 (50,51) have been shown to participate in DNA damage repair. These PARPs are potential candidate mediators of the observed PARPi-mediated restoration of TMZ sensitivity in MMR-deficient cells.

In summary, we demonstrate that PARPi combination with TMZ selectively restores chemosensitivity in MSH6-inactivated, MMR-deficient GBM cells. These results identify a genetically-defined subgroup of recurrent gliomas which may benefit from combination therapy of TMZ and PARPis. Our findings therefore support the assessment of TMZ combination with veliparib, or olaparib which is also brain penetrant(52), in glioma patients selected for MMR deficiency. From a mechanistic standpoint, we also show that this restoration effect does not require PARP1 and is independent of BER pathway, which has significant implications for our understanding of the pathways underlying the combination treatment effect. Further studies are needed to understand the precise mechanisms of PARPi chemo-restoration to dissect the functions of PARPs that are targeted by this PARPi effect.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance:

There is a keen interest to investigate the efficacy of PARPi combination with TMZ in gliomas, and multiple National Cancer Institute (NCI) consortia sponsored trials are underway. However, preclinical studies in gliomas have shown variable responses to the combination treatment, and molecular biomarkers to identify subgroups which benefit from PARPi combination with TMZ are still elusive. Meanwhile, multiple studies have identified MSH6 inactivation as a key molecular mechanism of acquired TMZ resistance in glioma cells *in vitro*, and the development of MMR deficiency in post-treatment gliomas in clinical tumor specimens. We here show MMR deficiency mediated by MSH6 inactivation is a unique molecular alteration predicting PARPi restoration of TMZ chemosensitivity. Furthermore, we demonstrate that the PARPi re-sensitization effect is independent of BER blockage, revealing a distinct therapeutic impact of PARPi.

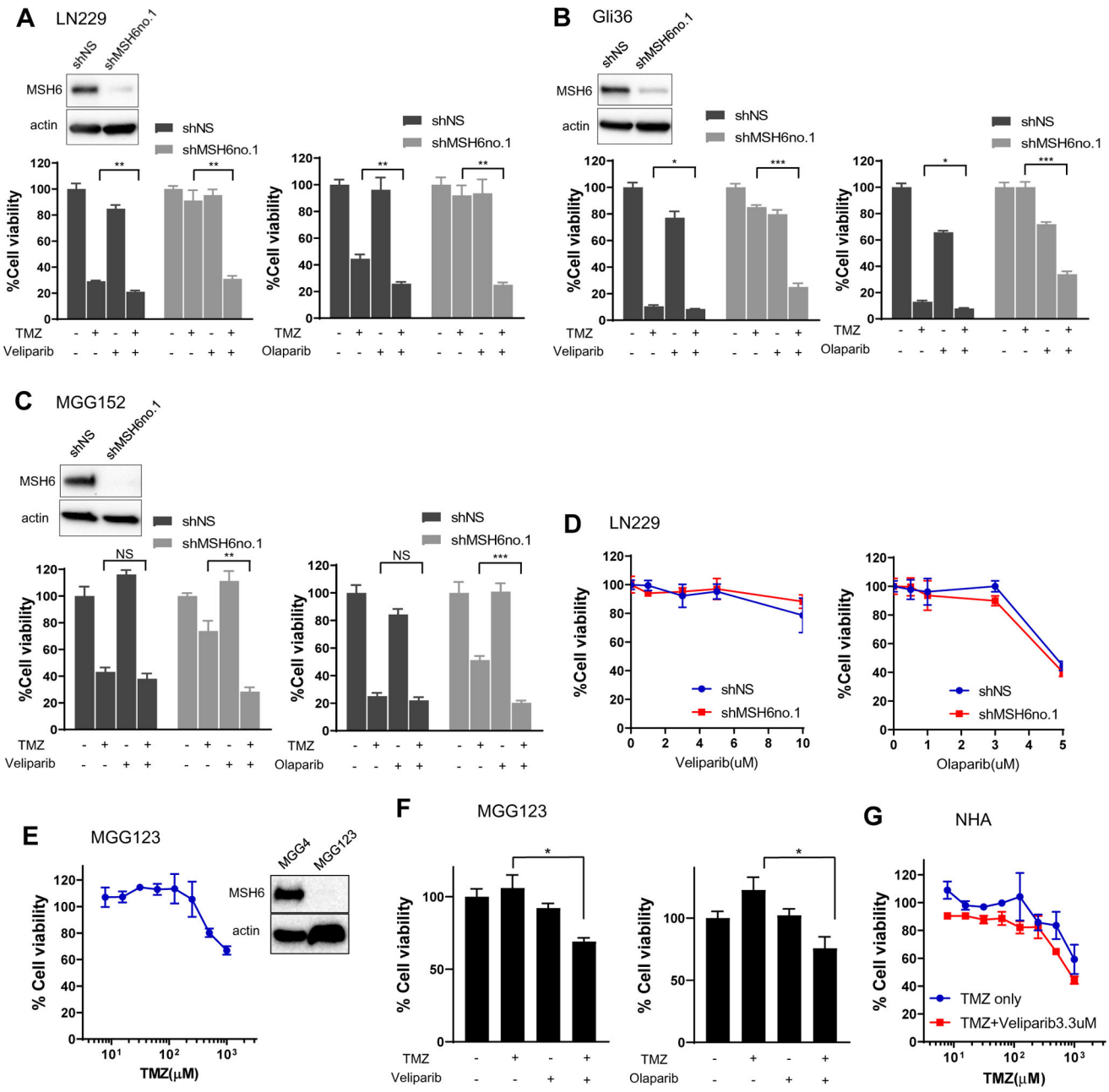

Figure 1. PARP inhibitors restore sensitivity to temozolomide in MSH6-inactivated, temozolomide resistant glioblastoma cells.

(A-D) Glioblastoma (GBM) cell lines (LN229 and Gli36) and patient-derived GBM sphere lines (MGG152) were engineered with a non-targeting shRNA (shNS) or MSH6-directed shRNA (shMSH6no.1) lentivirus. Immunoblot confirmed MSH6 knockdown, with Actin as a loading control. Cells were treated with temozolomide (TMZ), Veliparib/Olaparib or TMZ combination with Veliparib/Olaparib, and cell viability was evaluated by Cell Titer Glo on day 6. A: LN229 (TMZ 200 uM, Veliparib 3 uM, Olaparib 1uM) B: Gli36 (TMZ 30 uM, Veliparib 1 uM, Olaparib 0.5uM) C: MGG152(TMZ 200 uM, Veliparib 3 uM, Olaparib 1uM). * P<0.05, **P<0.001, *** P<0.0001 (student *t*-test). (D) Cell viability assay for

Veliparib(left)/Olaparib(right) dose response in LN229shNS and LN229shMSH6no.1. Cell viability was evaluated by Cell Titer Glo on day 6. (E) Immunoblot showing loss of MSH6 in MGG123 with MGG4 as a positive control. Cell viability assay to determine TMZ dose response in MGG123. Cells were treated with specified concentrations of TMZ, and cell viability was evaluated by Cell Titer Glo on day 6. (F) MGG123 cells were treated with TMZ, Veliparib/Olaparib, or TMZ combination with Veliparib/Olaparib, and cell viability was evaluated by Cell Titer Glo on day 6. ** $P<0.005$ (student *t*-test). (G) Cell viability assay for TMZ dose response with/without Veliparib (3.3 uM) in normal human astrocytes (NHA). Cell viability was evaluated by Cell Titer Glo on day 6.

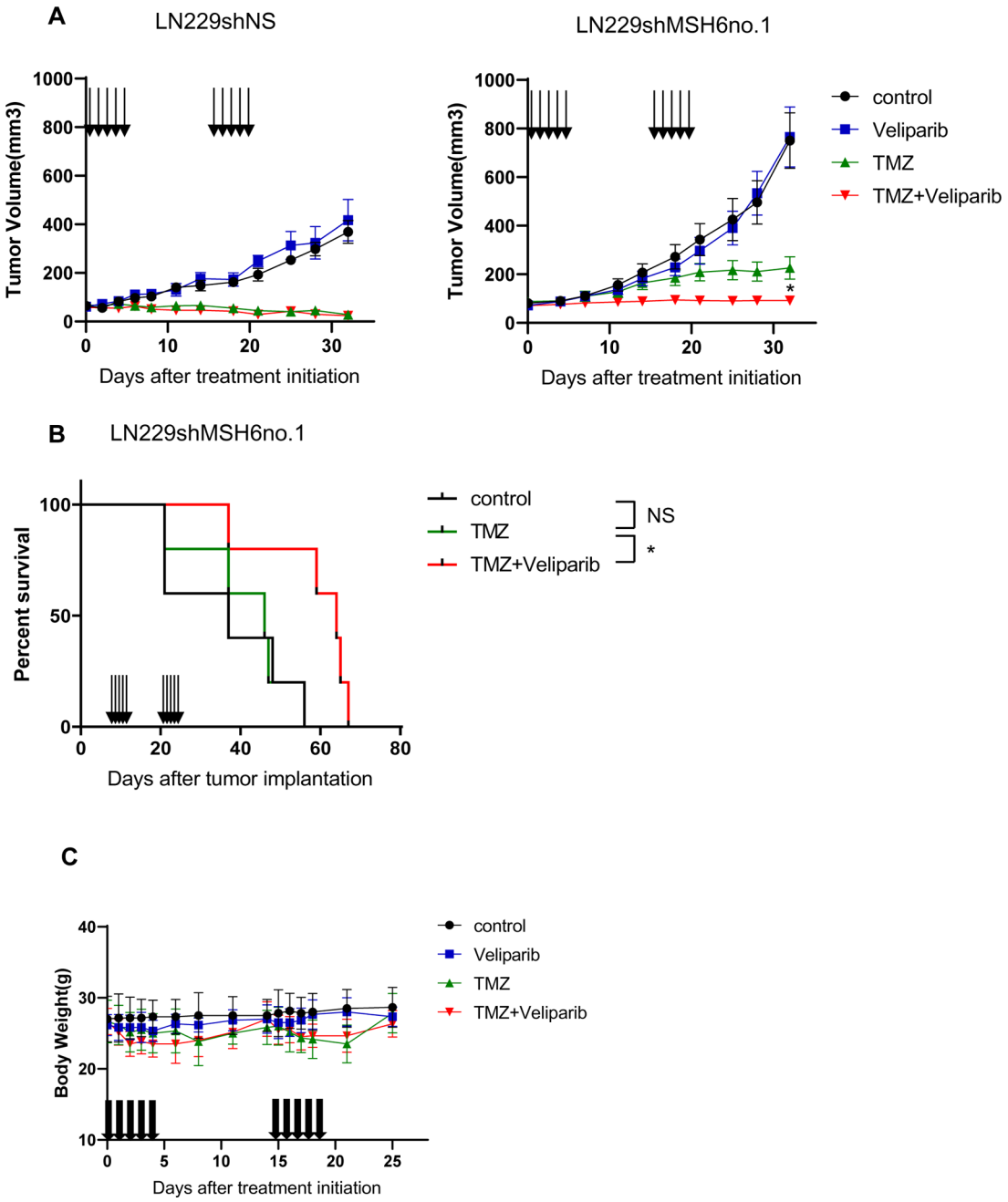

Figure 2. Veliparib combination treatment with TMZ delays tumor growth of MSH6-deficient glioblastoma.

(A) Tumor growth curves in the LN229shNS (left) and LN229shMSH6 (right) flank xenograft models. Animals were treated with PBS (n=6), Veliparib alone (50 mg/kg oral, 5 days/week × 2 cycles)(n=6), TMZ alone (50 mg/kg i.p., 5 days/week × 2 cycles) (n=6), and TMZ plus Veliparib (n=6). Data are presented as mean tumor volume and SEM in each group. (*P=0.019, TMZ vs TMZ + Veliparib on day 32, Student t- test) (B) Kaplan-Meier analysis of mice bearing LN229shMSH6 orthotopic glioblastoma that were treated with TMZ alone or TMZ combination with Veliparib. Animals were treated with PBS (n=5), TMZ alone (50 mg/kg i.p. 5 days/week × 2 cycles) (n=5), or TMZ plus Veliparib (50 mg/kg

oral, 5 days/week × 2 cycles) (n=5). *P=0.02, TMZ vs TMZ + Veriparib (log-rank test). NS, non-significant. (C) Median body weights of mice with flank LN229shNS tumors for each treatment groups (same experiment as A). Bars are SD. Weight was measured daily during treatments) or every two days after treatment.

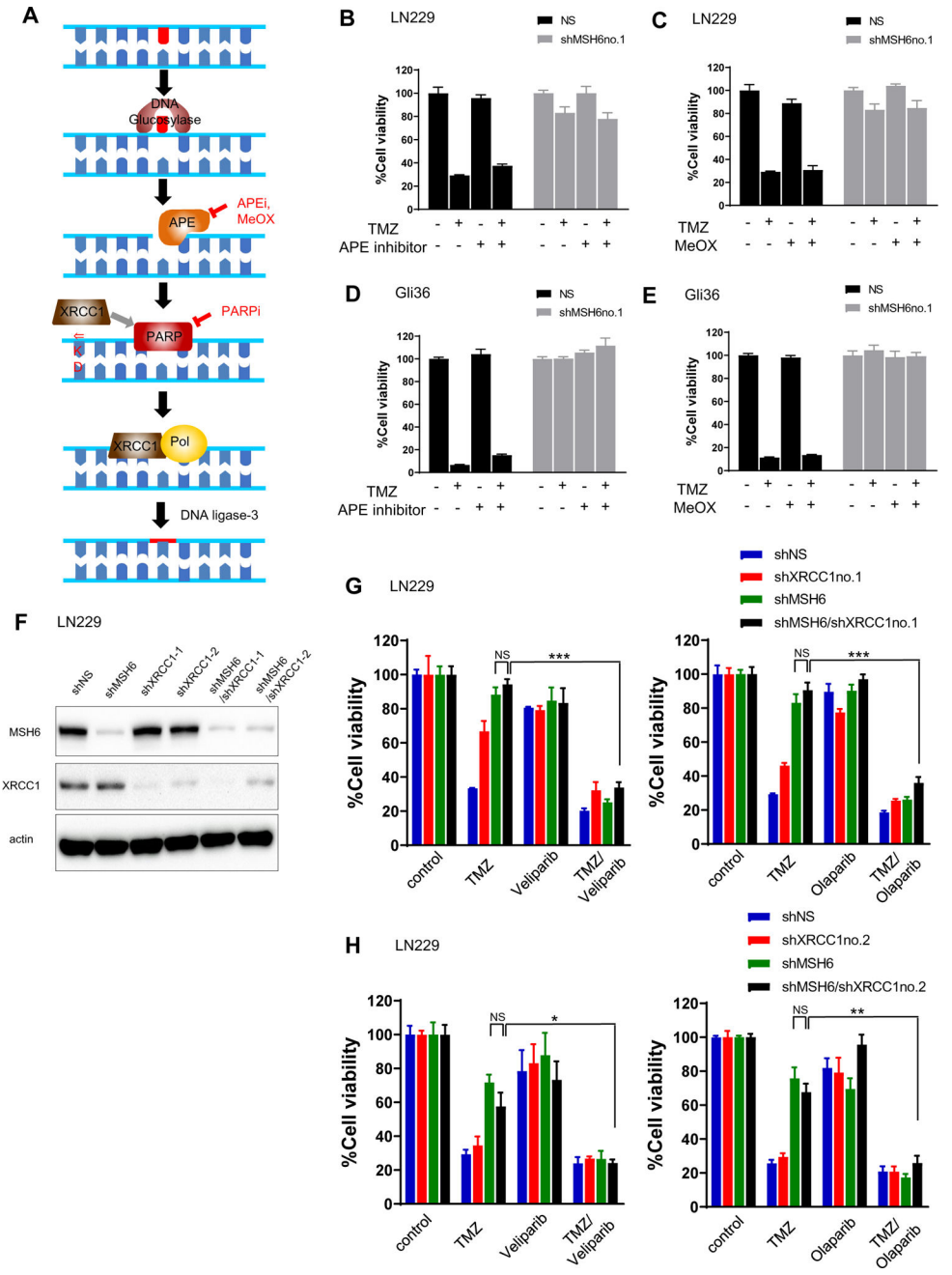

Figure 3. PARP inhibitor restoration of TMZ sensitivity is not through BER signaling blockage.

(A) A scheme of Base Excision Pathway showing PARPs, other key proteins and their inhibitors. (B, C) Non-targeting (shNS) and MSH6 knockdown (shMSH6no.1) LN229 cells were treated with TMZ (200 uM), APE inhibitor (1 uM, in B), MeOX (3 mM, in C), or TMZ combination with APE inhibitor (B) or MeOX (C). Cell viability was evaluated by Cell Titer Glo on day 6.(D,E) Same experiments as B and C using Gli36shNS and Gli36shMSH6no.1. TMZ (30 uM), APE inhibitor (1 uM, in D), MeOX (3 mM, in E) (F) LN229 cells were engineered with a non-targeting (shNS), MSH6-directed (shMSH6) or XRCC1-directed shRNA (shXRCC1–1, shXRCC1–2) lentivirus or both shMSH6 and shXRCC1. Immunoblot

confirmed knockdown of MSH6 and XRCC1. Actin was used as a loading control. (G) LN229shNS, LN229shMSH6, LN229shXRCC1–1, LN229shMSH6/XRCC1–1 cells were treated with TMZ (200 uM), PARP inhibitor (veliparib, 3 uM, in E; olaparib, 1 uM, in F) or TMZ combination with PARP inhibitor. Cell viability was evaluated by Cell Titer Glo on day 6. (H) Same experiments as E and F using a second shRNA, XRCC1–2. *P<0.005, **P<0.0005, ***P<0.0001 (student *t*-test).

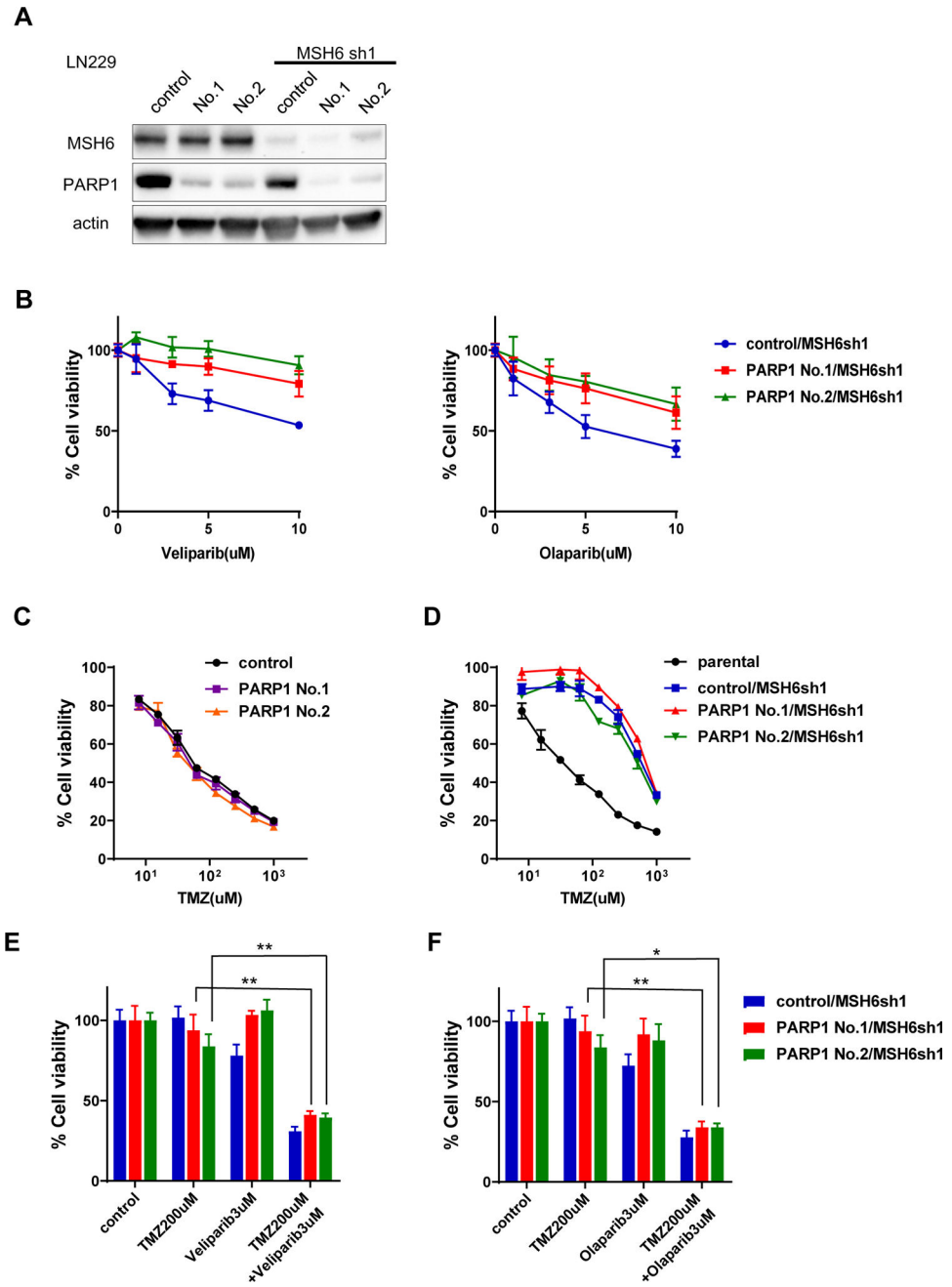

Figure 4. PARP inhibitors restore TMZ sensitivity in MSH6-deficient, PARP1 knockout glioblastoma cells.

(A) LN229 parental cells and LN229shMSH6 cells were engineered with a non-targeting CRISPR-Cas9 (control) or *PARP1* CRISPR-Cas9 (PARP1 no.1, PARP1 no.2) lentivirus. Immunoblot confirmed PARP1 knockout (KO). Actin was used as a loading control. (B) Veliparib (left) or Olaparib (right) dose response in shMSH6LN229 cells with CRISPR-Cas9 control, and PARP1 KO no.1 and no.2. (C, D) TMZ dose response in MSH6 intact LN229 cells, with and without PARP1KO no.1 and no.2 (C) and in MSH6 intact LN229 cells, and shMSH6LN229 cells with and without PARP1KO no.1 and no.2 (D). (E, F) shMSH6LN229 cells with control (CRISPR-Cas9), and PARP1KO no.1 and no.2 were treated with TMZ

(200 uM), PARP inhibitor or TMZ combination with PARP inhibitor. (E) Veliparib 3 uM. (F) Olaparib 3 uM. Cell viability was evaluated by Cell Titer Glo on day 6 in B-F. *P<0.005, **P<0.00 (student *t*-test).