Therapeutics, Targets, and Chemical Biology

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Mutant IDH1-Driven Cellular Transformation Increases RAD51-Mediated Homologous Recombination and Temozolomide Resistance

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Abstract

Isocitrate dehydrogenase 1 (IDH1) mutations occur in most lower grade glioma and not only drive gliomagenesis but are also associated with longer patient survival and improved response to temozolomide. To investigate the possible causative relationship between these events, we introduced wild-type (WT) or mutant IDH1 into immortalized, untransformed human astrocytes, then monitored transformation status and temozolomide response. Temozolomide-sensitive parental cells exhibited DNA damage (γ-H2AX foci) and a prolonged G_2 cell-cycle arrest beginning three days after temozolomide (100 μmol/L, 3 hours) exposure and persisting for more than four days. The same cells transformed by expression of mutant IDH1 exhibited a comparable degree of DNA damage and cell-cycle arrest, but both events resolved significantly faster in association with increased, rather than decreased, clonogenic survival. The increases in DNA damage processing, cell-cycle progression, and clonogenicity were unique to cells transformed by mutant IDH1, and were not noted in cells transformed by WT IDH1 or an oncogenic form (V12H) of Ras. Similarly, these effects were not noted following introduction of mutant IDH1 into Ras-transformed cells or established glioma cells. They were, however, associated with increased homologous recombination (HR) and could be reversed by the genetic or pharmacologic suppression of the HR DNA repair protein RAD51. These results show that mutant IDH1 drives a unique set of transformative events that indirectly enhance HR and facilitate repair of temozolomide-induced DNA damage and temozolomide resistance. The results also suggest that inhibitors of HR may be a viable means to enhance temozolomide response in IDH1-mutant glioma. *Cancer Res; 74(17); 4836–44. ©2014 AACR.*

Introduction

Isocitrate dehydrogenase 1 (IDH1) mutations are the earliest and most common mutations in lower grade glioma (1, 2). Mutations at the R132 codon in IDH1, and in particular R132H, are noted in roughly 75% of all lower grade glioma (2, 3), and more recent studies suggest that IDH mutation precedes other alterations common to these tumors including p53 mutation in astrocytomas and 1p/19q codeletion in oligodendrogliomas (4, 5). The *IDH1* gene encodes IDH1, which is involved in the cytoplasmic oxidative carboxylation of isocitrate to α-ketoglutarate (α-KG; ref. 6). The α-KG generated in turn contributes to the production of NADPH in the citric acid cycle as well as to the activity of α-KG–dependent enzymes including the 5-methylcytosine hydroxylases and histone methyltransferases that regulate the epigenetic state of the cell (7, 8). Mutations in the *IDH1* gene result in the expression of a mutant IDH1 that has the unique ability to convert α-KG to 2-hydroxyglutarate (2HG; ref. 9). The accumulation of 2HG in mutant tumors in turn competes with α-KG for binding and activation of α-KG–dependent enzymes, with the result being large-scale alterations in DNA methylation, acetylation, and gene expression that contribute to the tumorigenic process (10–12). IDH1 mutations are noted in lower grade astrocytomas, oligodendrogliomas, anaplastic oligodendrogliomas, and secondary glioblastoma, but are rare in pilocytic astrocytomas and primary glioblastoma (1, 2). Furthermore, because IDH1-mutant tumors have a very different pattern of genetic alterations than histologically comparable IDH1 wild-type (WT) tumors (13), a consensus has emerged that IDH1 mutation sets cells on a different path to tumorigenesis than cells transformed by other oncogenic insults (14, 15).

IDH1 mutational status also has consequences for therapeutic outcome. In multiple studies, IDH1 mutation correlates with increased overall patient survival and progression-free survival (1, 2, 4, 5, 16), implying that IDH1 mutation alters the underlying biology of the tumor, the chemo/radiosensitivity of the tumor, or both. The DNA methylating agent temozolomide is one of the most frequently used drugs in the therapy of glioma, and indirectly causes DNA double-strand breaks, which if not repaired by RAD51-driven homologous

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recombination (HR) lead to cell-cycle arrest and delayed cell death (17–20). A higher rate of response to up-front temozolomide has been reported in IDH1-mutant low-grade glioma patients relative to that noted in patients with histologically identical IDH1 WT tumors (21), implying that mutant IDH1 contributes to temozolomide sensitivity. Gliomas driven by IDH1 mutations, however, are widely recognized to differ in cell of origin and underlying biology from comparable IDH WT glioma (14, 15), complicating any analysis of the contribution of IDH1 mutation to drug sensitivity. Furthermore, these correlative studies do not address whether mutant IDH1 alters drug sensitivity, or merely sets in motion a series of events that lead to tumorigenicity and altered drug sensitivity. Where examined, exogenous expression of mutant IDH1 had no significant effect on temozolomide sensitivity (22). The established glioblastoma cell lines used (U87 and U373MG), however, evolved in the absence of IDH1 mutations (22) and as such may not represent optimal models for the study of the effect of IDH1 status on drug sensitivity.

To more accurately determine whether and how IDH1 mutation alters drug sensitivity, we here employed a system that has been widely used not only for the study of cellular transformation and gliomagenesis, but also for the study of IDH1 biology (12, 23, 24). This system consists of normal human astrocytes (NHA), which are immortalized by introduction of virally-encoded E6, E7, and hTERT (23). These immortalized cells can be transformed into cells that grow in an anchorage-independent manner by additional expression of oncogenic H-Ras (23), or alternatively by expression of mutant IDH1, which has previously been shown to change patterns of DNA methylation in a manner similar to that noted in lower grade glioma (12, 24). We therefore used these cells to determine whether mutant IDH1 expression and/or the transformative events it sets in place were sufficient to alter sensitivity to temozolomide.

Materials and Methods

Cell culture, creation of cell lines, and drug treatment

U87MG human astrocytoma cells and ductal carcinoma T47D cells were obtained from the UCSF Brain Tumor Research Center, and were grown in Dulbecco Modified Eagle H-21 Medium supplemented with 10% FBS and 1% penicillin/streptomycin (UCSF Cell Culture Facility) at 37°C in a 5% CO_2 atmosphere. NHAs were obtained and maintained in Astrocyte Growth Medium (Clonetics). The generation of U87 cells overexpressing MGMT and NHAs expressing E6/E7/hTERT, and E6/E7/hTERT plus H-RasV12 (Ras astrocytes) has been described previously (23, 25, 26). To generate IDH1-expressing cells, lentiviral constructs encoding GFP and either WT or mut (R132H) IDH1 (27) were cotransfected with VSVG, and ΔVPR plasmids (Open Biosystems) at a 1:0.9:0.1 ratio into 293T packaging cells using Fugene6 (Roche). Lentiviral supernatants were harvested at 48 and 72 hours posttransfection and used to infect U87 cells or NHAs expressing E6/E7/hTERT or E6/E7/hTERT plus H-RasV12. After 72 hours, cells expressing GFP were isolated by fluorescent-activated cell sorting and verified by Western blot analysis for expression of the target protein of interest. Unsynchronized cells were pretreated with DMSO (Sigma) or B02 (10 μmol/L, 1 hour, EMD Millipore), and then treated with temozolomide (100 μmol/L, 3 hours; Sigma) in the presence of drugs. The final DMSO concentration did not exceed 0.1% (vol/vol). After temozolomide treatment, cells were washed, incubated in fresh medium containing DMSO or B02 (10 μmol/L), then harvested at various time points. The identity of all cells was verified by DNA fingerprinting using the PowerPlex 20 HS Kit and comparison with published markers (28). All NHA-derived cultures were similarly verified to be derived from the same parental population.

Genetic suppression of RAD51

E6/E7/hTERT/IDH1 mut cells were plated at 10^5/mL in 6-well plates in DMEM cell growth media without antibiotics. Twenty-four hours later, the cells were transfected with an optimized amount (5 nmol/L) of siRNA targeting human RAD51 (SMARTpool, Dharmacon) or nontargeting siRNAs as a negative control, using DharmaFECT reagent (Dharmacon) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were washed, treated with temozolomide (100 μmol/L, 3 hours) in complete medium free of siRNA, washed again, and placed in temozolomide-free medium until harvested for analysis.

Protein extraction and immunoblot analyses

Cells were washed in cold PBS and lysed in NP40 cell lysis buffer (Life Technologies) supplemented with 1× PhosStop and protease inhibitor cocktail (Roche). The protein was quantitated and used (30 μg) for Western blot analysis using primary antibodies against IDH1 (Dianova), IDH1R132H (Dianova), H-Ras (Santa Cruz Biotechnology), RAD51 (EMD Chemicals, PC130), MGMT, or β-actin (Cell Signaling Technologies), and the appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). Antibody binding was detected using ECL reagents (Amersham Pharmacia Biotech).

Cell-cycle and immunofluorescence studies

At various time points following temozolomide exposure, attached and floating cells were collected and subjected to flow cytometry and analysis using a FACSCalibur (BD Biosciences) and FlowJo data analysis software (TreeStar; ref. 29). For immunofluorescence studies, cells were seeded onto 8-well glass coverslips, incubated with temozolomide or temozolomide + B02, washed in PBS, fixed with 4% paraformaldehyde in PBS (15 minutes, room temperature), rinsed with PBS, and blocked in PBS containing 0.1% Triton-X and 10% FBS (1 hour, room temperature). The cells were stained with anti-Ser-139–phosphorylated H2AX antibody (Cell Signaling Technology) at 1:500 dilution followed by Alexa596-conjugated secondary antibody (Life Technologies; 30 minutes, room temperature). Cells were washed, counterstained with 4 V,6 V diamidino-2-phenylindole (DAPI), and mounted with Prolong anti-fade reagent (Life Technologies). For RAD51 studies, cells were incubated in blocking solution (PBS containing 20% goat serum, 3% BSA and 0.1% Triton X-100, 1 hour), stained with 1:1000 anti RAD51 antibody in blocking solution (1 hour), then washed in PBS and stained with 1:500 Alexa594-conjugated

secondary antibody (Life Technologies; 1hour). For double staining, anti-Ser-139–phosphorylated H2AX antibody and anti-RAD51 antibody (Gene Tex) were used as primary antibodies and Alexia 568 and 647 were used as secondary antibodies. The number of nuclei containing >5 γ-H2AX or RAD51 foci was quantified by fluorescence microscopy.

Measurement of cell viability, colony formation efficiency, and *in vitro* transformation

Cell viability, colony formation efficiency, and *in vitro* transformation of control and temozolomide-treated cells were determined by Trypan blue exclusion counting, colony formation assay, and soft agar assay, as previously described (23, 30).

Measurement of HR efficiency

HR efficiency was calculated using a qPCR-based HR assay kit (Norgen Biotek Corp.; ref. 31). Briefly, the system contains two plasmids each with a different mutation in its lacZ coding region. The plasmids were cotransfected 48 hours, after which the total cellular DNA was isolated and used in a qPCR reaction containing a set of universal primers that amplify all plasmid DNA (and serve as a control for transfection efficiency) or a set of primers that only amplify plasmid DNA generated by HR of the transfected plasmids. The amount of recombinant product for each reaction was calculated by comparing the cycle number at the point of inflection of the amplification curve generated using the HR-specific primers to that using universal primers, and converting the difference in cycle number to a DNA amount by comparison with a standard curve generated using universal primers and different amounts of input DNA. This value was set at 1 in parental/vector cell populations, with the amount of recombinant DNA produced in experimental groups expressed as a HR ratio to this value.

Statistical analyses

Data are reported as mean ± SE of at least three experiments. When two groups were compared, the unpaired Student *t* test was applied (*P* value). When multiple groups were evaluated, the one-way ANOVA test with *post hoc* Turkey–Kramer multiple comparisons test was used. $P < 0.05$ was considered statistically significant.

Results

Mutant IDH1 expression changes temozolomide response and increases temozolomide resistance

To begin to understand whether and how IDH1 mutations influence drug sensitivity, NHAs immortalized by expression of E6, E7, and hTERT were first lentivirally infected with constructs encoding blank or mutant IDH1, after which the response to temozolomide (3 hour, 100 μmol/L) was examined. Parental blank vector E6/E7/hTERT cells are MGMT deficient (Fig. 1A, column 3) and as a result exhibited a typical pattern of response to temozolomide. Like other MGMT-deficient cells (17, 18), they underwent a G_2 arrest (here defined as occurring when the percentage of cells with 4N DNA content was greater than that of cells with 2N DNA content) beginning 3 days after drug exposure, and remained arrested at least 4 days following initiation of the arrest (shaded peaks, Fig. 1B). The G_2 arrest was also temporally accompanied by the appearance of γ-H2AX foci indicative of DNA double-strand breaks which persisted for the duration of the study following a range of temozolomide exposure (Fig. 1C). Consistent with these data, the number of viable cells did not significantly change between day 4 and day 7 after temozolomide exposure (Fig. 1D) and the temozolomide-treated cells exhibited a dose-dependent loss of clonogenicity (Fig. 1E). Introduction of a construct encoding WT IDH1 had no significant effect on any of these parameters (Fig. 1A–E). Introduction of a construct encoding mutant IDH1 (Fig. 1A) also had no significant effect on the timing of the onset of G_2 arrest following temozolomide exposure (Fig. 1B) or the percentage of γ-H2AX–positive cells at 3 days after temozolomide (Fig. 1C). Cells expressing mutant IDH1, however, exhibited a much shorter temozolomide-induced G_2 arrest that lasted less than 48 hours following the initiation of arrest (Fig. 1B). Consistent with these findings, the temozolomide-induced γ-H2AX foci disappeared significantly more rapidly (Fig. 1C) in mutant IDH1-expressing cells than in empty vector controls. Furthermore, the number of viable cells expressing mutant IDH1 nearly doubled between 4 days and 6 days after temozolomide exposure (Fig. 1D), and the mutant IDH1-expressing cells also exhibited increased clonogenicity relative to uninfected or blank vector controls (Fig. 1E). These results show that although the introduction of mutant IDH1 does not affect the extent of temozolomide-induced DNA damage, it significantly alters the processing of this damage, and consequently drug action.

The effects of mutant IDH1 on drug response are not a generalized result of cellular transformation

Tumor cells, as well as normal human cells made tumorigenic by exogenous expression of large T antigen, have both been reported to have an increased ability to repair DNA double-strand breaks relative to comparable nontransformed cells (32). We therefore questioned whether the effects of mutant IDH1 on temozolomide-induced DNA repair and drug action were simply a consequence of mutant IDH1-driven cellular transformation. The parental E6/E7/hTERT cells used were immortalized, but when analyzed for growth in soft agar (a hallmark of *in vitro* transformation; ref. 33) failed to form colonies (Table 1). The same cells, however, were transformed and readily formed colonies in soft agar after exogenous expression of mutant V12H-Ras (Fig. 1A; Table 1). Similarly, exogenous expression of either WT or mutant IDH1 resulted in *in vitro* transformation of the parental cells, with the mutant IDH1 cells forming fewer colonies than Ras-transformed cells, but more than those expressing WT IDH1 (Table 1). Although the cells expressing mutant H-Ras, WT IDH1, or mutant IDH1 were all transformed, the cells transformed by WT IDH1 were no different than parental cells with regard to the duration of temozolomide-induced G_2 arrest (Fig. 1B), persistence of γ-H2AX foci (Fig. 1C), and temozolomide sensitivity (Fig. 1D), whereas the cells transformed by mutant H-Ras exhibited a duration of G_2 arrest and γ-H2AX foci that was intermediate between that of the parental and mutant IDH1 cells (Fig. 1B and C) and a temozolomide sensitivity no different than

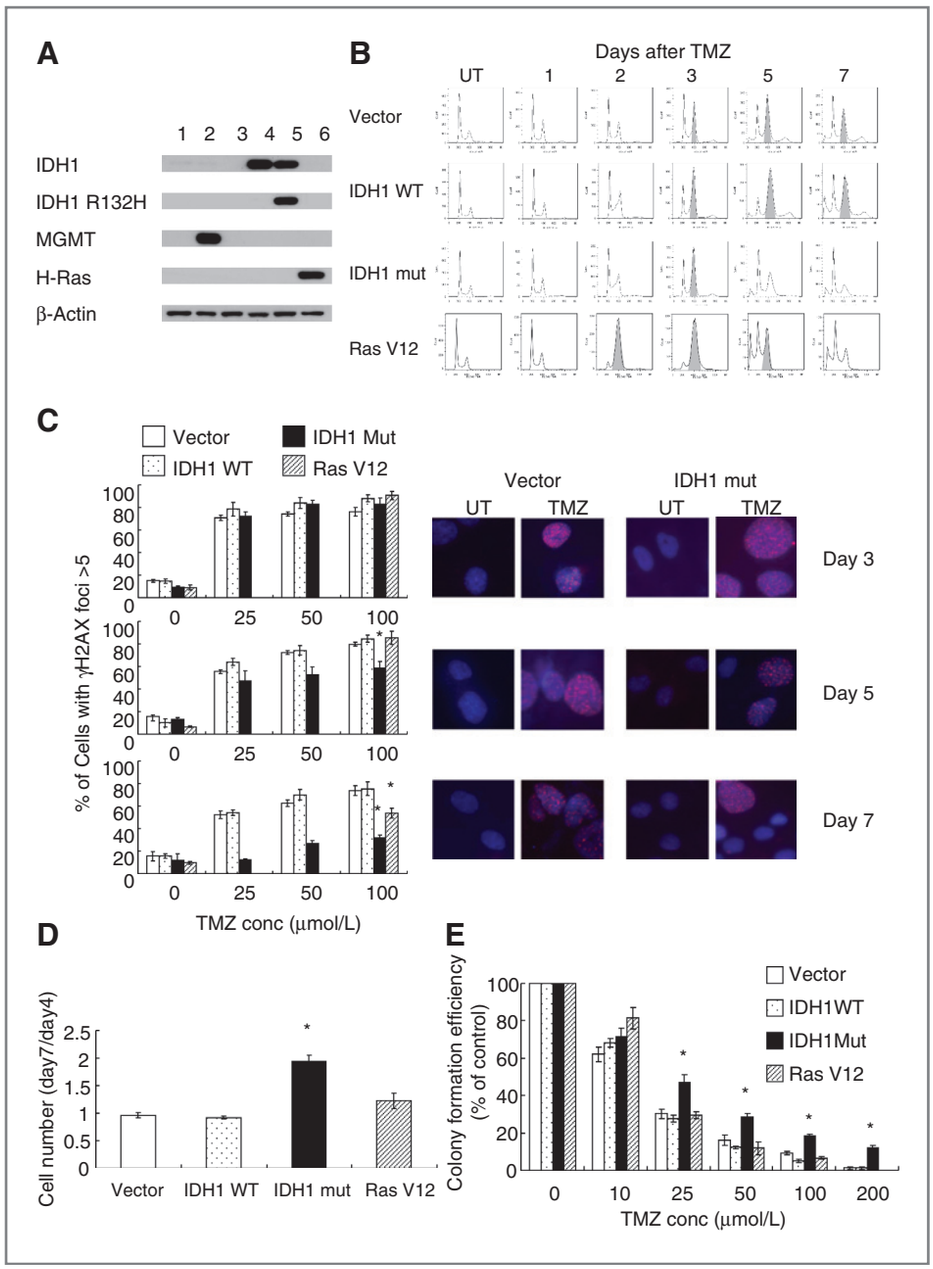

Figure 1. A, Western blot validation of the identity of the cell lines used in this panel. 1, U87; 2, U87+MGMT; 3, E6/E7/hTERT (vector); 4, E6/E7/hTERT+WT IDH1; 5, E6/E7/hTERT+R132H–mutant IDH1; 6, E6/E7/hTERT+RasV12. B, FACS analysis of cell-cycle distribution in various cell lines 1 to 7 days after temozolomide (100 μmol/L, 3 hours) exposure. Shading, cell populations exhibiting significant G_2–M arrest. C, right, immunofluorescence images of γ-H2AX foci (pink) in DAPI-stained (blue) control and drug-treated parental E6/E7/hTERT cells at various times after temozolomide exposure (100 μmol/L, 3 hours) and quantitation of data (left) in all cells studied. D, cell number day 7 versus day 4 following temozolomide exposure (100 μmol/L, 3 hours) in control and IDH1-modified cells. E, colony formation efficiency of cells studied following temozolomide exposure (0–100 μmol/L, 3 hours). *, $P < 0.05$. Values derived are representative of, or derived from, ≥3 independent experiments. TMZ, temozolomide.

parental cells (Fig. 1E). The effects of mutant IDH1 on temozolomide action were therefore not merely a general result of the transformation process.

The effects of mutant IDH1 on drug response are not mediated in a direct manner

The ability of mutant IDH1 to uniquely alter temozolomide response suggests that expression of mutant IDH1 might directly alter cellular mechanisms that contributed to temozolomide response. To address this possibility, a blank construct, or a construct encoding mutant IDH1 was lentivirally introduced into the E6/E7/hTERT cells already transformed by expression of mutant Ras, and also into MGMT-deficient tumorigenic U87 glioma cells (Fig. 2A), after which effects on temozolomide-induced G_2 arrest, γ-H2AX foci formation and persistence, and clonogenicity were examined. Both the parental E6/E7/hTERT+mutant Ras cells and the U87 cells exhibited the expected temozolomide-induced G_2 arrest beginning 2 to 3 days after drug exposure (Fig. 2B) in association with the formation and persistence of γ-H2AX foci (Fig. 2C) and dose-dependent loss of clonogenicity (Fig. 2D). Exogenous expression of mutant IDH1 (Fig. 2A) in these already transformed cells had no effect on the extent of temozolomide-induced G_2 arrest (Fig. 2B), γ-H2AX foci (Fig. 2C), or

Table 1. Transformation of NHAE6E7hTERT by introduction of IDH1 WT, IDH1 mut, or Ras V12

Cell types	Colony numbers
Vector	5 ± 1
IDH1 WT	85 ± 4
IDH1 mut	189 ± 14
Ras V12	1,191 ± 78

clonogenicity (Fig. 2D) in either cell type. These results show that mutant IDH1 does not alter temozolomide response in a direct manner, but rather does so only in connection with the mutant IDH1-driven events that lead to cellular transformation.

Mutant IDH1-driven transformation increases HR

Temozolomide-induced DNA double-strand breaks are repaired in human cells by the process of HR (19, 20). HR in turn requires the collective action of a variety of proteins including RAD51, which accumulates at the γ-H2AX–marked sites of DNA double-strand breaks and contributes to their repair (34, 35). To first determine whether alterations in HR contributed to the ability of mutant IDH1-expressing cells to escape from temozolomide-induced G_2 arrest, we monitored the formation and disappearance of RAD51 foci in temozolomide-treated E6/E7/hTERT cells lentivirally infected with a

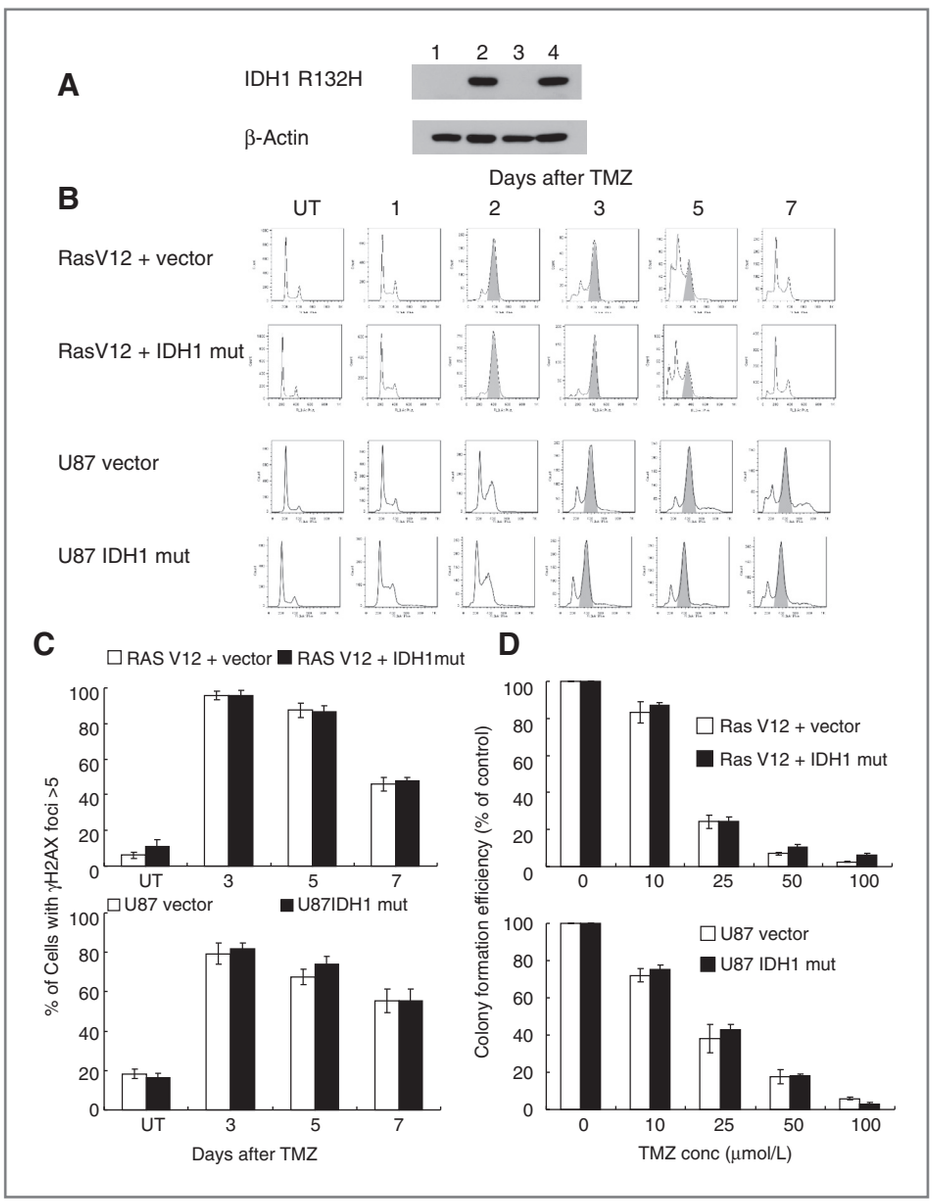

Figure 2. A, Western blot validation of the identity of the cell lines used in this panel. 1, E6/E7/hTERT+RasV12 (RasV12); 2, E6/E7/hTERT+RasV12+R132H–mutant IDH1; 3, U87; 4, U87+R132H–mutant IDH1. B, FACS analysis of cell-cycle distribution in various cell lines 1 to 7 days after temozolomide (100 μmol/L, 3 hours) exposure. Shading, cell populations exhibiting significant G_2–M arrest. C, quantitated data from immunofluorescence analysis of γ-H2AX foci in control and drug-treated parental and IDH1-modified cells at various times after temozolomide exposure (100 μmol/L, 3 hours). D, colony formation efficiency of parental and IDH1-modified cells following temozolomide (100 μmol/L, 3 hours) exposure. Values derived are representative of, or derived from, ≥3 independent experiments. TMZ, temozolomide.

blank vector or a construct encoding WT or mutant IDH1. As shown in Fig. 3A, the sites of temozolomide-induced RAD51 foci formation in parental cells overlapped those of γ-H2AX foci formation 3 days following temozolomide exposure, supporting the idea that RAD51 accumulates at sites of DNA double-strand breaks. All cell groups also exhibited similar percentages of cells with RAD51 foci 3 days after temozolomide exposure (Fig. 3B) mirroring the results derived from the temporal analysis of γ-H2AX foci appearance and disappearance in the same cells (Fig. 1). Seven days after temozolomide exposure, however, the cells expressing mutant IDH1 which had displayed fewer γ-H2AX foci and had escaped from cell-cycle arrest also were less likely to contain RAD51 foci, suggesting that an HR-related event was stimulated in the cells expressing mutant IDH1. To directly address this possibility, we used an *in vivo* plasmid recombination-based method (31) to determine whether exogenous expression of mutant IDH1 altered the ability of the cells to perform HR. As shown in Fig. 3C, control E6/E7/hTERT cells and E6/E7/hTERT cells expressing WT IDH1 had a measurable rate of HR that was significantly less than that of positive control T47D cells, which are known to have a high rate of HR (36). The cells expressing mutant IDH1, however, had a 3-fold higher rate of HR. These results show that mutant IDH1-driven transformation increases HR.

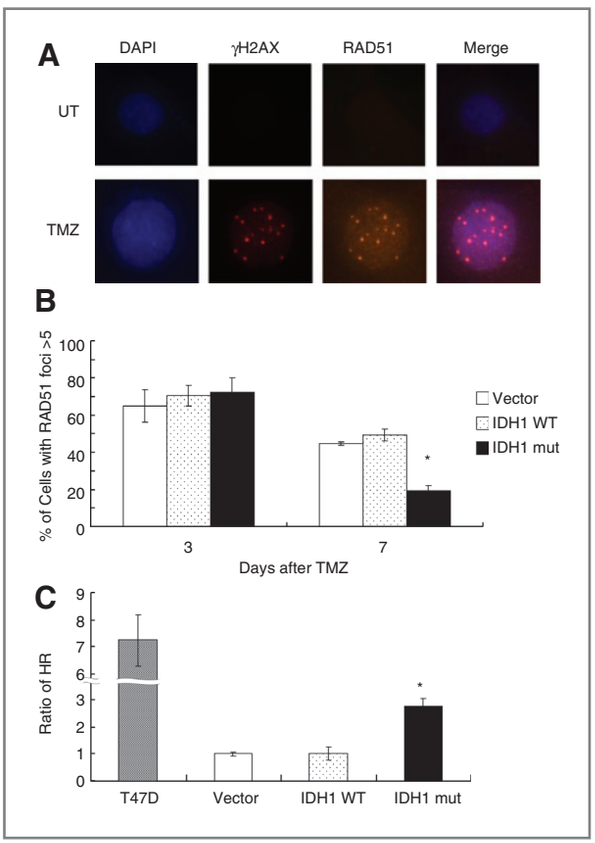

Figure 3. A, immunofluorescence analysis of the physical coincidence of γ-H2AX and RAD51 foci in E6/E7/hTERT parental cells 3 days following temozolomide exposure (0 or 100 μmol/L, 3 hours). B, quantitated data from immunofluorescence analysis of γ-H2AX foci in control and drug-treated parental (E6/E7/hTERT) and IDH1-modified cells at 3 and 7 days after temozolomide exposure (100 μmol/L, 3 hours). C, HR activity as determined by an *in vivo* plasmid-based recombination reporter assay. Value of the parental cells was set at 1; *, $P < 0.05$. Values derived are representative of, or derived from, ≥3 independent experiments. TMZ, temozolomide.

Inhibition of HR reverses the effects of mutant IDH1 expression on temozolomide resistance

Because the activity of RAD51 is critical for HR, further studies were also performed in which RAD51 activity was genetically or pharmacologically inhibited, after which the levels of HR and effects on temozolomide action were measured in the mutant IDH1-expressing cells. Expression of RAD51 siRNA, but not a scrambled control siRNA, in E6/E7/hTERT cells expressing mutant IDH1 suppressed levels of RAD51 by more than 85% (Fig. 4A), and also reduced HR (Fig. 4B) to levels comparable with those seen in parental E6/E7/hTERT cells in Fig. 3C. Introduction of the RAD51 siRNA also blocked the ability of mutant IDH1 to enhance the repair of temozolomide-induced DNA double-strand breaks (Fig. 4C), to shorten temozolomide-induced G_2 arrest (Fig. 4D), and to confer resistance to temozolomide (Fig. 4E).

To verify these results, similar experiments were performed using B02, a specific inhibitor of the DNA strand exchange activity of human RAD51 (37, 38). Incubation of E6/E7/hTERT cells expressing mutant IDH1 with non-toxic concentrations of B02 significantly reduced HR in these cells to levels comparable with those seen in control cells (Fig. 5A), significantly reduced the percentage of cells with RAD51 foci (Fig. 5B), and also significantly reduced the temozolomide resistance conferred by mutant IDH1 overexpression (Fig. 5C). These results show that the transformation process driven by mutant IDH1 contributes to enhanced HR and temozolomide resistance.

Discussion

The overwhelming majority of low-grade glioma and secondary glioblastoma express a mutant form of IDH1, and mutant IDH1 expression is thought to play a driving role in the genesis of these tumors (1, 2). Because IDH1-mutant glioma seems to be more chemosensitive than histologically identical but genetically distinct IDH1 WT tumors, it was suggested that IDH1 mutation conferred a degree of sensitivity to the drugs used in the treatment of these tumors, including temozolomide. The present results show that in an extensively used model of gliomagenesis, expression of mutant IDH1 confers temozolomide resistance rather than temozolomide sensitivity. This drug resistance is not a direct effect of mutant IDH1 expression but rather is an indirect effect of mutant IDH1-driven cellular transformation associated with changes in RAD51-driven HR, and can be reversed by exposure to inhibitors of the HR process.

The present results differ in important ways from those of previous studies of the effect of mutant IDH1 expression on drug sensitivity. In previous studies, introduction of mutant IDH1 into U87 and 373MG cells had no effect on temozolomide resistance (22). In the present studies, introduction of mutant IDH1 into cells transformed independently of mutant IDH1,

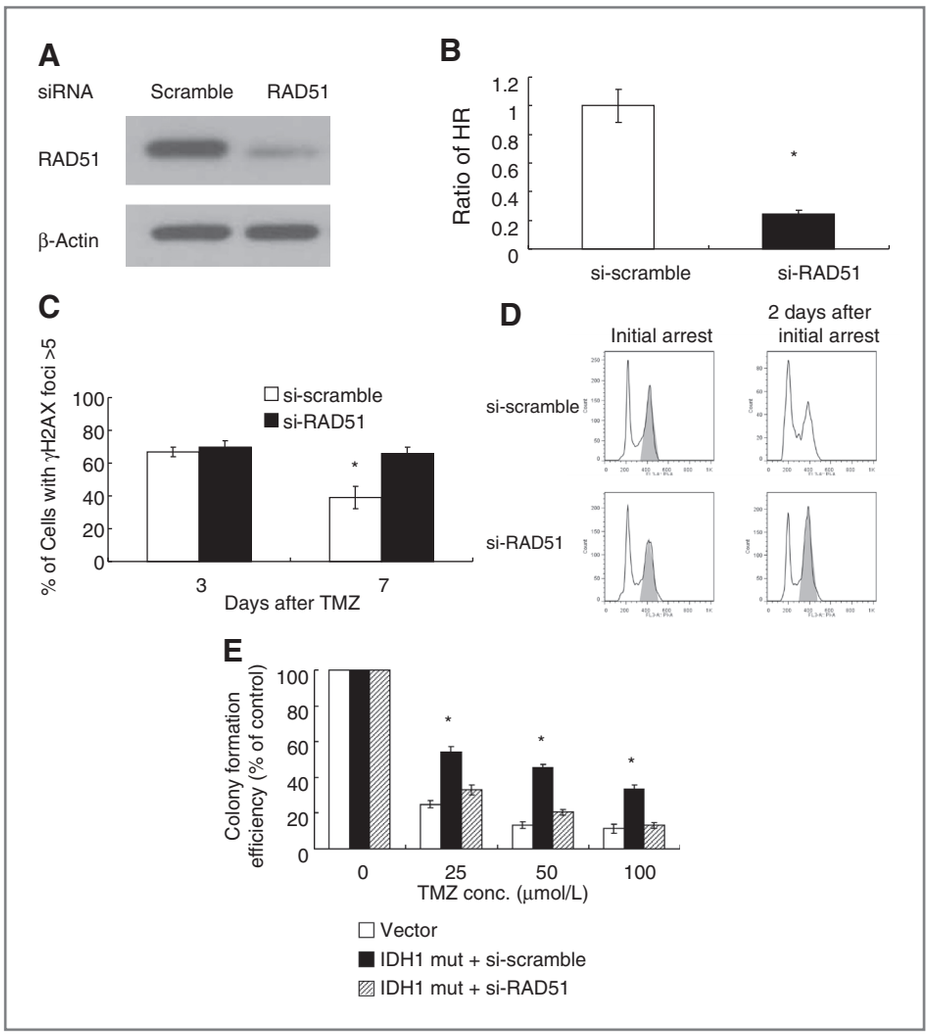

Figure 4. A, Western blot analysis of RAD51 and β-actin levels in E6/E7h/TERT+mutant IDH1 cells 3 days following exposure to scramble or RAD51-targeting siRNA. B, HR activity as determined by an *in vivo* plasmid-based recombination reporter assay. Value of the si-scramble cells was set at 1. C, quantitated data from immunofluorescence analysis of γ-H2AX foci in control and drug-treated parental and RAD51-suppressed cells 3 and 7 days after temozolomide exposure (100 μmol/L, 3 hours). D, FACS analysis of cell-cycle distribution in control and RAD51-suppressed temozolomide-treated (100 μmol/L, 3 hours) cells at the time of arrest and 2 days later. Shading, cell populations exhibiting significant G_2–M arrest. E, colony formation efficiency of control and RAD51-suppressed cells studied following temozolomide exposure (0–100 μmol/L, 3 hours). *, $P < 0.05$. Values derived are representative of, or derived from, ≥3 independent experiments. TMZ, temozolomide.

including U87, also did not increase temozolomide resistance. Because expression of mutant IDH1 drives the tumorigenesis process; however, cells transformed in an IDH1-independent manner are not an appropriate model in which to determine how mutant IDH1 function contributes to tumor behavior. Although cells derived from IDH1-mutant tumors could be used, IDH1-mutant tumor cells have proven to be extremely difficult to culture or grow as xenografts, frequently undergo loss of mutant IDH1, and do not allow for a direct isogenic comparison between WT and IDH1-mutant populations (39, 40). The immortalized human astrocyte cell system used in the present studies therefore represents perhaps the only viable approach to study the effects of mutant IDH1 expression on drug sensitivity, particularly as in these cells the exogenous expression of mutant IDH1 generates epigenetic alterations similar to those noted in IDH1-mutant lower-grade gliomas (12, 24), contributes to the transformation of the cells (12, 24), and generates cells which when injected intracranially in mice generate tumors which histologically resemble grade III astrocytomas (unpublished observation). In this proper context, mutant IDH1 expression was shown to increase, rather than decrease, temozolomide resistance, suggesting that the context in which IDH1 mutations occur are critical in determining the effect of the alteration on drug sensitivity.

The present results also show that the effects of exogenous mutant IDH1 on temozolomide sensitivity are not direct, but rather are part of a larger indirect cellular reprogramming. Exogenous expression of mutant IDH1 has been shown to alter the concentrations of a variety of metabolites and also to change the levels of various enzymes involved in energy regulation (11, 12, 27, 41). Furthermore, direct mutant IDH1-driven decreases in NADPH levels have been suggested to result in increased susceptibility to oxidative stress and drug-induced apoptosis (42). In the present study, however, such changes were not sufficient in and of themselves to alter temozolomide sensitivity. Rather, expression of mutant IDH1 only had effects on temozolomide sensitivity in cells that were also transformed by mutant IDH1. As such, the effects of mutant IDH1 on temozolomide sensitivity seem to be a result of the action of a larger indirect mechanism that contributes to transformation.

The results from the present studies show that expression of exogenous mutant IDH1 increases RAD51-driven HR, which in

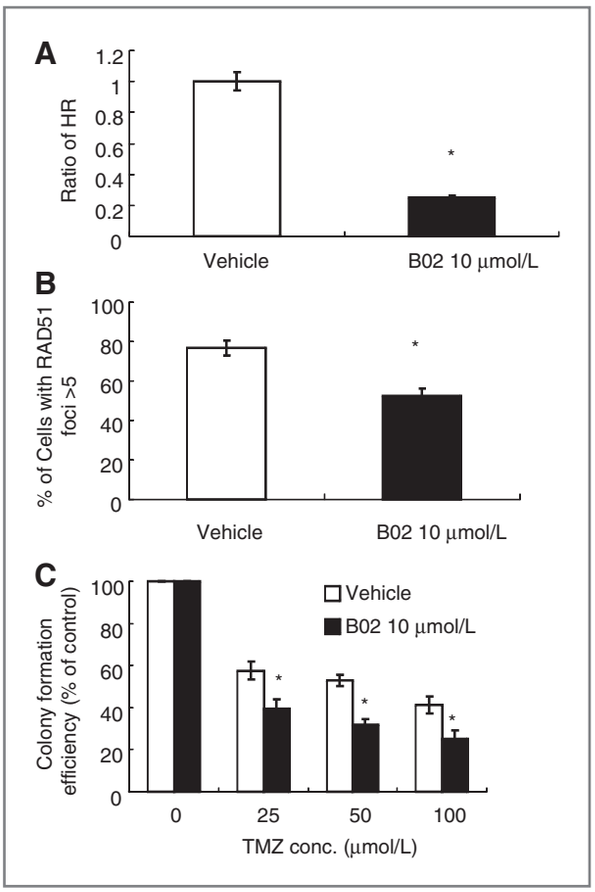

Figure 5. A, HR activity in vehicle or drug-treated (10 μmol/L B02, 48 hours) E6/E7/hTERT+mutant IDH1 cells as determined by an *in vivo* plasmid-based recombination reporter assay. Value of the vehicle cells was set at 1. B, quantitated data from immunofluorescence analysis of RAD51 foci in the same vehicle and drug-treated (B02, 10 μmol/L, 48 hours) cells 3 days after temozolomide exposure (100 μmol/L, 3 hours). C, colony formation efficiency of vehicle and drug-treated (B02, 10 μmol/L) cells after temozolomide exposure (0–100 μmol/L, 3 hours). *, $P < 0.05$. Values derived are representative of, or derived from, ≥3 independent experiments. TMZ, temozolomide.

turn contributes to increased temozolomide resistance as well as perhaps the transformation process itself. Although the mutant IDH1-driven changes in HR and temozolomide sensitivity occur in the absence of changes in the protein level of RAD51 (relative to WT IDH1 groups, not shown), a number of proteins contribute to HR, and modulation of any or all of these could lead to changes in the rate of HR. HR has been suggested to contribute to genomic stability and suppression of tumorigenesis (43), and as such it is not immediately apparent why the expression of transforming IDH1 mutants increases HR. It is worth considering, however, that mutant IDH1 tumor cells are typically also defective in telomere maintenance, and rely on an alternative (ALT) mechanism rather than telomerase upregulation to elongate their telomeres (13, 44). Because HR has been suggested to play a role in the ALT process (45), it may be possible that the increased HR driven by mutant IDH1 contributes to the early events required for gliomagenesis, whereas the epigenetic alterations driven by mutant IDH1 and 2HG substitute for the frank mutations and genomic instability that drive tumorigenesis in HR-deficient cells. These possibilities remain to be examined.

From a clinical perspective, the relative chemosensitivity of IDH1-mutant tumors has been a therapeutic bright spot, and there has been little consideration of means to increase drug sensitivity in IDH1-mutant tumors. The present study suggests that the chemosensitivity of mutant IDH1 tumors may be somewhat of an illusion, and that this sensitivity only exists in comparison with genetically unrelated but histologically identical IDH1 WT tumors. The present studies further suggest that targeting components of HR, including RAD51, may be as effective as targeting mutant IDH1 itself in increasing the effectiveness of standard therapy. A careful examination of the use of temozolomide, or perhaps other double-strand break-producing agents, in combination with inhibitors of HR might be an effective way to further improve response in IDH1-mutant tumors. The better understanding of the underlying mechanism of mutant IDH1 provided here may therefore pave the way for better combinatorial approaches in otherwise difficult to treat lower grade gliomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Ohba, J. Mukherjee, R.O. Pieper
Development of methodology: S. Ohba, J. Mukherjee
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Ohba
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Ohba, W.L. See, R.O. Pieper
Writing, review, and/or revision of the manuscript: S. Ohba, W.L. See, R.O. Pieper
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W.L. See, R.O. Pieper
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