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Original Article

BRCA1 identified as a modulator of temozolomide resistance in P53 wild-type GBM using a high-throughput shRNA-based synthetic lethality screening

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Abstract: Glioblastoma multiforme (GBM), the most common type of primary brain tumor, is universally fatal, with a median survival duration ranging from 12-15 months despite maximum treatment efforts. Temozolomide (TMZ) is the current standard of care for GBM patients; however patients usually develop resistance to TMZ and limits its benefit. The identification of novel synergistic targets in GBM will lead to the development of new targeted drugs, which could be combined with broad-spectrum cytotoxic agents. In this study, we used a high-throughput synthetic lethality screen with a pooled short hairpin DNA repair library, in combination with TMZ, to identify targets that will enhance TMZ-induced antitumor effects. Using an unbiased bioinformatical analysis, we identified BRCA1 as a potential promising candidate gene that induced synthetic lethality with TMZ in glioma sphere-forming cells (GSCs). BRCA1 knockdown resulted in antitumor activity with TMZ in P53 wild-type GSCs but not in P53 mutant GSCs. TMZ treatment induced a DNA damage repair response; the activation of BRCA1 DNA repair pathway targets and knockdown of BRCA1, together with TMZ, led to increased DNA damage and cell death in P53 wild-type GSCs. Our study identified BRCA1 as a potential target that sensitizes TMZ-induced cell death in P53 wild-type GBM, suggesting that the combined inhibition of BRCA1 and TMZ treatment will be a successful targeted therapy for GBM patients.

Keywords: Synthetic lethality, BRCA1, TMZ, apoptosis

Introduction

Grade IV astrocytoma (as defined by the World Health Organization), also called glioblastoma multiforme (GBM), is the most aggressive and common malignant brain tumor in adults despite optimal treatment, including radical surgical resection, followed by radiation therapy and temozolomide (TMZ), the median survival duration of GBM patients is only 12-15 months [1]. The limitations of these current standard therapies for GBM are TMZ resistance, incomplete tumor resection, an insufficient radiation dose to eradicate the tumor, blood-brain barrier disruption, and chemotherapy toxicities [2]. Overcoming any of the above limitations will lead to more effective therapeutics, benefitting GBM patients.

TMZ treatment results in a moderate prolongation of survival [1]. However, because of the acquisition of acquired resistance, its benefits are limited. The TMZ resistance process is not completely understood. Studies have shown that it is not mediated by a single molecular event but by multiple events; in most cases, this resistance is associated with the expression levels of DNA alkylating proteins and DNA damage repair (DDR) enzymes. The cytotoxicity of TMZ is mediated by its addition of methyl groups at N^7 and O^6 sites on guanines and the O^3 site on adenines in genomic DNA. In approximately 50% of patients, O^6-methylguanine (O^6-MetG) is rapidly removed by the enzyme O^6-methylguanine-DNA methyltransferase (MGMT), conferring resistance to chemotherapy. MGMT also plays a key role in repairing O^6-site

lesions induced by lomustine and carmustine, which are second-line chemotherapies for GBM. In the remaining 50% of GBM patients, MGMT expression is absent as a result of methylation of the MGMT promoter [3]. MGMT-mediated repair of O^6-MetG is deficient, and cells use a detour pathway to maintain genomic stability. The unrepaired O^6-MetG leads to stalled replication forks that result in DNA double-strand breaks (DSBs). These DSBs are repaired by two major mechanisms: non-homologous end-joining (NHEJ) and homologous recombination (HR). Ataxia-telangiectasia mutated serine/threonine protein kinase and Rad3-related signaling are activated to repair one-ended DSBs by HR, and ataxia-telangiectasia mutated deficiency is associated with increased sensitivity to TMZ.

Some molecules that are essential for the homologous recombination (HR)-dependent DNA repair pathway in mammalian cells have been reported to be involved in cellular resistance to alkylating agents, such as breast cancer 1 (BRCA1), BRCA2, and RAD51 [4-7]. BRCA1, a key player in DNA damage response, is critical for DNA repair, transcription, chromatin remodeling, and cell survival. In mammalian cells, BRCA1/2, FANCD2, and RAD51 protect the replication forks, preventing DSB repair protein MRE11 nuclease-mediated DNA strand degradation [8, 9]. The role of BRCA1 in cell cycle control involves its ability to interact with various cyclins and cyclin-dependent kinases, activate the cyclin-dependent kinase inhibitor p21-WAF-1, and P53. Several studies have shown that malignant gliomas exhibit constitutive activation of the DNA damage response, a network that has been implicated in the early stages of tumor progression [10, 11], as well as in tumor maintenance and response to therapeutics in later stages of cancer [12]. Because of the genomic instability of GSCs and consequent replication stress, glioma development may dependend on BRCA1; thus, BRCA1 may be a negative prognostic factor for glioma patient survival [13, 14].

The identification of novel synergistic targets for GBM will lead to the development of targeted drugs, which could be combined with broad-spectrum cytotoxic agents. In this study, we used high-throughput synthetic lethal screening to identify targets that could be used to enhance the antitumor effect of TMZ. Our unbiased screen identified synthetic lethality between TMZ and various DDR candidate genes. Validation of the top hits revealed that BRCA1 is the top gene candidate for further study. Knockdown of BRCA1, together with TMZ treatment, had a significant antitumor effect in P53 wild-type (wt) glioma sphere-forming cells (GSCs). TMZ treatment led to the activation of the BRCA1 DNA repair pathway, and the combination of TMZ and BRCA1 knockdown induced DNA damage and cell death in P53 wt GSCs. In this study, we identified a potential target BRCA1, whose knockdown sensitizes GBM to TMZ treatment.

Materials and methods

Cell lines and reagents

We used 14 patient-derived GSC lines that had been isolated from fresh surgical specimens of human GBM tissue from 2005 through 2008, as described previously [15]. Cells were authenticated by testing short tandem repeats using the Applied Biosystems AmpFISTR Identifier kit (Foster City, CA, USA). The last authentication test was performed on July 31, 2017. This study was approved by the institutional review board of The University of Texas MD Anderson Cancer Center (Houston, TX, USA). The GSC lines were cultured in DMEM/F12 medium containing B27 supplement (Invitrogen, Grand Island, NY, USA), basic fibroblast growth factor (20 ng/ml), and epidermal growth factor (20 ng/ml).

Pooled lentiviral shRNA library screening

The 3.5K DNA repair shRNA library plasmids were purchased from Cellecta (Mountain View, CA, USA); each plasmid contains 10 shRNAs per gene. The library contained 350 DNA repair genes in total. A total of 1×10^7 GSC11 cells were seeded into a 175 cm^2 cell culture flask for transduction. Cells were transduced with the lentiviral packaged modules at around 20% multiplicity of infection (MOI = 0.35) in DMEM/F12 medium containing 2.5 μg/ml Polybrene (Millipore, Burlington, MA, USA). We anticipated that the majority of transduced cells would carry only one integrated provirus from the 3.5K DNA repair shRNA library plasmids. The viral supernatant was replaced after a 24-hour cell transduction, and the cells were incubated with fresh DMEM/F12 medium (no Polybrene

or virus). The transduced cells were selected by 1 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO, USA) for 3 days.

Next, 3×10^6 cells were collected as a reference sample for transduction quality control. Duplicated 2×10^6 cells were seeded in 25-cm^2 cell culture flasks as untreated and TMZ IC_{10} (100 μM)-treated groups. After a 3-day cell culture (one cell doubling time), cells were counted and diluted, and the cells in each flask were treated with fresh TMZ IC_{10}. After eight cell doubling time, the cells (untreated × 2 and TMZ treated × 2) were harvested for DNA extraction and barcode analysis.

Barcode analysis of sequencing data

Genomic DNA extraction, barcode amplification, and sequencing library preparation were performed according to our previously published protocol [16]. Illumina-based sequencing results were processed using CASAVA (v.1.8.2), and generated reads were processed using our in-house pipeline. Raw FASTQ files were filtered for a 4-bp spacer (CGAA) starting at the 18th base, allowing for one mismatch, such that only reads amplified using the above-mentioned PCR steps were used for further processing. We then extracted 23-44 bp of the above reads and aligned them to their respective library (3.5k DNA repair library) using Bowtie (2.0.2) [17]. We used SAMtools to count the number of reads aligned to each barcode [17, 18].

For each sample, the log2 fold-change (FC) was calculated by comparing the collected endpoints to the reference pellet. A summary measure per condition was derived using the median of the quantile-transformed log2 FC across replicates. Thereafter, a modified version of the Redundant siRNA activity (RSA) algorithm was used to derive the gene-level summary measure per condition. Specifically, at least two hairpins were used when calculating the minimum *p*-value (in RSA). In addition, hairpins that ranked above luciferase controls were not used in choosing the minimum *p*-value. The quantile rank of luciferase control barcodes was evaluated across all experiments. They ranked a mean of > 0.6 (on the quantile-transformed log2 FC scale). Therefore, hairpins with quantile-transformed log2 FC > 0.6 were not used for the gene-level RSA score (log value) [19].

Cell proliferation assay

The CellTiter-Blue (Promega, Madison, WI, USA) viability assay was used to estimate cell proliferation. Cells were treated in triplicate with either TMZ alone, BRCA1 siRNA/shRNA alone, or both for 5 days. Cell viability was calculated and Graphpad Prism 7 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to calculate IC_{50} values.

Western blot analysis

A Western blot analysis was carried out as previously described [20]. The following primary antibodies were used for protein expression detection: anti-BRCA1, anti-P53, anti-rH2AX, anti-PARP, anti-caspase 3 (Cell Signaling, Boston, MA, USA), anti-FANCD2, and anti-Rad51 (Abcam, Cambridge, MA, USA). Anti-β-actin antibody was purchased from Sigma (St. Louis, MO, USA) and used as the loading control.

Flow cytometric analysis of apoptosis and cell cycle

Cell apoptosis was detected using the Annexin-V-Fluor staining kit (Roche, San Francisco, CA) according to the manufacturer's instructions. In brief, for apoptosis detection, 1×10^6 cells were collected and resuspended in 100 μL of binding buffer. FITC annexin V (5 μL) and 7-amino-actinomycin D (7-AAD) (5 μL) were added, and cells were incubated for 15 minutes in the dark at ambient temperature. Samples were tested using BD FACSCelesta (BD Biosciences, Franklin Lakes, NJ) and analyzed using FlowJo software version 10.3.

Small interfering RNA (siRNA) gene silencing and CRISPR knockout

GSCs were transfected with 40 nM siRNA against human LIG1, FANCB, PPM1D, TERF2, POLD1, POLR2G, BRCA1, and negative control siRNA (Qiagen, Hilden, Germany) using Lipofectamine 2000 transfection reagent, according to the manufacturer's instructions (Invitrogen Life Technologies). The knockdown efficiency was checked by measuring the protein expression of the target gene transcript by immunoblotting.

For the CRISPR-Cas9-mediated knockout of P53 in GSC11 cells, P53 double nickase plasmid (sc-416469-NIC, Santa Cruz, Dallas, TX,

USA) and control double nickase plasmid (sc-437281, Santa Cruz) were transfected into GSC11 cells using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) for 48 hours. GSC11 cells with green fluorescent protein were sorted by flow cytometry and plated into 96-well plates to form single colonies. After 4-5 weeks, single colonies were transferred into 24-well plates to determine cell proliferation. A reverse transcription-polymerase chain reaction was performed, followed by sequencing to confirm complete allelic knockout.

Plasmids and transfection

Lentiviral vector pLKO-mediated expression of shRNAs for BRCA1 (clone ID TRCN0000244986 and TRCN0000244984) were purchased from Sigma-Aldrich. Human embryonic kidney 293FT cells were used to produce lentiviral particles with the mixed set of packing plasmids, and the viruses were concentrated and titered as described previously [15].

Immunofluorescence staining of γH2AX

Cells were seeded onto Lab-Tek II tissue culture slides (Thermo Fisher, Waltham, MA, USA) and treated with TMZ, BRCA1 siRNA, or both. Cells were fixed with 4% PFA, permeabilized with 0.2% Triton X-100, blocked with 5% goat serum in PBS, and stained overnight at 4°C with γH-2AX antibody (Cell Signaling, Boston, MA, USA). Cells were then washed with PBS and stained with secondary antibody (Alexa Fluor 594 goat anti-mouse immunoglobulin G; Invitrogen, Grand Island, NY, USA) for 2 hours. Cells were counterstained with Vecta shield sealant containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). The percentage of γH-2AX-positive cells was quantified by counting five random fields in three independent experiments.

Statistical analysis

Statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA) and GraphPad Prism software 8.0.0 (GraphPad Software, San Diego, CA, USA). D'Agostino-Pearson test is used to determine if a data set is well-modeled by a normal distribution. F-test is used to test if the variances of two populations are equal. Whether two sets of data were significantly different was determined by unpaired two-tailed Student t-tests (for data sets with normal distribution and equal variance), Welch's t-test (for data sets with normal distribution and unequal variances), or Wilcoxon rank-sum test (for data sets without normal distribution). The mean values shown represent the mean of at least three independent experiments, and the error bars represent the standard deviations. The survival analysis was performed using Graphpad Prism7 software. Differences were considered significant at $P < 0.05$ for all comparisons.

Results

Screening of shRNA library against DDR genes identified synthetic lethal targets with TMZ

We screened 14 GSC lines to generate IC_{50} profiles for TMZ treatment using a cell proliferation assay. A waterfall plot was generated (**Figure 1A**), which showed that GSC11 was the most TMZ-resistant GSC line. Using GSC11 as the working model, we used an approach that combined a pooled 3500 shRNA DNA repair library, silencing 350 DNA repair genes, in the presence or absence of TMZ treatment, with an average of 10 shRNAs per gene. Transduction with low viral titer (MOI = 0.35) ensured one-virus integration into the cell. That is, only one gene was knocked down per target cell. After transduction, cells were selected with 1 μg/ml puromycin for 3 days and then split into two groups: with and without TMZ (100 μM) treatment with 2×10^6 cells per condition. After a 3-day cell culture (one cell doubling time), cells were counted and diluted, and 2×10^6 cells were added to each 25-cm^2 cell culture flask, with fresh TMZ. After eight cell doubling time, the cells were harvested (untreated × 2 and TMZ treated × 2) for DNA extraction and barcode analysis (**Figure 1B**). The gene barcode signals, which were lower in the TMZ treatment group than in the control group, were identified as synthetic lethal candidate genes.

Synthetic lethal screening data analysis

We obtained a RSA *p*-value for TMZ-treated and control untreated samples (**Figure 2A**). We ranked genes for treated and control samples in ascending order (1 to n) according to the log *p*-value, with the highest ranked genes being the most significant (lower log *p*-values). TMZ treatment-related genes and essential genes

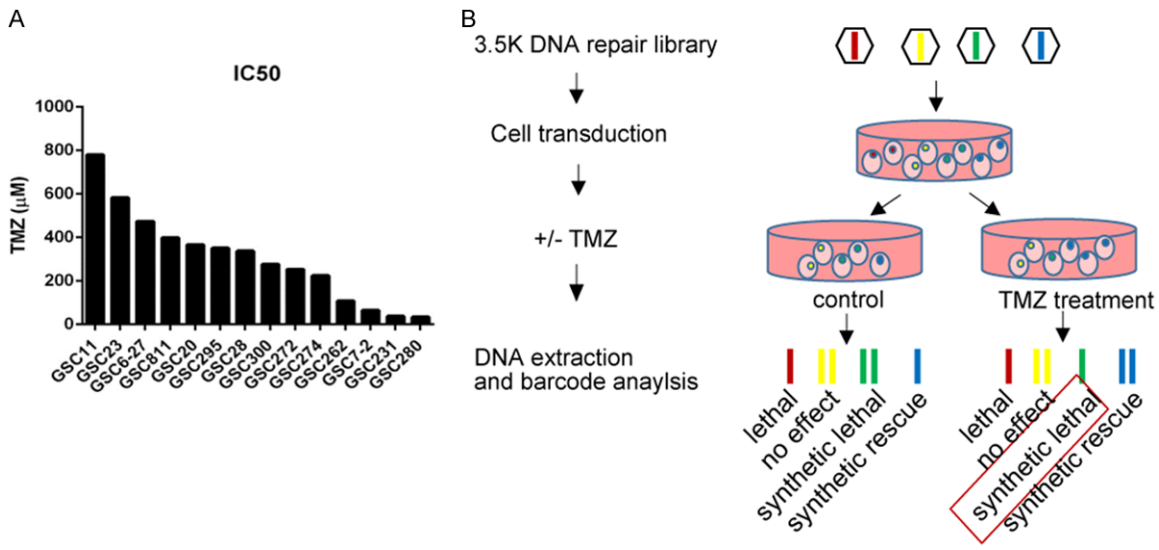
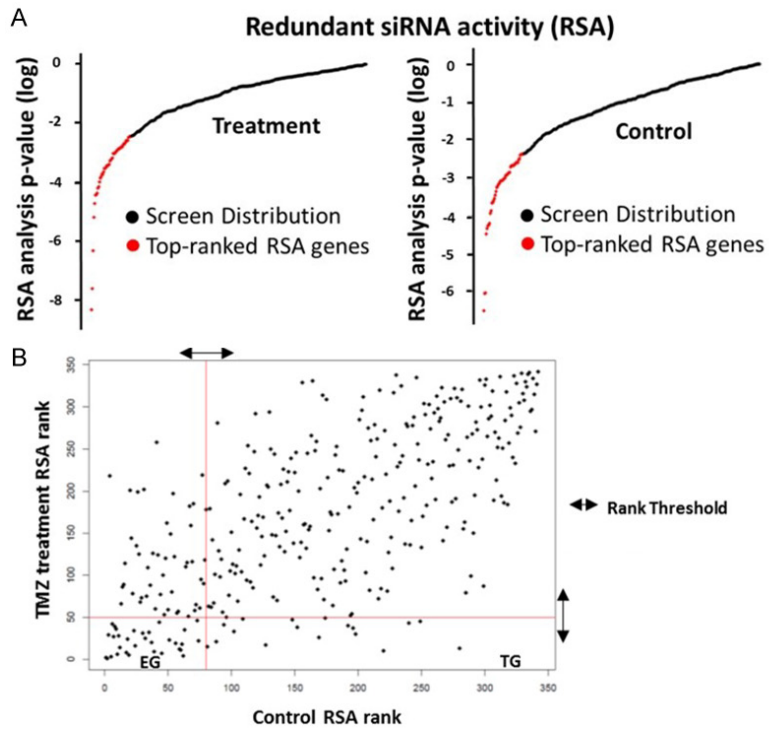


Figure 1. Synthetic lethality Screening using TMZ with DDR genes. A. GSCs were screened for TMZ sensitivity using cell titer blue analysis. B. shRNA library against 3.5 kb DNA damage repair genes was transduced in presence and absence of TMZ to identify synthetic lethal genes.



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Treatment-relevant genes (TG)

Gene	Control Rank	Treatment Rank	Rank Difference
LIG1	280	13	267
FANCB	220	10	210
PPM1D	249	45	204
BRCA1	240	43	197
FANCM	198	30	168
CDC6	196	37	159
TERF2	192	40	152
POLD1	169	26	143
HDAC4	174	49	125
SLX4	152	38	114
FANCD2	127	17	110
POLD3	148	47	101
RFC4	102	33	69
POLR2G	88	21	67
SMC6	81	15	66
XRCC5	109	48	61
FANCG	94	41	53
POLA1	96	50	46

Figure 2. Identifying synthetic lethal genes using RSA analysis. A. Redundant siRNA activity (RSA) for all genes in TMZ treated (treatment) and untreated (control) samples are ranked according to *p*-value. B. Scater plot showing distribution of shRNA, screening hits were ranked in order of the combined FC (endpoint/baseline) values of the three shRNAs of each target gene, from lowest (most depleted) to highest (most enriched). Indicated TMZ treated-specific hits selected for further validation (red) displayed lower ranking in TMZ treated GSCs compared to untreated GSCs, whereas shRNAs targeting the known essential genes were strongly depleted in all cultures. B. Scater plot showing distribution of Treatment-relevant genes (TG) whose RSA rank is high for treated samples and low for control samples and essential genes (EG) are low ranked or depleted in both. Rank difference is obtained by calculating the absolute value from the subtraction of control samples rank from treated samples rank. C. Table showing top depleted genes with TMZ treatment.

were identified by comparing and plotting the RSA *p*-value ranks using certain thresholds (X = 80, Y = 50). TMZ treatment-related genes were top ranked by TMZ treatment RSA and had the highest RSA rank difference compared to controls, while essential genes were top ranked for both treatment and control samples (**Figure 2B**). Of the 350 genes analyzed, the top 18 treatment-relevent genes are listed in **Figure 2C**, with RSA ranks.

Hit target validation identified BRCA1 as the synthetic lethal target with TMZ treatment

We selected 18 top genes for further validation; we examined the top seven targets with known functions of these genes (LIG1, FANCB, PPM1D, BRCA1, TERF2, POLD1, and POLR2G) using gene knockdown by gene-specific siRNA. Selected siRNA knockdown was tested for cell viability in the presence of TMZ in a cell proliferation assay in two TMZ-resistant GSC lines: GSC11 and GSC23. We did not see any synthetic lethal effects with LIG1, FANCB, PPM1D, TERF2, POLD1, and POLR2G after TMZ treatment (Figure S1). The siRNAs targeting BRCA1 had the strongest synthetic lethal effects after TMZ treatment in GSC11 compared to the other candidate genes (**Figures 3A** and S1). However, we did not observe synthetic lethal effects in the GSC23 cell line (**Figure 3A**).

To further validate BRCA1 as a synthetic lethal target, and explore why GSC23 did not show synthetic letal effects, we examined another four GSC lines, GSC295, GSC6-27, GSC811, and GSC272, upon BRCA1 knockdown. Interestingly, only two cell lines, GSC295 and GSC6-27, showed TMZ-induced synthetic lethality. Further characterization of the cell lines revealed that GSC11, GSC295, and GSC6-27 are all P53 wt cells and GSC23, GSC811, and GSC272 are all P53 mutant (mut) cells (**Figure 3B**). These findings suggest that synthetic lethal sensitivity to TMZ treatment and BRCA1 knockdown is associated with P53 wt function.

BRCA1 knockdown selectively induces synthetic lethality with TMZ in P53-wt cells

We performed a cell apoptosis analysis using annexin 5 staining by flow cytometry in two P53 wt cell lines (GSC11 and GSC295) and two P53 mut cell lines (GSC23 and GSC811). As shown in **Figures 4A** and S2, the combination of two BRCA1 shRNAs and TMZ induced significantly more apoptosis in in GSC11 cells (33% and 60%, respectively) in comparson to shRNA/TMZ alone ($P < 0.05$); and 64% and 62% in GSC295 cells compared to shRNA/TMZ alone ($P < 0.01$); However apoptosis was not significant in P53 mutant cells lines GSC23 and GSC811 cells (**Figures 4A** and S3).

We further confirmed apoptosis at the protein level by checking cleaved caspase 3 and PARP expression. The data revealed increased cleaved caspase 3 and PARP expression in P53 wt GSC11 cells after BRCA1 knockdown and TMZ treatment and lower expression of cleaved caspase 3 and PARP expression in P53 mut GSC23 cells (**Figure 4B**). Overall, these results suggest that synthetic lethality is present between BRCA1 knockdown and TMZ treatment in P53 wt GSCs but not in P53 mut GSCs. Taken together, these findings demonstrate a potent reduction of cell viability and apoptosis induction (synthetic lethality) in P53 wt GSCs upon genetic knockdown of BRCA1 and TMZ treatment.

To confirm that wt P53 plays an essential role in the synthetic lethality between BRCA1 knockdown and TMZ, we used CRISPR-Cas9 gene editing to knockout wt TP53 in GSC11 cells (**Figure 5A**). Knockout of P53 was confirmed by Western blot analysis (**Figure 5A**) for P53 protein. We then examined the effect of BRCA1 knockdown and TMZ treatment on apoptosis. The depletion of wt P53 rendered GSC11 ineffective at inducing apoptosis after BRCA1 knockdown and TMZ combination treatment (**Figure 5B** and **5C**), as detected by annexin V staining. Further, cleaved caspase 3 and PARP were not detected in P53 knockout GSC11 cells after BRCA1 knockdown and TMZ treatment (**Figure 5D**). These findings further suggest that p53 has a role in inducing synthetic lethality upon BRCA1 knockdown and TMZ treatment.

Synthetic lethality in P53 wt GSCs was correlated with increased DNA damage and increased DNA repair

To assess why P53 wt cells showed more TMZ-induced synthetic lethality, we evaluated the DNA damage response and DDR repair capacity of these GSCs upon TMZ treatment. We first treated cells with 100 μM TMZ for different

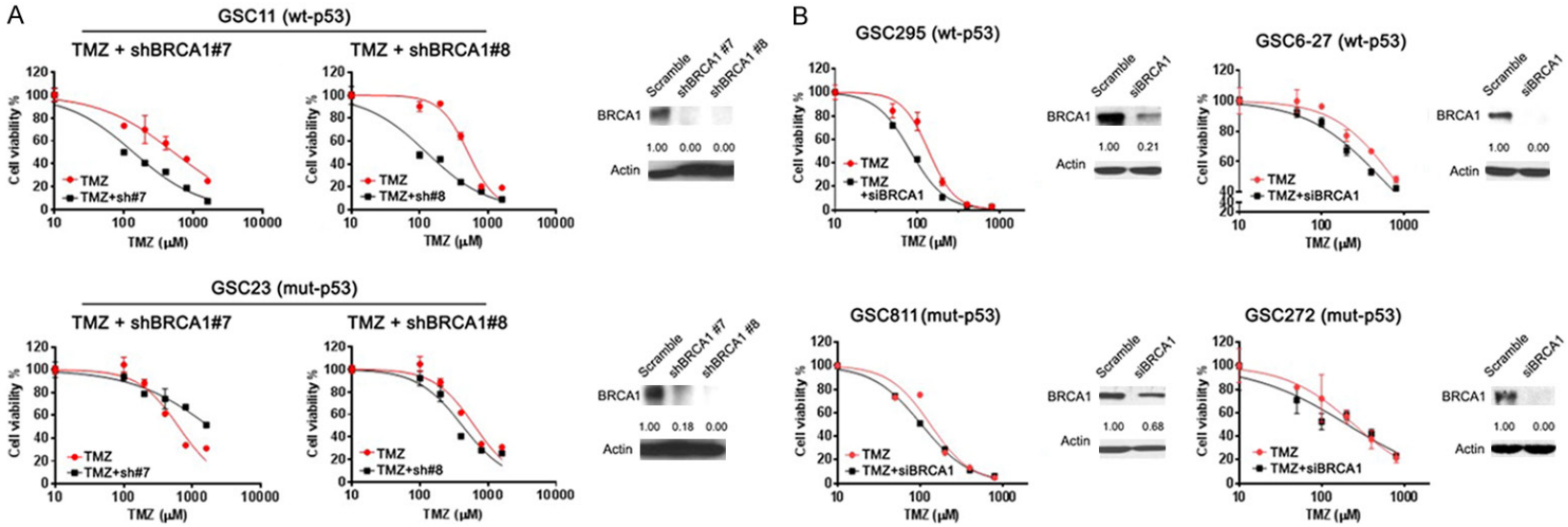

Figure 3. BRCA1 knockdown sensitizes GSCs to TMZ induced synthetic lethality selectively in p53 wild type cells. A and B. Cell viability of GSCs upon TMZ treatment upon BRCA1 kncockdown using ShRNA/siRNA against BRCA1 incomparon to scramble control. Cell viability was determined by cell titer blue assay (after 5 days of treatment) of the GSCs after BRCA1 knockdown in presence of TMZ, n = 3/data point; mean ± SD. Right panel western blot showing knock down of BRCA1 protein.

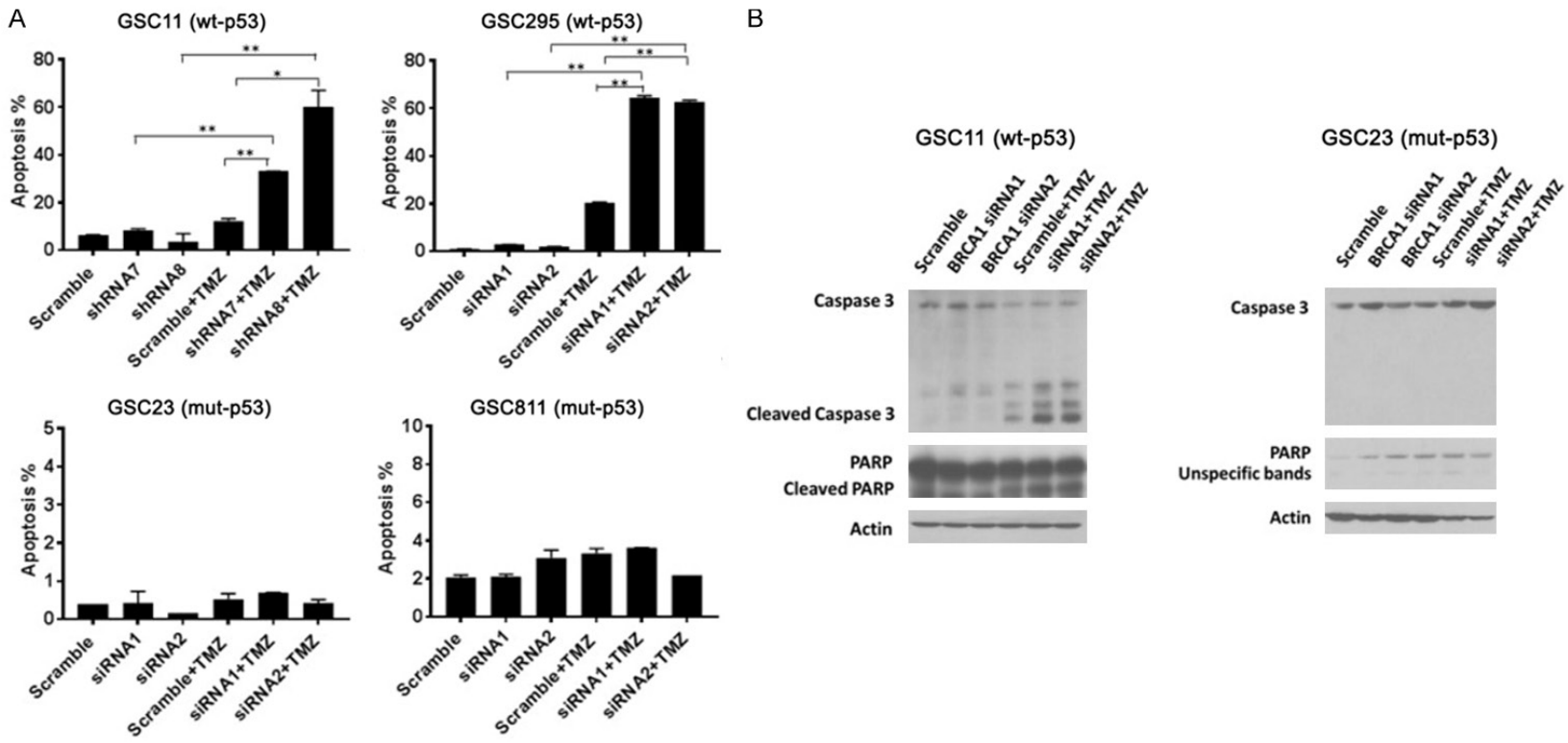

Figure 4. p53 is necesssray to induce synthetic lethality. A. Increased cell death observed in p53 wt cells (GSC11 and GSC295) in BRCA1 knockdown cells in presence of TMZ in comparison to either TMZ alone or BRCA1 knockdown alone by annexin V staining. B. Western blot analysis showing increased protein expression of cleaved caspase 3 and cleaved PARP in p53 wild type cells GSC11 cells after BRCA1 knockdown in presence of TMZ.

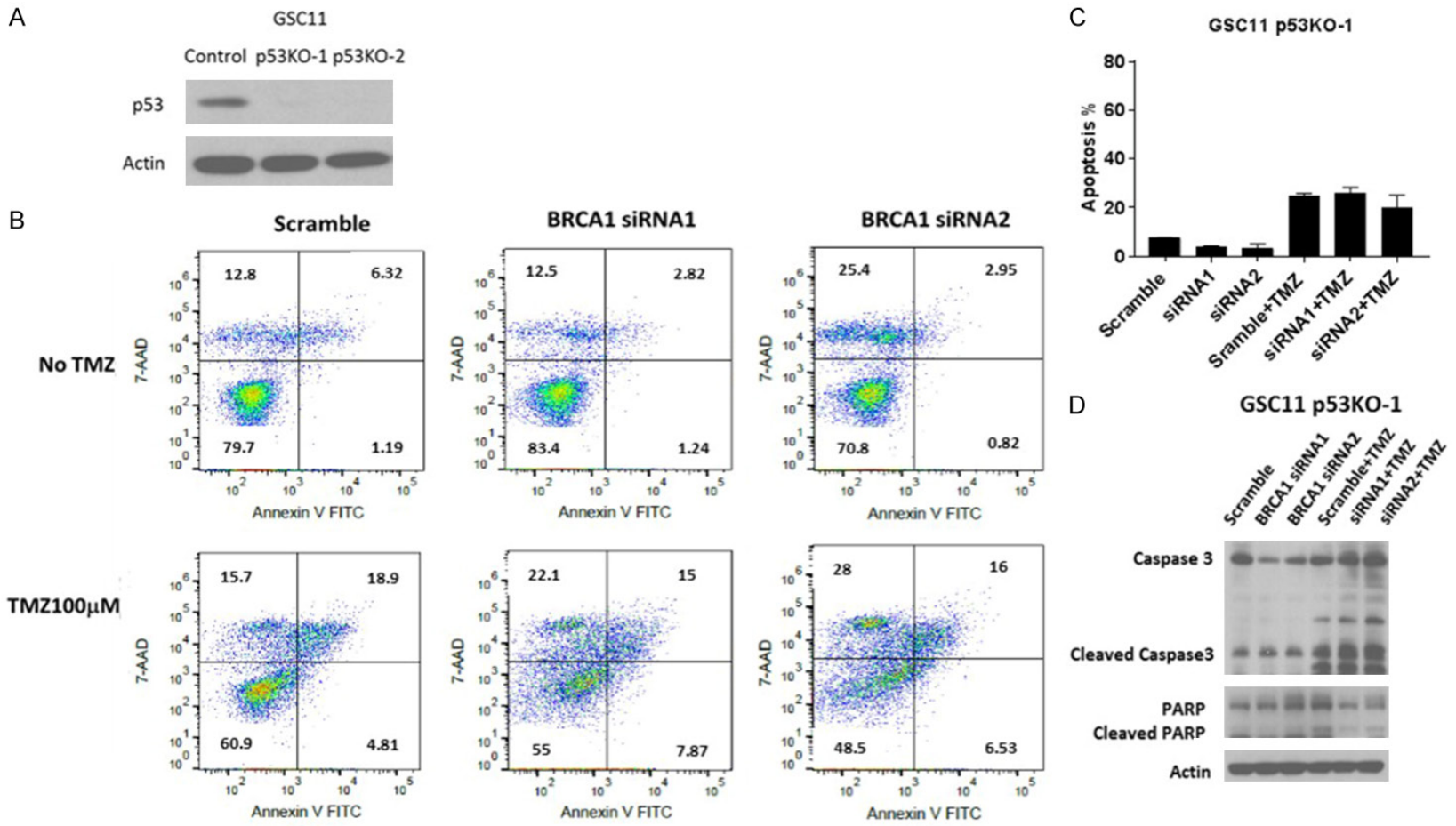

Figure 5. P53 knockdown abolishes apoptotic capacity. A. Western blot showing knock out of p53 in GSC11 (p53 wt) cells. B and C. Flow cytometry analysis showing knock down of BRCA1 in presence of TMZ failed to induce apoptosis when p53 is knocled out in p53 wt cells. D. Western blot showing no increase of caspase 3 cleavage an PARP clevage by knockdown of BRCA1 and TMZ when p53 is knocked out in wt cells.

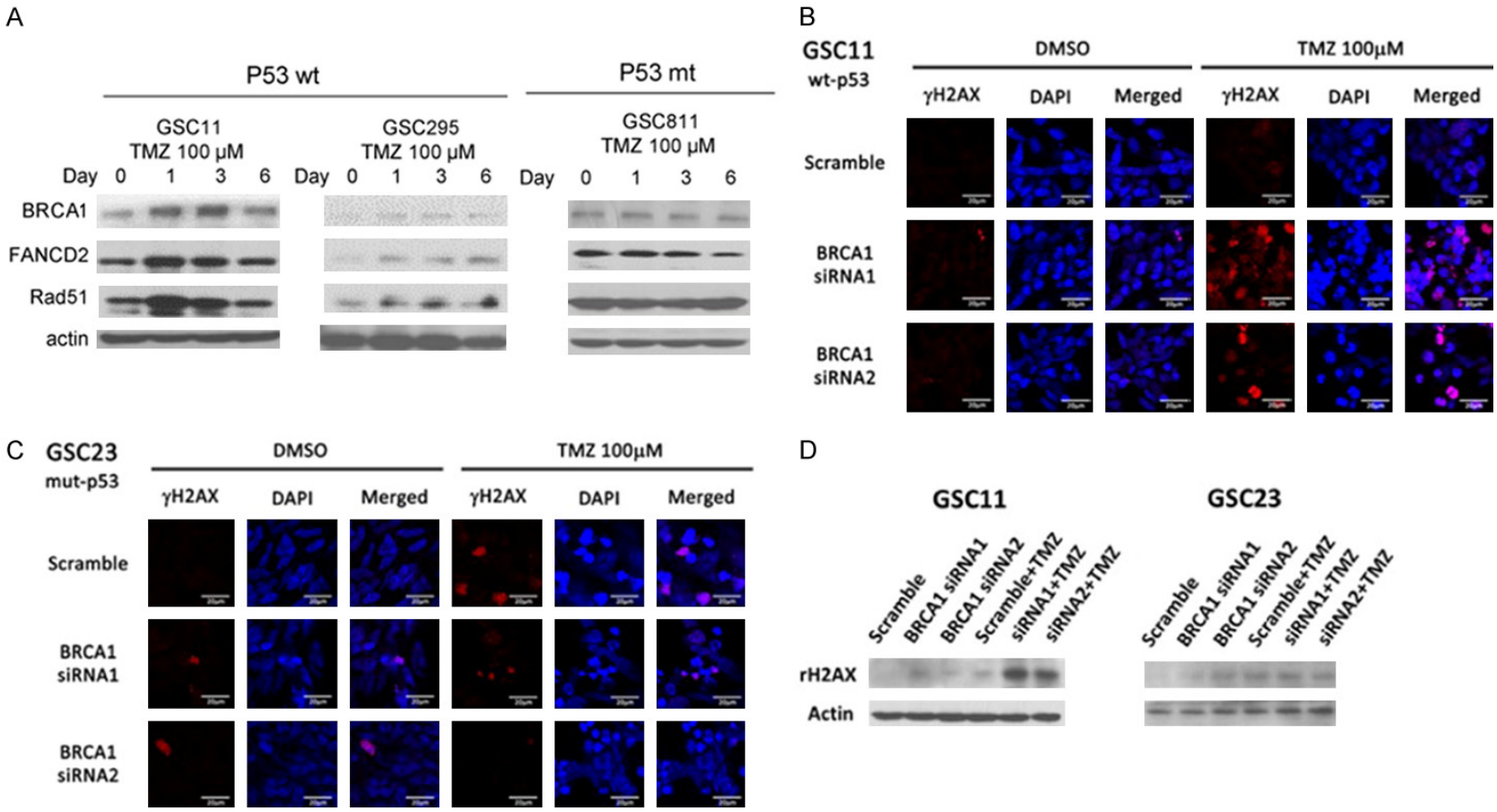

Figure 6. Increased DNA damage response (DDR) in p53 wt cells coorelates with synthetic lethality. A. Western blot analysis with the indicated antibodies of protein lysates from p53 wt and p53 mut GSCS showed increased DDR protein expression in p53 wt cells than in mut p53 cells. B and C. Immunofluorescence of p53 wt and p53 mut GSCs treated with 100 μM of TMZ and siRNA BRCA1 knockdown by for 72 hours and DNA damage was evaluated by γH2AX (red). D. Western blot showing increased γH2AX protein in p53 wt cells (GSC11) thank in p53 myt (GSC23) cell lines.

time periods. After TMZ treatment, the expression of BRCA1 and several BRCA1 downstream signaling DNA repair proteins, including RAD51 and FANCD2, were all markedly increased in a time-dependent manner (**Figure 6A**). Of interest, only the P53 wt GSC11 and GSC295 cell lines showed increased DNA repair capacity; P53 mut GSC811 cells did not show any increase in BRCA1, RAD51 and FANCD2.

We speculated that the increase in DNA repair protein expression after TMZ treatment was intended to increase the DNA damage repair response [21]. Immunofluorescence studies revealed that the levels of DNA damage response protein γH2AX foci in GSC11 and GSC23 (**Figure 6B** and **6C**) was increased after exposure to the combination of TMZ and BRCA1 siRNA in comparison to TMZ or BRCA1 siRNA alone in P53 wt GSC11 cells, while the intensity of γH2AX foci was not enhanced in P53 mut GSC23 cells. Consistent with this finding, the γH2AX protein level was significantly increased in GSC11 cells, but not GSC23 cells, after treatment with TMZ and BRCA1 knockdown (**Figure 6D**). The increase in γH2AX foci formation and the higher γH2AX protein level indicated that TMZ and BRCA1 siRNA result in more DNA damage in TP53 wt GSCs, causing synthetic lethality.

Discussion

The use of DDR inhibitors is an attractive approach to chemo-sensitization or radio-sensitization [22-25]. In this study, we tested shRNA-mediated downregulation of DNA repair library genes; and we show that downregulation of BRCA1 induced apoptosis in GSCs and enhanced cell sensitization to the chemotherapeutic agent TMZ.

We determined whether BRCA1 knockdown affects GBM resistance to TMZ. The DDR has several components that determines the fate of a damaged cell involving signaling events and enzyme activities that result from the induction and detection of DNA damage. These include cell cycle check point, regulation of DNA replication, and the repair or bypass of DNA damage. The lack of DNA repair leads to an unsupportable level of genomic instability; DDR can also affect downstream cell fate decisions, such as cell death or senescence [26, 27]. The results of previous studies have shown that there are at least 450 proteins in DDR genes [28]; the optimal drug target will depend on what type of DNA damage needs to be inhibited. Therefore, classification of GBM patients on the basis of their gene or protein profiling data could be beneficial for selecting drugs for DNA damage treatment.

Here, we used a synthetic lethality platform and a high-throughput shRNA library against DDR genes to identify the repair pathways used by GSCs to repair DNA damage upon TMZ treatment. Our hypothesis was that inhibiting DNA repair pathways will potentiate the efficacy of TMZ in GBM and form a basis for targeted therapies as a strategy to increase the effectiveness of TMZ.

Accumulating data have shown that BRCA1 plays an important role in DNA damage response and that it functions as a DDR protein in various cellular pathways that maintain genomic stability. On the basis of its associations and physical interactions with other proteins, BRCA1 plays an essential role as a multifunctional protein, mediating HR, non-homologous end-joining, and single-strand annealing repair via a variety of mechanisms, including transcriptional regulation, ubiquitination, and mRNA splicing. Various binding partners of BRCA1 can regulate its function in repair pathways; therefore, the identification of novel interactors of BRCA1 will provide information about its mechanistic mode of action and lead to the identification of novel therapeutic targets for the treatment of BRCA-associated tumors.

Here, we identified BRCA1 was crucial for inducing TMZ lethality in GSCs, using high-throughput synthetic lethality screening. In the search for synthetic lethal targets in TMZ-treated GSCs, we used an unbiased approach: we screened genes using shRNAs against the DDR library to identify the important players in DDR and define the roles of these proteins in tumor suppression. BRCA1, RAD51, and XRCC3, which are some of the major determinants of TMZ-induced cytotoxicity that are involved in HR, were shown to be regulated in malignant glioma at variable levels. We also observed that the knockdown of genes, including LIG1, FANCB, PPM1D, BRCA1, TERF2, POLD1, and POLR2G, led to TMZ-induced cytotoxicity. However, further validation with BRCA1 knockout the presence of TMZ had cytotoxic effects

in the GSCs tested and revealed new options for concomitant treatment via HR-related DNA repair. Further validation with BRCA1 knockdown showed that only P53 wt GSCs showed synthetic lethality with TMZ; such cytotoxicity was not observed in P53 mut GSCs.

There have been mixed results concerning the association between TMZ resistance and gene mutation [29-31]. P53 is the only gene that has been shown to be associated with TMZ resistance. However, while some reports have shown that P53 wt GBM cells were resistant to TMZ, others have shown that P53 mut cells were resistant. Based on previous reports, the suppression of MGMT reporter gene activity by wild-type p53 predicts a higher efficacy of TMZ in P53 wild-type than p53 mutant tumours [32, 33]. In addition, a P53 mediated cell cycle arrest after TMZ treatment may also be necessary for TMZ induced cytotoxicity [34, 35].

In this study, P53 status seemed to be associated with the induction of synthetic lethality with TMZ and blocking DDR by BRCA1 deficiency. Therefore, it is important to continue elucidating BRCA1-dependent pathways to design specific therapies that can target these specific tumors. In addition, targeting BRCA1 function in combination with chemotherapy is an effective second line of therapy against GBM that has become resistant to these treatments; it increases the sensitivity of tumor and overcomes its resistance. An increasing amount of data support the key role of DNA repair in targeted cancer therapy, therefore using personalized therapy of high-grade gliomas with genotoxic drugs is a growing and effective field of research.

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Disclosure of conflict of interest

W.K. Alfred Yung discloses a conflict of interest as a consultant with DNATrix. The rest of the authors have no conflicts of interest to disclose.

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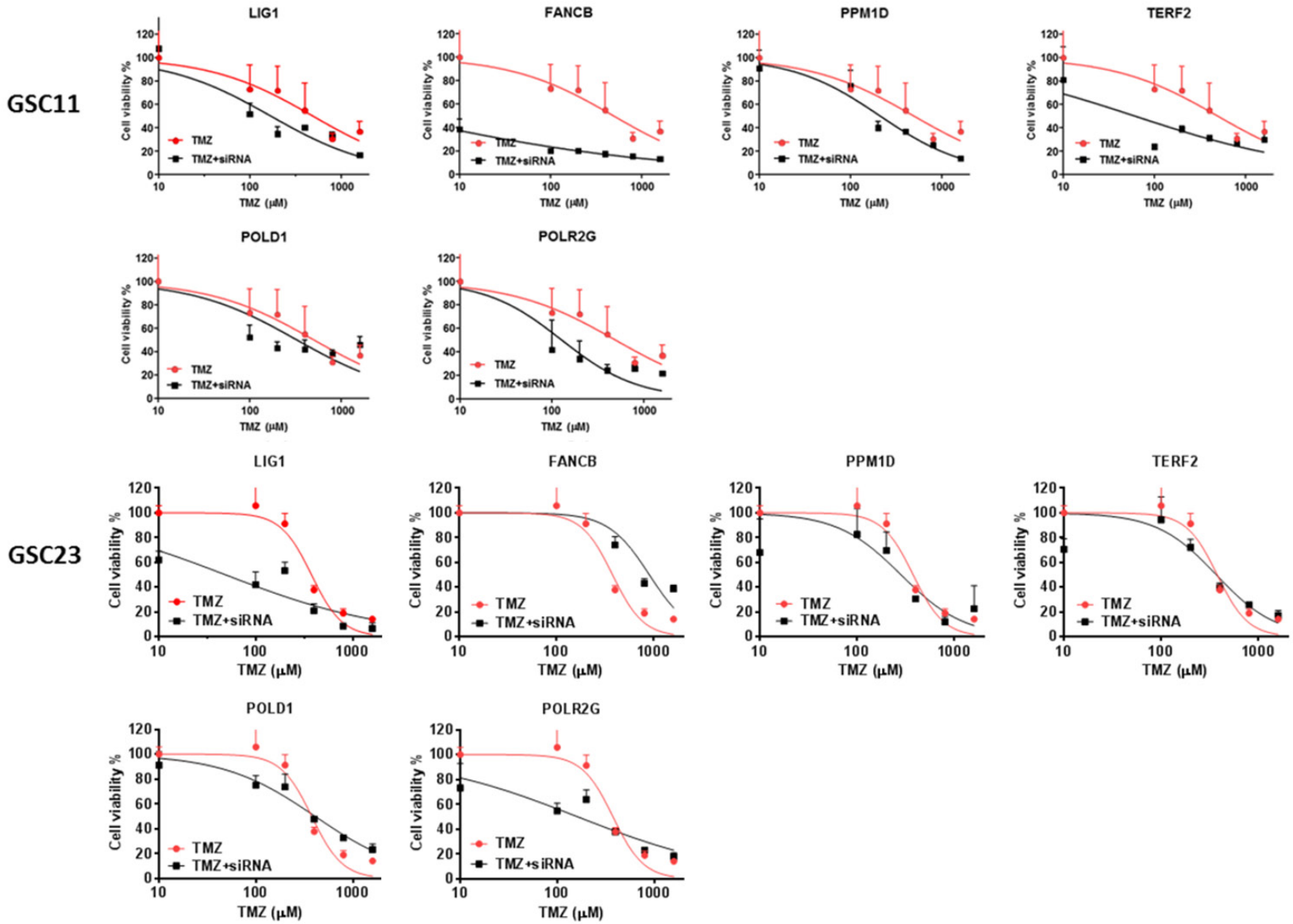

Figure S1. Effect of Knock down of various genes by SiRNA in combination with TMZ on cell viability as detected by cell titer blue assay. Cells were treated with specific siRNA and varying doses of TMZ for 72 hrs and cell viability was determined after 72 hrs.

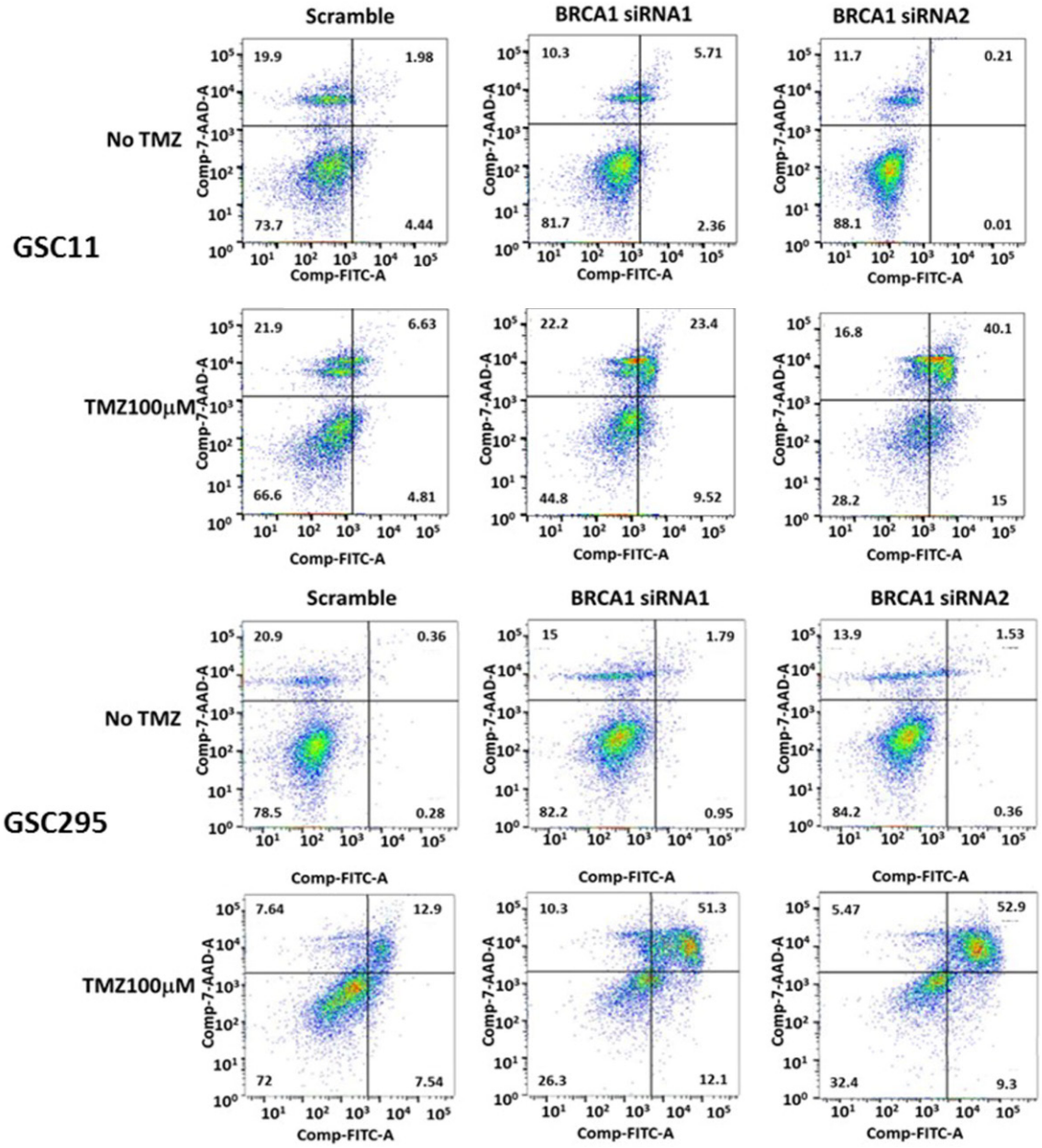

Figure S2. BRCA1 knockdown and TMZ combination induced more apoptosis in wt-p53 GSCs. GSCs were treated for 72 hours and then stained with FITC-annexin V/7-AAD.

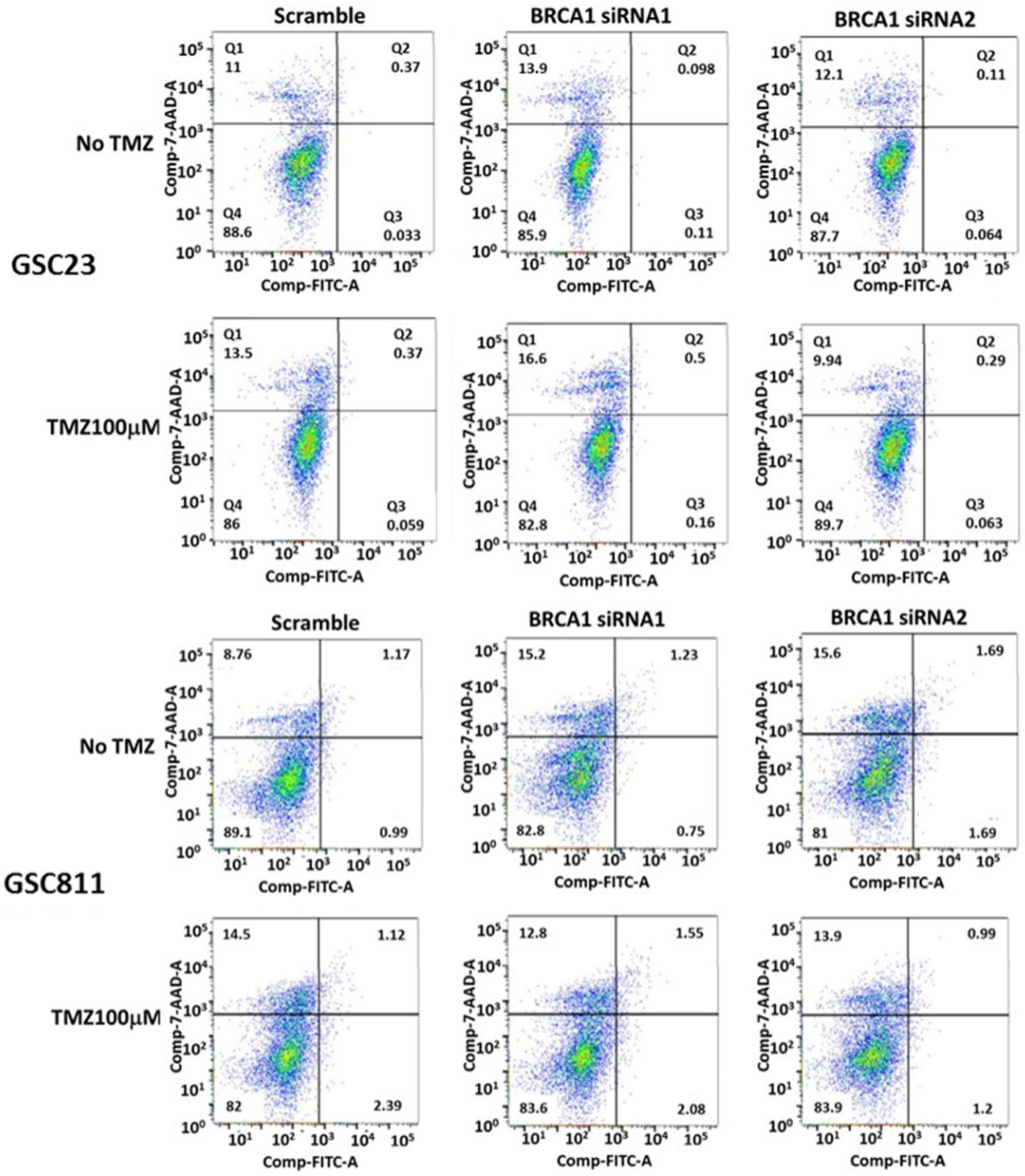

Figure S3. BRCA1 knockdown and TMZ combination did not induced more apoptosis in Mut-p53 GSCs. GSCs were treated for 72 hours and then stained with FITC-annexin V/7-AAD.