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Synthetic mRNA-based gene therapy for glioblastoma: TRAIL-mRNA synergistically enhances PTEN-mRNA-based therapy

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Glioblastoma (GBM) is characterized as having high molecular heterogeneity and complexity, which can be well revealed by genomic study. A truly effective treatment for GBM should flexibly address its heterogeneities, complexity, and strong drug resistance. This study was performed to explore the effectiveness of an mRNA-based therapeutic strategy using *in vitro* synthesized PTEN-mRNA and TRAIL-mRNA in tumor cells derived from PTEN-deletion patients. The PTEN gene alterations were revealed by whole-exome sequencing of three paired clinical GBMs and selected as the therapy target. Patient-derived primary glioblastoma stem cells (GBM2) and a DBTRG-cell-derived xenograft were used to detect mRNA's cytotoxicity *in vitro* and tumor suppression *in vivo*. Following the successful *in vitro* synthesis of PTEN-mRNA and TRAIL-mRNA, the combinational treatment of PTEN-mRNA and TRAIL-mRNA significantly suppressed tumor growth compared with treatment with PBS (96.4%), PTEN-mRNA (89.7%), and TRAIL-mRNA (84.5%). The combinational application of PTEN-mRNA and TRAIL-mRNA showed synergistic inhibition of tumor growth, and the JNK pathway might be the major mechanism involved. This study provided a basis for an mRNA-based therapeutic strategy to be developed into an effective patient-tailored treatment for GBM.

Exon analysis of paired glioma samples can find reliable therapeutic targets. PTEN-mRNA and TRAIL-mRNA combined therapy can reduce drug resistance and enhance antitumor activity; the JNK pathway is the main mechanism for the observed synergy.

INTRODUCTION

Although surgical techniques and adjuvant therapies have made significant progress over the past few decades, the treatment for glioblastoma (GBM) remains poor.[1] The high degree of heterogeneity and invasiveness and the existence of blood-brain barrier (BBB) all contribute to the difficulty in GBM treatment. As a result, the survival rate for GBM patients within five years after diagnosis is less than 5%.[2]

With improved understanding of the molecular biology of GBM, targeted therapies have become a new hope in improved treatment of the disease. Molecularly targeted therapies and gene therapies have shown promise in preclinical trials, but the results of clinical trials of various targeted agents have shown only modest therapeutic benefit, and many of these therapies demonstrated no therapeutic benefit.[3–5] The lack of clinical benefit is, at least in part, likely due to the high heterogeneity and complexity of GBM. An ideal treatment for GBM should adequately address its heterogeneity and complexity and its resistance to the standard therapies. Whole-exome sequencing exhibited the gene mutation landscape that provides precise information to guide targeted gene therapy. mRNA has a variety of unique biological characteristics, such as easy manipulation, rapid and transient expression, and adaptive convertibility without mutagenesis.[6] Therefore, mRNA-based therapeutic strategies have great potential and can be developed to be the most effective treatment for GBM.

On the basis of confirming the significance of phosphatase and tensin homolog on chromosome ten (PTEN) by whole-exome sequencing of paired clinical GBM samples and gene analysis using The Cancer Genome Atlas (TCGA) data, we choose PTEN as the therapeutic target.

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PTEN functions as the central negative regulator of the PI_3K-AKT-mTOR (phosphoinositide 3-kinase–protein kinase B–mammalian target of rapamycin [mTOR]) pathway in controlling apoptosis.[7] PTEN induces cell apoptosis through intrinsic pathways. Almost all patients with gliomas are associated with absent or low activity of PTEN, and up to 40% of gliomas are caused by PTEN mutations.[8,9] Many studies revealed that PI_3K pathway inhibitors may be effective alone or in combination with chemotherapy or radiation therapy or biological therapy, such as tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL) or monoclonal antibodies.[10,11] Some researchers provided evidence supporting PTEN upregulating TRAIL sensitivity in tumor cells.[12,13]

TRAIL is an apoptotic gene, whose protein product can selectively induce tumor cell apoptosis through an extrinsic pathway without damaging normal cells.[14,15] Its specificity is determined by the specific expression of death receptors DR4 and DR5 on tumor cells.[16,17] Previous studies showed that the separate use of TRAIL or PTEN has only limited antitumor effect on gliomas,[18–21] and the clinical trials have revealed only little therapeutic benefit.[5]

During the past twenty years, various targeted therapies have been explored to treat GBM based on the study of genomic tools, such as inhibitors of multiple tyrosine kinase pathways,[22] poly (ADP-ribose) polymerase 1/2 (PARP1/2) inhibitors,[23] mTOR inhibitors,[24] histone deacetylase inhibitors,[25,26] and proteasome inhibitors.[27,28] But these treatments are unable to target the GBM patients' genomic and molecular characteristics sufficiently. In this work, we sequenced the whole exon of three paired GBM samples and a matched blood control and found that the PTEN's deletion or mutation play an essential role in these three GBM patients. Then, PTEN-mRNA and TRAIL-mRNA were synthesized *in vitro* and intracranially delivered. Interestingly, the efficacy of PTEN-targeted therapy can be improved through co-targeting TRAIL *in vitro* and *in vivo*. Lastly, we studied the mechanism of the combination effect through RNA sequence and protein array and found that the synergistic effect is mediated by JNK. We expect an mRNA-based therapeutic strategy to be developed into an effective patient-tailored treatment for GBM.

RESULTS

Whole-exon sequencing identified PTEN as a promising molecular target

We recruited three GBM patients from the Taihe Hospital and performed whole-exon sequencing (WES) on primary tumor tissues, recurrent tumor tissues, and blood controls. The resulting sequencing coverage was 100-fold for the tumor samples and 50-fold for the blood samples. In total, 130 functional driver sSNVs/indels were detected across these samples. The most frequent drivers were PTPRT (50%), GNAS (36%), CIC (33%), EP300 (33%), and FLT3 (33%). The top 13 mutation genes were presented in Figure 1A. The GBM purity of P1,P2 and P3 were 0.28, 0.43, and 0.65, respectively. At the chromosomal level, 2 primary GBM samples harbored loss of the entire of chromosome 10, including the PTEN locus losses (Figure 1E). Another primary samples included two somatic mutations in PTEN (chr10:89720649A>T and chr10:89720726G>T). Meanwhile, the corresponding cellular fraction (CCF), which represents the fraction of cancer cells that contain a given alteration, showed that these mutations were higher than other alterations (Figures 1B–1D). The high mutation frequency of PTEN in GBM was directly correlated with a low survival rate (Figure S1). On these grounds, the patient2-derived cell line, GBM2, was isolated from a GBM patient (patient 2, with complete deletion of PTEN) for the therapeutic experiments in this study.

TRAIL-mRNA synergistically enhances PTEN-mRNA treatments *in vitro*

The schematic of the process that mRNA synthesized *in vitro* and expressed *in vivo* is presented in Figure S2A. The expression of synthetic Luc-mRNA was stable after intracranial injection, and the duration of luciferase expression was more than 60 h (Figure S2B). Synthetic mRNA has been identified as an effective gene transfection agent and widely applied in the therapy of different diseases.[29]

PTEN-mRNA and TRAIL-mRNA were synthesized for the study of an mRNA-based therapeutic strategy. The right sizes of synthesized PTEN-mRNA and TRAIL-mRNA were confirmed using thermal asymmetric interlaced (TAIL)-PCR (Figure 2A). The expressions were verified in 293T cells by immunoblotting (Figure 2B). We measured cell viability of DBTRG-Luc cells with an RTCA after PTEN-mRNA and TRAIL-mRNA transfection. The combinational treatment further reduced cell viability compared with each single mRNA (Figure 2D). We used the additive model to evaluate the nature of combined treatment.[30] According to this method, we found a synergistic reduction in the combined mRNA group (observed/expected ratio = 0.46; Table 1). We also measured the effect on GBM2, which was isolated from human GBM tissue, and validated the specific markers (Figure S3). The mRNA-induced cell viability was also observed in GBM2 (Figure 2C).

Tumor cell death was analyzed by flow cytometry in PTEN-mRNA- and TRAIL-mRNA-transfected GBM2 and DBTRG-Luc cells. The combined treatment showed much stronger cytotoxicity compared with each mRNA alone and untreated cells (Figures 2E–2H). These results exhibited that PTEN-mRNA and TRAIL-mRNA could inhibit GBM cell growth in a synergistic manner.

Determination of the synergistic effect of PTEN-mRNA and TRAIL-mRNA treatments *in vivo*

To prove the therapeutic effects of combinational application of PTEN-mRNA and TRAIL-mRNA *in vivo*, nude mice were randomly divided into four groups (n = 5) and intracranially implanted with DBTRG-Luc cells, which were pretransfected with PBS, PTEN-mRNA, TRAIL-mRNA, and PTEN-mRNA + TRAIL-mRNA, respectively. Mice were disposed as exhibited in Figure 3A. The antitumor gene transfection did not reduce the luciferase radiance, indicating that the cell viability of the tested tumor cells was not altered by synthetic mRNA transfection within 6 h (Figure S4). The results of *in vivo* imaging system (IVIS)-monitored tumor growth demonstrated that the combinational treatment of PTEN-mRNA and TRAIL-mRNA significantly

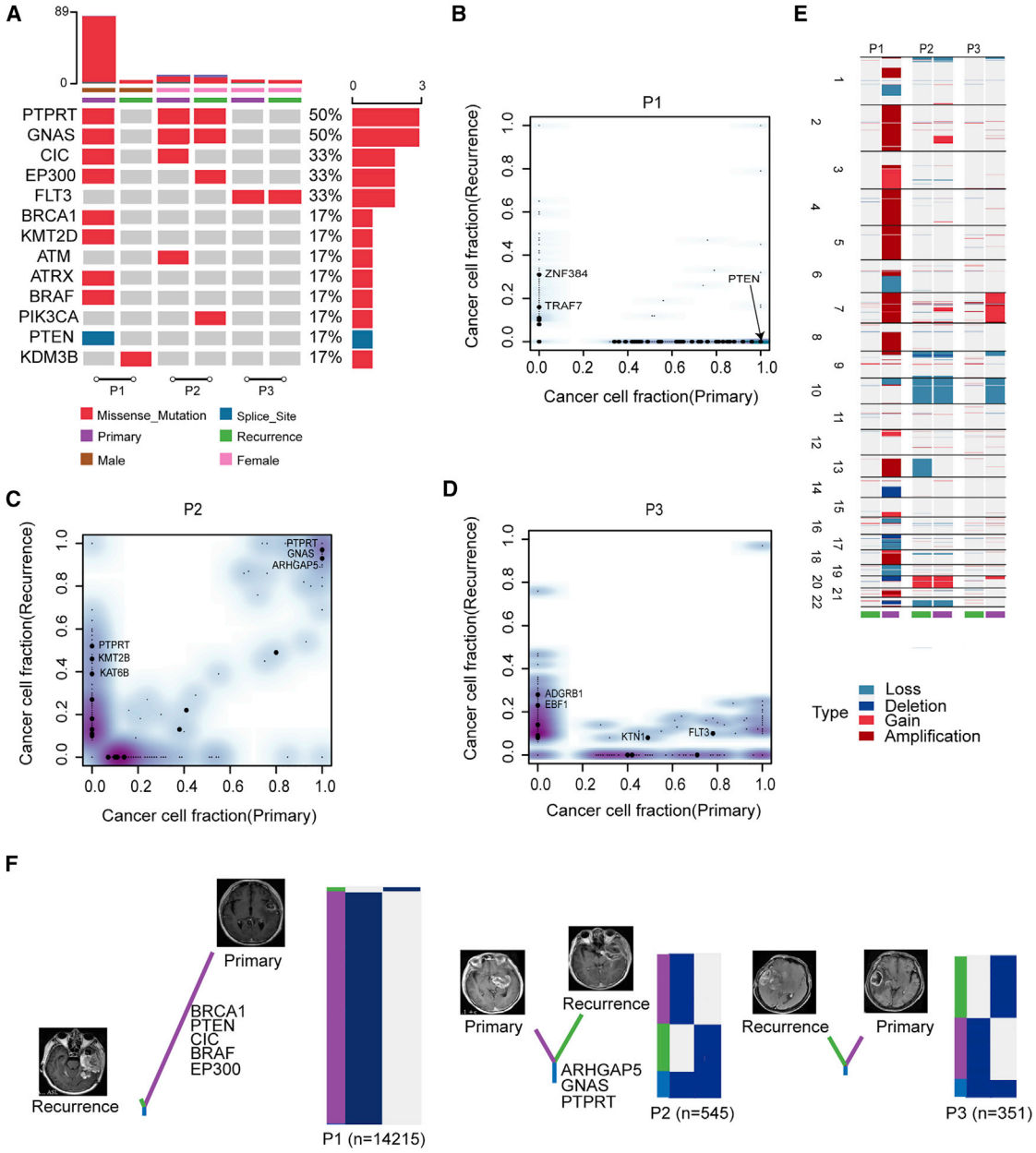

Figure 1. The mutational landscape in paired primary GBMs and recurrence

(A) Oncoplot of mutation. Each column represents a sample, and each row represents a different gene. The top barplot has the counts of mutations for each patient, while the right bar plot has the counts of mutations in each gene. (B–D) Cellular prevalence of individual SNVs was plotted for each paired sample. (E) Chromosomal gains and losses in primary and relapsed tumors. (F) Phylogenetic tree analysis for three paired samples.

suppressed the tumor growth compared with the treatment with PBS (96.4%), PTEN-mRNA (89.7%), and TRAIL-mRNA (84.5%) at day 60 (Figure 3C). Figure 3B showed representative mouse images at day 0, 14, 28, and 60. Compared with other treatment, the survival time of mice in the combinational treatment group was significantly prolonged ($p < 0.05$, Figure 3D). The mouse weights in the control and the single gene group were obviously descended compared with the combinational treatment group (Figure 3E). The representative magnetic resonance images (MRIs) (Figure 3F) and isolated tumors (Figure 3G) revealed the largest inhibition of the combined treatment group. Ki67 staining showed decreased cell density of mRNA-treated tumors compared with control (Figure 3H). Thus, PTEN-mRNA and TRAIL-mRNA could inhibit GBM cell growth *in vivo* and TRAIL-mRNA synergistically enhances the PTEN-mRNA effect.

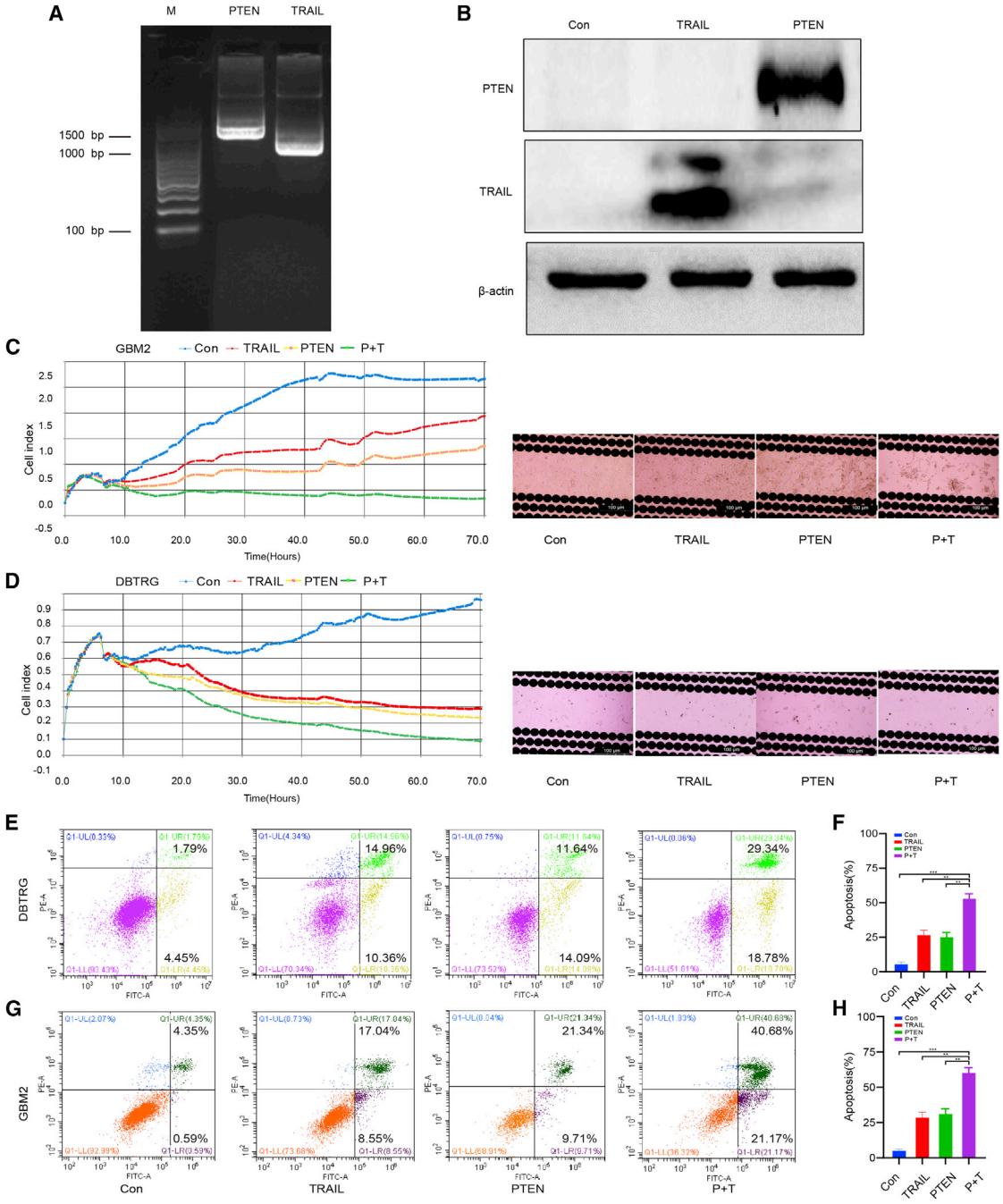

Figure 2. Analysis of cell death induced by mRNA treatment

(A) Determination of synthesized PTEN-mRNA and TRAIL-mRNA by agarose gel electrophoresis. (B) Immunoblotting analysis of PTEN and TRAIL expression in 293T cells. Cells were harvested at 24 h after PTEN-mRNA and TRAIL-mRNA transfection. (C) GBM2 cells viability detected by RTCA at an interval of 5 min until the end of 72 h. After the experiment, the cells that stayed in the E-plate culture plate were observed under the microscope; scale bar, 100 μm. (D) DBTRG-Luc cell viability detected by RTCA in GBM2 cells; scale bar, 100 μm. (E and F) Flow cytometry analysis (E) and quantitative analysis (F) of synthetic mRNA-induced apoptosis in DBTRG-Luc cells using annexin V-FITC/PI. (G and H) Flow cytometry analysis (G) and quantitative analysis (H) of synthetic mRNA induced apoptosis in GBM2 cells by annexin V-FITC/PI. GBM2 and DBTRG cells were transfected with 0.1 μg PTEN-mRNA and/or TRAIL-mRNA. Data are presented as mean ± SD; n = 3 independent experiments; $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 1. The synergistic effect of TRAIL-mRNA and PTEN-mRNA in DBTRG cells

Treatment	Cell viability (% of control)	Cell viability/100
TRAIL	29	0.29
PTEN	46	0.46
TRAIL + PTEN combined—expected	0.29*0.46	0.13
TRAIL + PTEN combined—observed	6.2	0.06
Observed/expected		0.46

0.46 < 0.8 synergistic effect

The nature of the interaction between TRAIL-mRNA and PTEN-mRNA was analyzed using the additive model. According to the model, a ratio between the observed and the expected viability of the cells was calculated for the combined treatment, and a ratio less than 0.8 was considered to be synergistic.

Analysis of possible synergistic mechanism

The immunoblotting analysis showed that the expressions of PTEN and TRAIL were much upregulated in the combined gene group compared with the single gene group, both in GBM2 and DBTRG-Luc cells (Figures 4A and 4B). As shown in Figures S5, 4C, and 4D, molecular function mainly enriched in protein kinase activity, mitogen-activated protein kinase (MAPK) kinase activity, and phosphatase binding. The KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway revealed that the MAPK signaling pathway was the most enriched pathway, followed by the tumor necrosis factor (TNF) signaling pathway. According to RNA sequence results, phosphorylated protein array was performed to further analyze the possible regulating molecules in the MAPK signaling pathway. Phosphorylated P38, P53, JNK, ERK1/2, and several other genes were differentially expressed and might play important roles (Figure 4E). The KEGG pathway also indicated that the MAPK signaling pathway was the most enriched pathway (Figure 4F). By review, ERK1/2 (extracellular signal-regulated kinases 1/2), P38-MAPKs, JNK-1/2/3 (c-Jun NH2-terminal kinases), and ERK5 (extracellular signal-regulated kinase 5) are the four main transduction pathways of MAPK signaling cascades.[31] In view of JNK regulating many death factors, we thought that JNK might be the key gene adjusting synergistic effect.

JNK mediates the synergistic effect of PTEN-mRNA and TRAIL-mRNA treatments

To confirm JNK's critical role in upregulating combined antitumor effect, the expression of related proteins was assessed using immunoblotting analysis. Compared with the single-mRNA-transfected group and untreated group, the expression of apoptosis-related genes Bax, cleaved Caspase 3, and phosphorylated (p)-JNK increased in the combined group, whereas oncogene Bcl-2 declined dramatically (Figures 5A–5D). In the concentration testing experiment, 20 μmol/L of JNK inhibitor SP600125 could significantly inhibit p-JNK expression (Figure S6), and this concentration of SP600125 was applied in the combinational treatment study. As shown in Figures 5E, 5F, and 5I, the expressions of p-JNK, PTEN, TRAIL, p-P53, Bax, and Bcl-2 were apparently offset by SP600125, and the same for the tumor cell killing effect. In GBM2, the expression of apoptosis-related proteins was mainly regulated by p-JNK (Figures 5G, 5H, and 5J). Meanwhile, TRAIL death receptors DR4 and DR5 were detected by flow cytometry. The PTEN-mRNA-induced upregulation of DR5 and these two mRNA-induced upregulations of DR4 at 12 h were observed in both GBM2 and DBTRG cells (Figure S7). According to these results, we speculated that JNK plays a key role in the synergy between PTEN and TRAIL in terms of glioma tumor cell cytotoxicity (Figure 5K).

To verify JNK-mediated cytotoxic effect of PTEN-mRNA and TRAIL-mRNA *in vitro*, the cell viability of GBM2 and DBTRG-Luc cells was detected with RTCA and flow cytometry after the cells were treated with JNK inhibitor (SP600125, 20 μmol/L). With the pretreatment of JNK inhibitor, the enhanced tumor killing effect of the combined treatment was evidently reversed (Figures 6A–6D). The RTCA results clearly exhibited SP600125 could enhance the cell index and reduce the cytotoxic effect of combined treatment with PTEN-mRNA and TRAIL-mRNA (Figures 6E and 6F). The immunohistochemical staining analysis of tumor tissue formalin-fixed, paraffin-embedded (FFPE) sections showed much higher expression of p-JNK and lower expression of p-AKT in the combinational treatment mice than those in the single gene treatment mice (Figure 3H), which also supports the view that JNK plays an important role in the synergistic effect.

DISCUSSION

We have shown that the PTEN's loss and/or mutation play an essential role in GBM patients and any PTEN-mRNA-based therapy rescues PTEN function in PTEN-null GBM, effectively suppress tumor progression. Especially, the combinational application of PTEN-mRNA and TRAIL-mRNA showed synergistic inhibition of tumor growth and synergistic effect is mediated by the JNK pathway.

Along with the deepening research of glioma genomics, we realize that different glioma patients, even the same type, have different mutant genes and driving genes, and the same mutant gene also has different functions. A transcriptomics study of isocitrate dehydrogenase (IDH) wild type in new-onset and recurring GBMs confirmed that only 55% of new-onset GBMs maintained the same expression pattern at the time of recurrence,[32,33] which is consistent with the paired gene analysis results in the present study (Figure 1). Owing to mRNA's unique biomedical features, synthetic mRNA-based gene therapy is promising for developing into the most effective treatment for GBM. This study was intended to use synthetic TRAIL-mRNA and PTEN-mRNA as experimental samples to reveal the feasibility of this mRNA-based therapeutic strategy through their alternate or combined application on GBM cells and xenografted animal models. Given that TRAIL and PTEN could induce tumor cell apoptosis through extrinsic and intrinsic pathways, respectively,[7,14] theoretically, alternate or combined application of two or more anticancer drugs, which work through completely different mechanisms, can reduce the possibility of tumor cells' tolerance and thus enhance

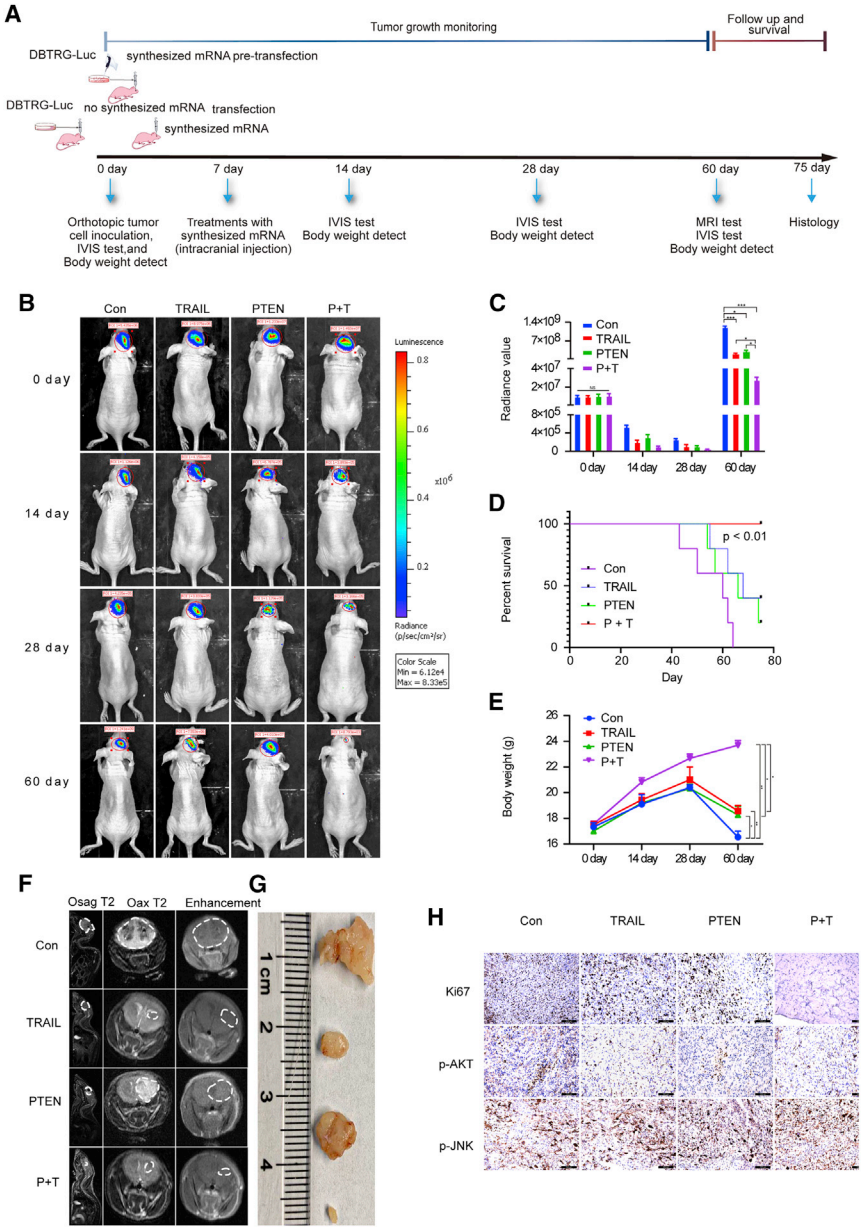

Figure 3. *In vivo* antitumor efficacy of PTEN-mRNA combined with TRAIL-mRNA

(A) Trial design for testing PTEN-mRNA and TRAIL-mRNA combination efficacy in the xenografted orthotopic DBTRG-Luc model. The tumor cells were pretreated with PBS or appropriate synthetic mRNA for 6 h before inoculating. (B and C) Representative IVIS images and corresponding quantitative analysis for each treatment group (n = 5) at day 0, 14, 28, and 60 post tumor inoculation were presented in (B) and (C), respectively. P + T represented TRAIL-mRNA and PTEN-mRNA combination. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (D) Kaplan-Meier survival graph. Log rank test; $p < 0.05$ for the combined mRNA treatment compared with all other treatment groups. (E) Body weight during the treatment. Data are shown as mean ± SD (n = 5), *$p < 0.05$. (F and G) Representative brain MRI scans and isolated tumor samples from each treatment group on day 60. (H) Ki67, p-AKT, and p-JNK immunohistochemistry analysis (scale bar, 200 μm) of tumor tissues after various treatments.

their antitumor ability. This was investigated using human glioma stem cells (GBM2) and the DBTRG-cell-derived xenografted glioma model.

When one synthetic mRNA was used alone, a similar apoptotic effect of PTEN-mRNA and TRAIL-mRNA was observed in both GBM2 and DBTRG cells. However, the synthetic mRNA-induced cytotoxicity was significantly strengthened by the combined use of these two mRNAs (Figure 2). The simultaneous application of the two seems to have a synergistic effect. The mechanism of synergy between PTEN-mRNA and TRAIL-mRNA may involve several aspects. First, as shown in Figure 4, the combined use of PTEN-mRNA and TRAIL-mRNA could enhance each other's protein expression through an unknown mechanism. Second, some key factors in the apoptosis pathway were upregulated by the simultaneous use of TRAIL-mRNA and PTEN-mRNA. As shown in Figure 5, single use of PTEN-mRNA or TRAIL-mRNA resulted in a moderate elevation of p-JNK, Bax, Caspase 3, and cleaved Caspase 3 and a moderate reduction of Bcl-2 in both GBM2 and DBTRG cells, whereas remarkable changes were caused by their combinational use. These results indicate the involvement of the JNK pathway in this synthetic mRNA-induced apoptosis. This view was supported by the application of SP600125, a JNK inhibitor. Finally, the synergy of these two mRNAs may also be manifested through their mutual sensitization. A number of reported studies have demonstrated that PTEN sensitizes cancer cells to TRAIL through upregulating the expression of death receptors.[34–36] In the present study, the PTEN-mRNA-induced upregulation of death receptor, especially DR5, was also observed in both GBM2 and DBTRG cells (Figure S6). It is also believed that the over-activated PI3K/Akt/mTOR pathway increases TRAIL resistance; nevertheless, PTEN is a negative regulator of the PI3K-Akt pathway, which indirectly conquers tumor cell's resistance to TRAIL.[37,38]

In summary, PTEN gene mutations played an important role in the three pairs of GBM samples, which was confirmed by WES. The GBM2 used in this study were derived from a primary GBM sample with harbored loss of the entire of chromosome 10, including the PTEN locus losses. Based on the premise that the *in vitro* synthesized

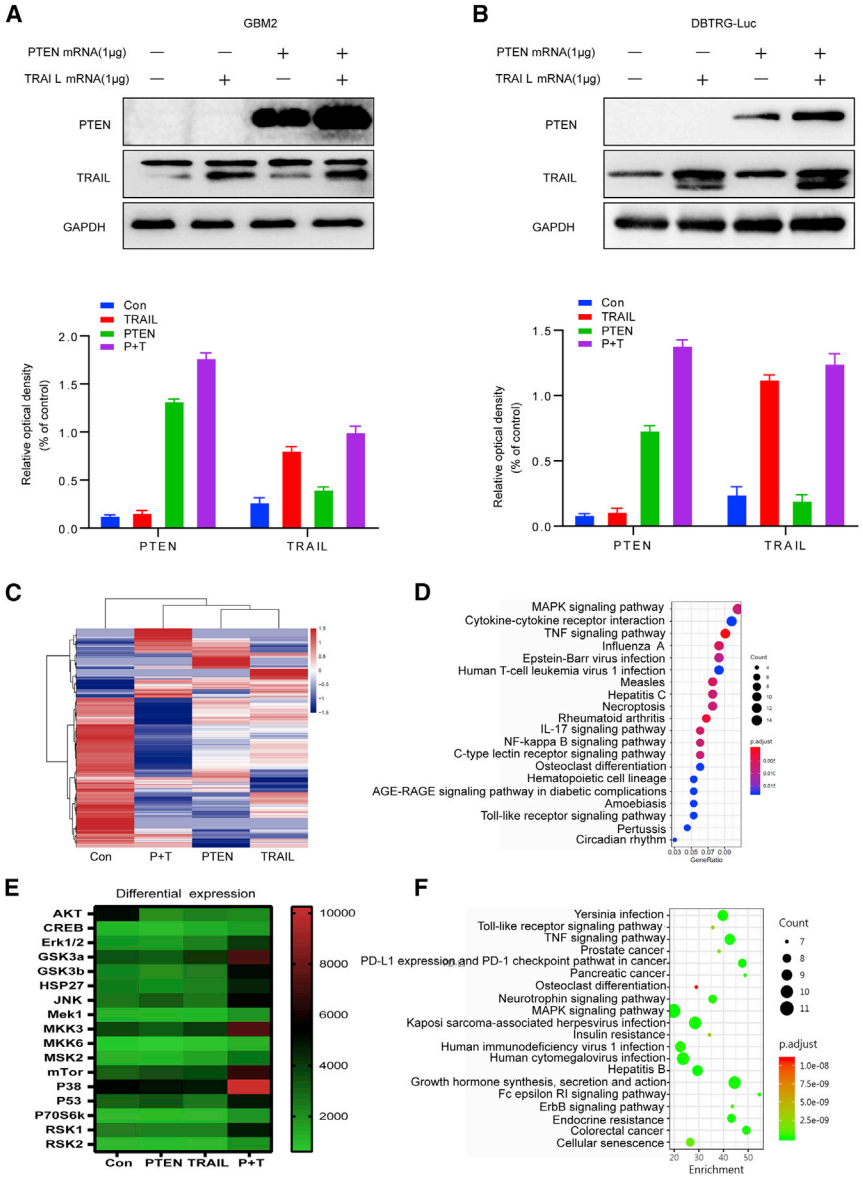

Figure 4. Mechanistic study of synergistic effect of combined treatment with PTEN-mRNA and TRAIL-mRNA

(A and B) Immunoblot analysis of PTEN and TRAIL expression in GBM2 (A) and DBTRG-LUC cells (B) at 24 h after PTEN-mRNA and/or TRAIL-mRNA transfection. (C and D) RNA sequence analysis of the main apoptosis pathway in the combined mRNA group compared with single mRNA groups. (E and F) Phosphorylated protein array analysis of the differential expression proteins (E) and enrichment pathway (F).

mRNAs can be effectively expressed, the therapeutic effect of mRNA-based therapy on GBM was explored through the use of PTEN-mRNA and TRAIL-mRNA alone and their combination in patient-derived GBM tumor cells and a xenografted GBM animal model. The separate application of PTEN-mRNA or TRAIL-mRNA could lead to a certain degree of cytotoxicity and tumor growth inhibition, but their combinational use showed most inhibition of tumor growth. Meanwhile, a synergistic effect of PTEN-mRNA and TRAIL-mRNA was observed under present experimental conditions. The JNK pathway might be the major mechanism involved in this synthetic mRNA-induced apoptosis. In view of the ease of handling and replacement of synthetic mRNA, an mRNA-based therapeutic strategy is bound to develop into an effective patient-tailored treatment for GBM. The main drawback of this potent therapeutic modality is the need for more precise and quicker genomic tools, mainly applicable to GBM patients with insufficient tumor suppressor genes.

MATERIALS AND METHODS

Ethics statement

All experimental procedures and animal protocols were approved by the review comments of medical research ethics committee of Taihe Hospital (no. 201816) and we tried our best to minimize the suffering of the study animals.

We confirm the participants provided written informed consent to take part in the study.

Whole-exome sequencing

Total genomic DNA from GBM tissues and peripheral blood mononuclear cells (PBMCs) were extracted using the QIAamp DNA Mini Kit (Qiagen, Dusseldorf,German), according to the manufacturer's instructions. DNA samples were subjected to DNA library preparation, and whole-exome capture was performed using SureSelect Human All Exon V6 kits (Agilent, Santa Clara,USA). Then, the captured DNA library was sequenced on the HiSeq 4000 sequencing platform (Illumina), according to the manufacturer's instructions for paired-end 150 base-pair (bp) reads (Novogene, Beijing, China). The sequencing depth was 100×. Paired-end clean reads in FASTQ format generated by the Illumina pipeline were aligned to the reference human genome (UCSC hg19) by a Burrows-Wheeler Aligner (v.0.7.17) to get the original mapping results stored in Binary Alignment/Map (BAM) format. Samtools v1.9 and Genome Analysis Toolkit (GATK) v4.1.4.0 were used to sort BAM files and do sorting, duplicate marking, and base quality recalibration to generate final BAM files for downstream analysis (no. PRJNA689871). The somatic single-nucleotide variations (sSNVs) were detected by MuTect (v.1.1.4) with paired tumor and normal sequencing data. sSNVs that failed to pass the internal filters of MuTect had fewer than 10 total reads or 3 variant reads in the tumor sample and fewer than 10 reads in the normal sample.

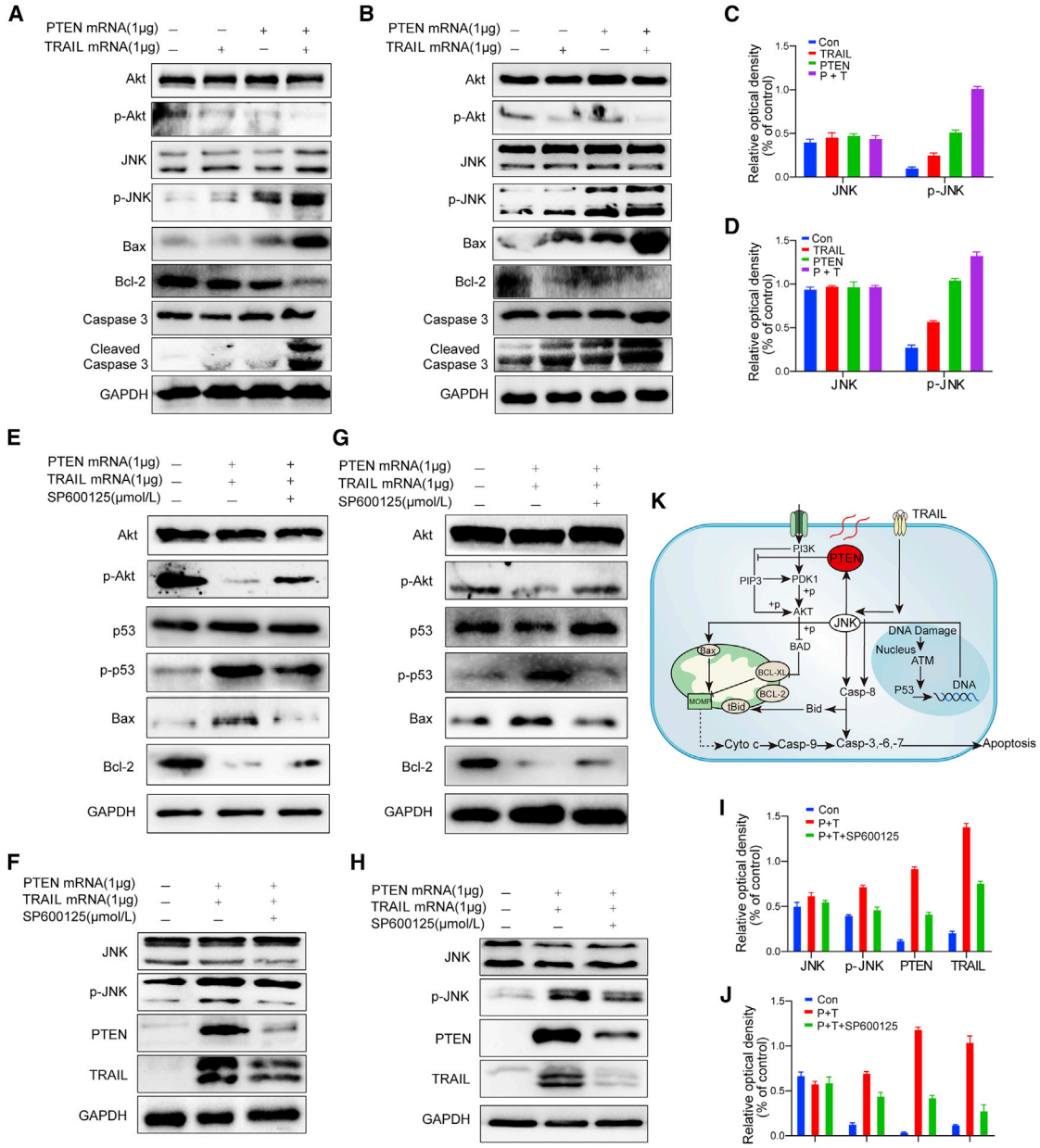

Figure 5. JNK signaling pathway-regulated synergistic killing effect in tumor cells

(A and B) Immunoblot for expression of apoptosis-related proteins in GBM2 (A) and DBTRG-LUC cells (B). Cells were treated with 0.1 μg PTEN-mRNA, TRAIL-mRNA, and PTEN + TRAIL-mRNA for 24 h. (C and D) Quantitative analysis of p-JNK expression in each group. (E–J) The expression levels of related proteins detected by western blot in DBTRG-LUC cells (E, F, and I) and GBM2 (G, H, and J) treated with or without JNK inhibitor (SP600125) at 20 μmol/mL in the combined mRNA treatment. (K) Schematic diagram of a possible synergistic regulation in combined treatment.

Additional VarScan (v.2.4.2) filters were applied to remove sSNVs with low average variant base qualities, low average mapping qualities among variant-supporting reads, strand bias among variant-supporting reads, and high average mismatch base quality sums among variant-supporting reads. Small indels were called with Strelka (v.2.9.2). ANNOVAR was performed to do annotation for VCF (Variant Call Format). Facets (v.0.16.0) was utilized to detect somatic copy number variants (CNVs). Mutational CCFs were estimated, as described in the previous study.[39] A phylogenetic tree was constructed through PHYLIP (v.3.698).

mRNA synthesis

PTEN-mRNA and TRAIL-mRNA were synthesized *in vitro*, as previously described.[40] Briefly, the human 5′UTR with Kozak sequence and 3′UTR sequence were commercially synthesized by Integrated

DNA Technologies (Coralville, Iowa,USA) and sub-cloned into pcDNA3.3. The MEGAscript T7 Kit (**Thermo Fisher**Waltham) was used to synthesize mRNAs, whereas m7GpppG was replaced with Anti-Reverse Cap Analog (ARCA) cap analog (New England Biolabs) and cytidine and uridine were replaced with 5-methylcytidine triphosphate and pseudouridine triphosphate (TriLink BioTechnologies), respectively. Reactions were sustained for 5 h at 37°C followed by DNase treatment. Then, the reactions were treated with Antarctic Phosphatase (New England Biolabs) for 2 h at 37°C to remove residual 5′-triphosphates. The synthesized mRNAs were purified with MEGAclear spin columns (Ambion) and quantitated with NanoDrop (Thermo Scientific).

Cell culture

The human glioma cell lines, DBTRG-Luc, were obtained from our laboratory. The cell lines were maintained in DMEM supplemented with 50 U/mL penicillin, 50 mg/mL streptomycin, and 10% fetal bovine serum (FBS) in a humidified incubator at 37°C with 5% CO_2. Glioma stem cells (GBM2) were isolated from human GBM tumor tissues, which were collected from a GBM patient at Taihe Hospital, with informed consent. The tumor tissues were processed immediately after surgery, and tumor cells were washed three times with DMEM/F12 and cultured in DMEM with 2% B-27 Supplement (Gibco), 20 ng/mL epidermal growth factor, and 10 ng/mL basic fibroblast growth factor (Invitrogen).

Identification of primary glioma cells

To identify the GBM2 isolated from glioma patients, the sample cells were incubated with anti-CD133 (1:200, Beyotime,Shanghai,China) and anti-glial fibrillary acidic protein (GFAP) (1:100, Beyotime,Shanghai,China) antibodies, or anti-CD31 antibody (rabbit immunoglobulin [IgG], 1:1000; Abcam) and anti-Ki67 (1:500, Abcam) antibodies. The cultured cells were fixed with 4% paraformaldehyde (Beyotime, Shanghai, China) for 10 min and washed three times with PBS. The cells were permeabilized with 0.1% Triton-X 100 (Beyotime, Shanghai, China) for 20 min, and blocked with 2% BSA at room temperature (RT) for 60 min. The cells were then incubated with the primary antibodies for 60 min at RT. After rinsing with PBS three times, cells were incubated with the following secondary antibodies for 60 min: Alexa 488-conjugated goat antirabbit IgG and Alexa 594-conjugated goat antimouse IgG (1:2000, Abcam,Cambridge,UK) at RT. Cell nuclei were counter-stained with DAPI (Beyotime, Shanghai, China). Cells were examined under a Confocal Microscope (Olympus,Tokyo, Japan).

Cell viability assay

Cell viability was detected by real-time assessment using the xCELLigence real-time cell analyzer (RTCA) (Roche, Sweden), as previously described.[40] Background measurements were taken from the wells by adding 100 μL of the same medium to the E-Plate 16. A volume of 100 μL of cell suspension (3×10^3 cells) was added to the wells to make a final volume of 200 μL. All cells were allowed to settle at the bottom of the wells at RT for 15 min and incubated at 37°C and 5% CO_2. The impedance signals were recorded every 15 min for 6 h. After 6 h of base line measurement, 0.1 μg synthetic TRAIL-mRNA mixture and/or PTEN-mRNA mixture was added into each well. The impedance signals were recorded using the same time intervals until the end of the experiment (up to 72 h). Cell index (CI) value represents cell status, which is defined as relative change in measured impedance to background impedance.

Flow cytometry analysis

Flow cytometry was used to determine the apoptosis of glioma cells. Briefly, 1×10^6 DBTRG-Luc and GBM2 cells were seeded in six-well plates and left 24 h in an incubator to resume their exponential growth. Synthetic TRAIL-mRNA, PTEN-mRNA, and PTEN-TRAIL-mRNA were transfected into DBTRG-Luc and GBM2 cells and incubated for 24 h. The cells were then harvested and washed with PBS, and the extent of apoptosis was measured using the Annexin V-FITC Apoptosis Detection Kit (Byotiome, Shanghai,China) and analyzed by flow cytometry software (Beckman, Brea,USA). The upper right part represents apoptotic cells undergoing secondary necrosis at the last stage or dead cells (annexin-V and propidium iodide [PI] double-positive), and the lower right part represents the early-stage apoptotic cell population (annexin-V-positive and PI-negative). Death receptor DR4 and DR5 were also detected by flow cytometry at 12 h and 24 h after TRAIL-mRNA and/or PTEN-mRNA transfected into DBTRG-Luc and GBM2 cells.

The assessment of JNK effect on PTEN-mRNA and TRAIL-mRNA induced cytotoxicity

To explore the role of JNK in the PTEN and TRAIL pathways, before PTEN-mRNA and TRAIL-mRNA transfection into glioma cells, cells were pretreated with JNK inhibitor SP600125 (Sigma,St.Louis, USA) for 2 h, followed by RTCA and flow cytometry, detecting the apoptosis induced by PTEN and TRAIL. The changes of JNK-related protein expression were assessed by immunoblotting analysis.

RNA sequence and protein array

GBM2 and DBTRG-Luc cells were transfected with PTEN-mRNA and TRAIL-mRNA for 24 h and washed 3 times with PBS. For the purpose of RNA sequencing, the cells were harvested using Trizol reagent (Sigma,St.Louis, USA) and stored at −80°C until sending the samples to Biotecan (Shanghai, China). For the purpose of protein array, the mRNA-transfected cells were harvested using a cell scraper and stored at −80°C until sending to RayBiotech (Guangzhou, China).

Immunoblotting analysis

PTEN-mRNA and TRAIL-mRNA were transfected into DBTRG-Luc and GBM2 cells for 24 h. Immunoblotting analysis was used to detect the cellular expressions of PTEN and TRAIL as well as related proteins, including AKT, p-AKT, JNK, p-JNK, caspase-3, Bcl-2, and Bax. Briefly, the mRNA-transfected GBM2 and DBTRG-Luc cells were washed three times with PBS and collected with the cell lysis RIPA buffer (CoWin Biosciences, China). The cell lysates were incubated on ice for 30 min. Protein concentration was determined using BCA Protein Assay reagents (Beyotime, Shanghai,China), according to the manufacturer's protocol.

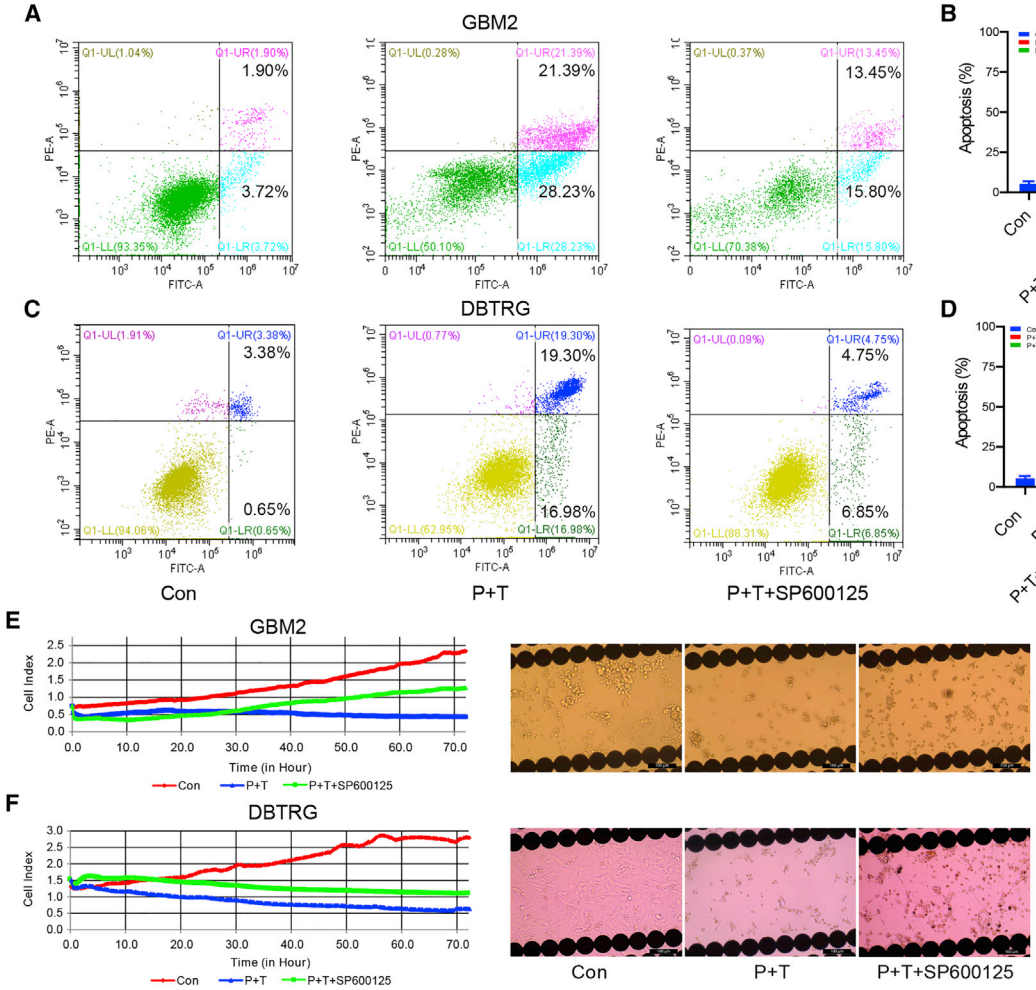

Figure 6. JNK inhibitor reverses the killing effect of combined treatment

(A and B) Flow cytometry (A) and quantitative analysis (B) were performed for apoptosis determination in GBM2 treated with 0.1 μg PTEN-mRNA and 0.1 μg TRAIL-mRNA for 24 h followed by treatment with or without 20 μM JNK inhibitor (SP600125) for 2 h. (C and D) Flow cytometry analysis (C) and quantitative analysis (D) were performed for apoptosis determination in DBTRG-Luc cells. The treatment was the same with GBM2. Data are presented as mean ± SD; n = 3. *$p < 0.05$. (E and F) RTCA was used to detect the cell viability in GBM2 (E) and DBTRG-Luc (F) cells treated with 0.1 μg PTEN-mRNA and 0.1 μg TRAIL-mRNA for 72 h followed by treatment with or without 20 μM JNK inhibitor (SP600125) for 2 h. After the experiment, the cells stayed in the E-plate culture plate were observed under a microscope; scale bar, 200 μm.

Equal amounts of protein (50 μg/each sample) were loaded onto each lane and separated by electrophoresis in 10% or 12% SDS polyacrylamide gel and electrotransferred to nitrocellulose membranes. The membrane was put in blocking buffer for 1 h at RT followed by overnight incubation at 4°C with appropriate primary PTEN antibody (1:1000, CST, Danvers,USA), TRAIL antibody (1:1000, CST, Danvers,USA), AKT antibody (1:1000, CST, Danvers,USA), p-AKT antibody (1:1000, CST,Danvers, USA), JNK antibody (1:1000, CST, Danvers,USA), p-JNK antibody (1:1000, CST, Danvers,USA), caspase-3 antibody (1:1000, CST, Danvers,USA), Bax antibody (1:1000, Proteintech, China), Bcl-2 antibody (1:1000, Proteintech, China), and GAPDH antibody (1:4000, Proteintech, China). The blots were rinsed with TBST three times and incubated with horseradish peroxide (HRP)-conjugated secondary antibody (1:2000) for 60 min and detected by chemiluminescence using enhanced chemiluminescence (ECL) Hyperfilm (Bio-rad,-Hercules, USA).

Immunohistochemical staining

Briefly, 5 μm serial sections of tumor tissues were dewaxed in xylene and rehydrated through graded alcohols. Endogenous peroxidases were blocked with 3% H_2O_2 for 30 min, and antigens were retrieved by microwaving slides. After cooling and washing, slides were blocked with goat serum for 30 min (1:10, Zymed Antibody Diluent). The sections were incubated with primary antibodies anti-Ki67 (1:50, Abcam,Cambridge,UK) and anti-p-AKT (1:200, CST, Danvers,USA), p-JNK (1:500, CST, Danvers,USA) at 4°C overnight and incubated with HRP-conjugated secondary antibodies followed by the Liquid DAB Substrate Chromogen System, according to the manufacturer's

instruction. The sections were examined under a fluorescence microscope (Olympus, Tokyo, Japan).

In vivo models

Before establishing the orthotopic glioma model, PTEN-mRNA or/and TRAIL-mRNA were pretransfected into DBTRG-Luc cells for 6 h and detected the viability of cells with an IVIS. A total of 3×10^5 DBTRG-Luc cells was implanted into the right caudate nucleus of each nude mice. This xenografted tumor model was used to test the therapeutic effect of synthetic PTEN-mRNA and TRAIL-mRNA through intracranial injection. Seven days after *in situ* implantation of DBTRG-Luc cells, 20 μL of a cocktail solution (containing 1 μg PTEN-mRNA and/or TRAIL-mRNA) were intracranially injected to each mouse. The bioluminescence and body weight were determined at day 0, 14, 28, and 60 using an IVIS spectrum system. And last, overall survival of mice was analyzed, and the tumors were resected at day 75.

Intracranial injection of synthetic mRNA in mice

The nude mice were anesthetized by intraperitoneal injection of 1% sodium pentobarbital. A sagittal incision (1.0–1.5 cm) was made on the scalp, and the calvarium was exposed by blunt dissection. A tiny parietal hole was created on the sagittal suture of skull. The microinjector was positioned at the right caudate nucleus (1 mm forward and 2 mm right side of the anterior fontanelle) and vertically punctured 3 mm. The PTEN-mRNA and/or TRAIL-mRNA solution (20 μL) were injected at a rate of 2 μL/min through the microinjector. The *Trans*IT-mRNA kit-mediated mRNA solution was composed of 1 μL of synthetic mRNA (1 μg/μL PTEN-mRNA or/and 1 μg/μL TRAIL-mRNA), 15 μL of Opti-MEM, 2 μL of Boost reagent and 2 μL of *Trans*IT-mRNA. After injection, the microinjector was kept in place for 10 min.

MRI test

To further explore the size of tumors in mice, mice were examined by MRI at day 60 of tumor implantation. Briefly, experiments were performed at a clinical 3.0T MRI scanner (GE Healthcare, Chicago,USA) using a 19F volume-resonator human head coil (GE healthcare, Chicago,USA), tunable to 1H and 19F nuclei channels. Morphological 1H MRIs of the brain were acquired with a coronal spinal echo T1- and T2-weighted imaging (T1WI/T2WI) sequence. After acquisition, the resonator was tuned to the 19F channel, and 19F images were acquired.

Statistical analysis

In general, unpaired two-tailed Student's t test and one-way ANOVA were used to make inter-group comparisons. The Kaplan-Meier method was used to estimate overall survival. All statistical analyses were performed with SPSS (v.16.0) and GraphPad (v.5.0). Data were presented as the mean ± SD. Statistical analyses were performed with Student's t test unless noted otherwise. $p < 0.05$ was considered statistically significant.

DATA AVAILABILITY

The data associated with this study can be found at the website: http://www.cbioportal.org/. Our WES data have been uploaded to https://www.ncbi.nlm.nih.gov/(No.PRJNA689871).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2022.01.013.

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AUTHOR CONTRIBUTIONS

Q.C., J.L., and L.D. contributed to the conception. X.T., H.P., P.X., R.F., J.L., and H.T performed all experiments and constructed the manuscript. P.X. and H.P. analyzed all the data; X.G., Z.D., and L.Z. supported the experimental techniques. X.T., P.X., and H.P. drafting the manuscript; L.D. and Q.C. helped critically reviewed the manuscript and provided intellectual input; and H.C. and K.H. participated in MRI testing and FFPE staining. Q.C., J.L., and L.D. supervised all studies. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors have declared that no competing interest exists.

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