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TGF-β1 modulates temozolomide resistance in glioblastoma via altered microRNA processing and elevated MGMT

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Abstract

Background. Our previous studies have indicated that miR-198 reduces cellular methylguanine DNA methyltransferase (MGMT) levels to enhance temozolomide sensitivity. Transforming growth factor beta 1 (TGF-β1) switches off miR-198 expression by repressing K-homology splicing regulatory protein (KSRP) expression in epidermal keratinocytes. However, the underlying role of TGF-β1 in temozolomide resistance has remained unknown.

Methods. The distribution of KSRP was detected by western blotting and immunofluorescence. Microarray analysis was used to compare the levels of long noncoding RNAs (lncRNAs) between TGF-β1–treated and untreated cells. RNA immunoprecipitation was performed to verify the relationship between RNAs and KSRP. Flow cytometry and orthotopic and subcutaneous xenograft tumor models were used to determine the function of TGF-β1 in temozolomide resistance.

Results. Overexpression of TGF-β1 contributed to temozolomide resistance in MGMT promoter hypomethylated glioblastoma cells in vitro and in vivo. TGF-β1 treatment reduced cellular MGMT levels through suppressing the expression of miR-198. However, TGF-β1 upregulation did not affect KSRP expression in glioma cells. We identified and characterized 2 lncRNAs (H19 and HOXD-AS2) that were upregulated by TGF-β1 through Smad signaling. H19 and HOXD-AS2 exhibited competitive binding to KSRP and prevented KSRP from binding to primary miR-198, thus decreasing miR-198 expression. HOXD-AS2 or H19 upregulation strongly promoted temozolomide resistance and MGMT expression. Moreover, KSRP depletion abrogated the effects of TGF-β1 and lncRNAs on miR-198 and MGMT. Finally, we found that patients with low levels of TGF-β1 or lncRNA expression benefited from temozolomide therapy.

Conclusions. Our results reveal an underlying mechanism by which TGF-β1 confers temozolomide resistance. Furthermore, our findings suggest that a novel combination of temozolomide with a TGF-β inhibitor may serve as an effective therapy for glioblastomas.

Key Points

1. TGF-β1 confers temozolomide resistance through the miR-198–MGMT signaling pathway.
2. TGF-β1 regulates miR-198 switching through promoting coupling of KSRP with lncRNAs.

Importance of the Study

Previous studies have indicated that TGF-β1 inhibits follistatin-like 1 (FSTL1)/miR-198 switching through the miR-181a–KSRP signaling pathway in human epidermal keratinocytes. In human epidermal keratinocytes, TGF-β1 binds to type I and type II TGF-β1 receptors and promotes Smad2/3 phosphorylation. Activated Smad2/3 proteins associate with Smad4, translocate to the nucleus, and bind to the promoter of miR-181a to promote transcription. However, in glioblastomas, high levels of H3K27me3 are found in the miR-181a promoter regions. The trimethylation of H3K27 inhibits Smad4 from binding to the promoter region of miR-181a. TGF-β1 treatment does not affect the expression of miR-181a or KSRP in gliomas. However, TGF-β1 stimulation leads to a significant increase in FSTL1 protein and a decrease in miR-198 expression in GBM cells. Moreover, KSRP knockdown abolishes the function of TGF-β1 on FSTL1, MGMT, and miR-198 expression. Our present study reveals a novel pathway by which TGF-β1 regulates FSTL1/miR-198 switching. TGF-β1 enhances the expression levels of H19 and HOXD-AS2 (HOXD Cluster Antisense RNA 2), which competitively bind to KSRP and prevent KSRP from participating in FSTL1/miR-198 switching. This pathway has not been reported previously. Additionally, we found that TGF-β1 confers temozolomide resistance through this pathway, suggesting that TGF-β1 signaling may be a target for the development of therapeutic strategies against glioblastomas.

Temozolomide (TMZ) is widely used to treat human glioblastoma multiforme (GBM). However, 40% of glioma patients exhibit resistance against TMZ.[1] Methylguanine DNA methyltransferase (MGMT) expression is the most direct mechanism of drug resistance.[2] MGMT reverses DNA alkylation through removing methyl groups from TMZ-induced O^6-methyguanine lesions.[3] Patients with low MGMT levels exhibit more efficacious outcomes from TMZ chemotherapy compared with those with higher MGMT levels.[4] Multiple factors are involved in the regulation of MGMT expression, including micro (mi)RNAs, transcription factors, histone acetylation, and promoter methylation. MiR-198, originating from the untranslated region (UTR) of follistatin-like 1 (FSTL1), is downregulated in human glioblastoma tissues.[5,6] Upregulation of miR-198 reduces cellular MGMT levels through binding to the 3′-UTR of MGMT to enhance TMZ sensitivity.[7] As the primary miRNA of miR-198, FSTL1 strengthens TMZ resistance through increasing MGMT levels.[8] Alternatively spliced FSTL1 mRNA generates drug-sensitive miR-198 or drug-resistant FSTL1 protein; however, the mechanisms that regulate this splice shifting are incompletely understood.

K-homology (KH) splicing regulatory protein (KSRP; also known as KHSRP) in both the cytoplasm and nucleus is an important negative regulator of *a*denylate-uridylate (AU)–rich element (ARE)–containing mRNAs.[9] In the cytoplasm, KSRP promotes RNA degradation by specifically binding to ARE regions.[10] In the nucleus, KSRP binds to primary miRs and promotes the maturation of miRs.[11] KSRP shears FSTL1 mRNA to generate miR-198 by binding to its 3′-UTR.[5] Furthermore, TGF-β1 inhibits miR-198 expression by repressing KSRP expression in human epidermal keratinocytes.[5] The increased expression of TGF-β1 observed in malignant gliomas indicates that it is an oncogene.[12,13] TGF-β1 plays pivotal roles in glioma cell invasion, angiogenesis, and proliferation.[14,15] Additionally, GBM patients with high TGF-β1 activity have a poor prognosis.[16] However, the underlying role of TGF-β1 in TMZ resistance has remained unknown. In the present study, we found that, in MGMT-promoter hypomethylated glioblastoma cells, TGF-β1 was associated with TMZ resistance. TGF-β1 upregulation led to an increased expression of long noncoding RNAs (lncRNAs) that exhibited competitive binding to KSRP and prevented KSRP from participating in miR-198 switching, thus decreasing miR-198 levels and increasing the expression of MGMT.

Materials and Methods

Cell Counting Kit 8 (CCK-8) proliferation assays and flow cytometry assays were performed as described in a previous study.[8]

Human Glioma Specimens and Cell Lines

Five nontumorous brain tissue (NBT) specimens and 41 tumor samples were obtained for analysis from patients registered in the Department of Neurosurgery at The First

Affiliated Hospital of Nanjing Medical University. This study was approved by the institutional review board and the research ethics committee of Nanjing Medical University. Informed consent was obtained from each participant. All methods were performed in accordance with the approved guidelines. Details of the tissue samples and cell lines that we used have been described previously[8]; see the Supplementary Material for details.

Plasmid Construction, Transfections, and Establishment of Stable Cells

See the Supplementary Material for details on plasmid construction, transfections, and establishment of stable cells.

Western Blot Analysis

Western blot analysis was performed as described previously.[16] The corresponding images were captured by a Bio-Rad ChemiDoc XRS+ (Bio-Rad). The primary antibodies are listed in Supplementary Table 1.

Quantitative Real-Time PCR

RNA was isolated using TRIzol (Invitrogen). First-strand cDNA was synthesized using the PrimeScript RT Master Mix (TaKaRa). Real-time PCR was performed using SYBR Green Master Mix (Roche Applied Science). The forward and reverse PCR primers are shown in Supplementary Table 2.

RNA Immunoprecipitation Assays

RNA immunoprecipitation (RIP) assays were performed using an Imprint RNA Immunoprecipitation Kit (Sigma-Aldrich) according to the methods described in a previous study.[17] Briefly, cell lysates were incubated with primary antibodies and immobilized protein-G magnetic beads (Cell Signaling Technology), while rotating overnight at 4°C. Beads were washed 4 times with a buffer containing 150 mM NaCl, 0.5% Triton X-100, 50 mM Tris·HCl (pH 8.0), and 1 × CompleteProtease Inhibitors (Roche). Total RNA prepared from immunocomplexes was isolated, reverse transcribed, and amplified by quantitative real-time (qRT) PCR. The primer sequences are shown in Supplementary Table 2.

Long Noncoding RNA Microarray Analysis

GBM cells were treated with vehicle or recombinant TGF-β1 (10 ng/mL) for 48 hours. RIP assays were performed using an Imprint RNA Immunoprecipitation Kit (Sigma-Aldrich) according to the manufacturer's protocol. Cell lysates were incubated with an anti-KSRP antibody and protein-G magnetic beads. Total RNA was extracted using TRIzol reagent. Total RNA was converted to complementary RNA and labeled using the Quick Amp Labeling kit (Agilent). Labeled cRNA was hybridized to microarrays overnight at 65°C using a Human LncRNA Array v2.0 (8 x 60K, ArrayStar). Microarrays were scanned using the Agilent Scanner G2505B and were analyzed by Agilent Feature Extraction software. Differentially expressed lncRNAs were identified as those with fold changes >1.50 or ˂0.67, and with a *P*-value <0.01.

Immunohistochemistry

The protein levels of TGF-β1, FSTL1, MGMT, γ-H2AX, and cleaved caspase-3 in tumor tissues were detected using immunohistochemistry (IHC). The steps were performed as described previously.[8]

RNA Fluorescent In Situ Hybridization

Fluorescent in situ hybridization (FISH) was performed using a FISH Tag RNA Green Kit with Alexa Fluor 488 dye (Invitrogen). Probes targeting lncH19, lncHOXD-AS2, and miR-198 were used according to the manufacturer's protocol.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed using an EZ-magna ChIP kit (Millipore), according to the manufacturer's instructions. See the Supplementary Material for details.

Orthotopic and Subcutaneous Xenograft Studies

Nonobese diabetic severe combined immunodeficient (NOD/SCID) mice were provided by the Model Animal Research Center of Nanjing University. The mice were randomly divided into 10 mice per group. For subcutaneous xenograft studies, D54 cells (1×10^7) (stably expressing H19, HOXD-AS2, TGF-β1) or an empty vector was inoculated subcutaneously into 4- to 6-week-old NOD/SCID mice. Owing to individual differences and surgical risks, some replicates died within 24 h. Next, we randomly selected 6 replicates per group for the following experiments. Mice were oral-gavaged with a TMZ suspension on the twenty-first day after inoculations. TMZ was given at 66 mg/kg/day for 5 days per week for 3 cycles. The lengths and widths of tumors were measured each week. All mice were sacrificed on the forty-second day after inoculations. Studies using NOD/SCID mice were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. Orthotopic human GBM murine xenograft models were established as described previously.[8] See the Supplementary Material for further details.

Statistical Analysis

All experiments were performed 3 times independently. GraphPad Prism 5 software, SPSS v17.0 software, and Microsoft Office Excel 2010 were used to analyze acquired data. Student's *t*-tests and one-way ANOVAs were used to determine inferential statistics. Quantitative data are

presented as the mean ± standard error of the mean (SEM). The statistical significance level was set at $P < 0.05$.

Results

TGF-β1 Confers TMZ Resistance Through MGMT

The increased expression of TGF-β1 observed in malignant gliomas indicates that it is an oncogene.[18,19] TGF-β1 plays pivotal roles in glioma cell invasion and proliferation.[14,15] Additionally, GBM patients with high TGF-β1 activity have a poor prognosis.[16] Blocking TGF-β receptor 1 kinase dramatically elevates gamma (γ)-H2AX levels and reduces glioma radiotherapy resistance and drug resistance.[20] However, the underlying role of TGF-β1 in TMZ resistance has remained unknown. Our previous studies have indicated that FSTL1 confers TMZ resistance through upregulating MGMT,[8] and miR-198 enhances TMZ sensitivity by targeting MGMT.[7] TGF-β1 promotes FSTL1 expression and suppresses miR-198 expression by repressing KSRP in human epidermal keratinocytes.[5] In the present study, we found that the FSTL1 and MGMT levels were significantly upregulated, while miR-198 expression was decreased in primary GBM2 (P-GBM2) and D54 cells (MGMT-promoter hypomethylation with MGMT activity[8]) after TGF-β1 treatment (Fig. 1A, B and Supplementary Figure 1). Of note, overexpression of miR-198 reversed the TGF-β1–induced upregulation of MGMT (Fig. 1C). Flow cytometry assays revealed that the pro-apoptotic effect of TMZ was reversed by TGF-β1 in P-GBM2 and D54 cells, but not in U87 cells (MGMT-promoter methylation with little MGMT activity[8]) (Fig. 1D and Supplementary Figure 2A). Notably, the levels of γ-H2AX and cleaved caspase-3 were downregulated by TGF-β1 after TMZ treatment (Fig. 1E). Next, MGMT-specific RNA interference (short hairpin [sh] MGMT) and O^6-benzylguanine (a specific MGMT inhibitor) were used to block MGMT signaling. Disruption of MGMT signaling observably abrogated TGF-β1–induced TMZ resistance (Supplementary Figure 2B). These observations suggest that TGF-β1 upregulation contributes to TMZ resistance through MGMT.

Next, NOD/SCID mice were intracranially injected with P-GBM2 cells stably expressing TGF-β1 or an empty vector. After confirmation of GBM engraftment, tumor-bearing mice were treated with placebo or TMZ (66 mg/kg/day) by oral gavage 5 times a week for 3 cycles at 7 days after inoculations. Mice bearing P-GBM2 cells transfected with a vector control had a significant decrease in tumor volume after TMZ treatment, whereas tumor growth in TMZ-treated mice implanted with TGF-β1–infected cells was not noticeably affected (Fig. 1F). In the presence of TMZ, the median survival of mice implanted with TGF-β1-infected cells was 39.3 days, whereas that of the vector-treated group was 59.5 days ($n = 8$/group, $P < 0.01$; Fig. 1G and Supplementary Table 3). A subcutaneous xenograft study also showed that upregulation of TGF-β1 resulted in larger tumors than in the control group (Supplementary Figure 3). Consistent with the in vitro data, xenografts overexpressing TGF-β1 had significantly decreased levels of γ-H2AX and cleaved caspase-3 in the presence of TMZ (Fig. 1H).

The Cancer Genome Atlas GBM RNA-sequencing data revealed that TGF-β1 levels were higher in GBMs than in NBTs (Fig. 1I). Next, we analyzed the overall survival in 90 GBM patients following TMZ therapy. Survival curves showed that patients with high-level TGF-β1 expression did not benefit from TMZ therapy (Fig. 1J).

TGF-β1 Mediates MGMT Expression Through KSRP

TGF-β1 stimulation enhances the expression of miR-181a that binds to the 3′-UTR of KSRP and represses its expression.[5] However, we did not detect any changes in KSRP or miR-181a in GBM cells treated with TGF-β1 (Fig. 2A, B). As an RNA-binding protein, KSRP binds to FSTL1 mRNA and promotes mature miR-198.[5] In our present study, the expression levels of FSTL1, miR-198, and MGMT were significantly altered in cells transfected with KSRP-specific small interfering (si)KSRP or KSRP plasmids (Supplementary Figure 4). In addition, KSRP knockdown blocked the functions of TGF-β1 on FSTL1, miR-198, and MGMT expression (Fig. 2C). Furthermore, TGF-β1 stimulation induced FSTL1 mRNA dissociation from KSRP (Supplementary Figure 5). As a component of Drosha and Dicer complexes, KSRP promotes miR maturation by binding with high affinity to the terminal loop of primary miRs.[11] Notably, the binding of KSRP and Drosha/Dicer complexes was weakened by TGF-β1 (Fig. 2D). These data imply that TGF-β1 mediates MGMT expression through KSRP, but not by affecting KSRP expression. Next, we extracted and separated total, cytoplasmic, and nuclear fractions of KSRP and found that total KSRP levels were not affected by TGF-β1 in GBM cells. However, cytoplasmic KSRP was increased significantly, while nuclear KSRP was reduced in cells treated with TGF-β1 (Fig. 2E, Supplementary Figure 6). Collectively, these results suggest that TGF-β1 induces MGMT expression by influencing KSRP nucleocytoplasmic trafficking.

TGF-β1 Regulates Subcellular Localization of KSRP Through Promoting Coupling Between KSRP and lncRNAs

The distribution of KSRP is regulated and restricted by many factors. KSRP interacts with lncRNAs in the cytoplasm that favor KSRP binding to mRNA targets and promote mRNA degradation.[21] To systematically explore the roles of lncRNAs in the distribution of KSRP, we performed RIP to collect KSRP-interacting lncRNAs from cells treated with TGF-β1 or vehicle. Microarray analysis was used to compare the levels of lncRNAs between TGF-β1 treated and untreated cells. Heatmaps were generated to show the upregulated and downregulated lncRNAs (Fig. 3A, B). We found 3 upregulated and 5 downregulated lncRNAs ($P < 0.01$; Fig. 3C). Next, we specifically focused on upregulated lncRNAs-H19 and HOXD-AS2, which are each known to competitively bind to KSRP. TGF-β1 treatment enhanced the expression levels of H19 and HOXD-AS2 (Fig. 3D), while Smad2/3 knockdown blocked TGF-β1–induced lncRNA expression (Fig. 3E). Furthermore, qRT-PCR analysis demonstrated binding of KSRP with FSTL1, H19,

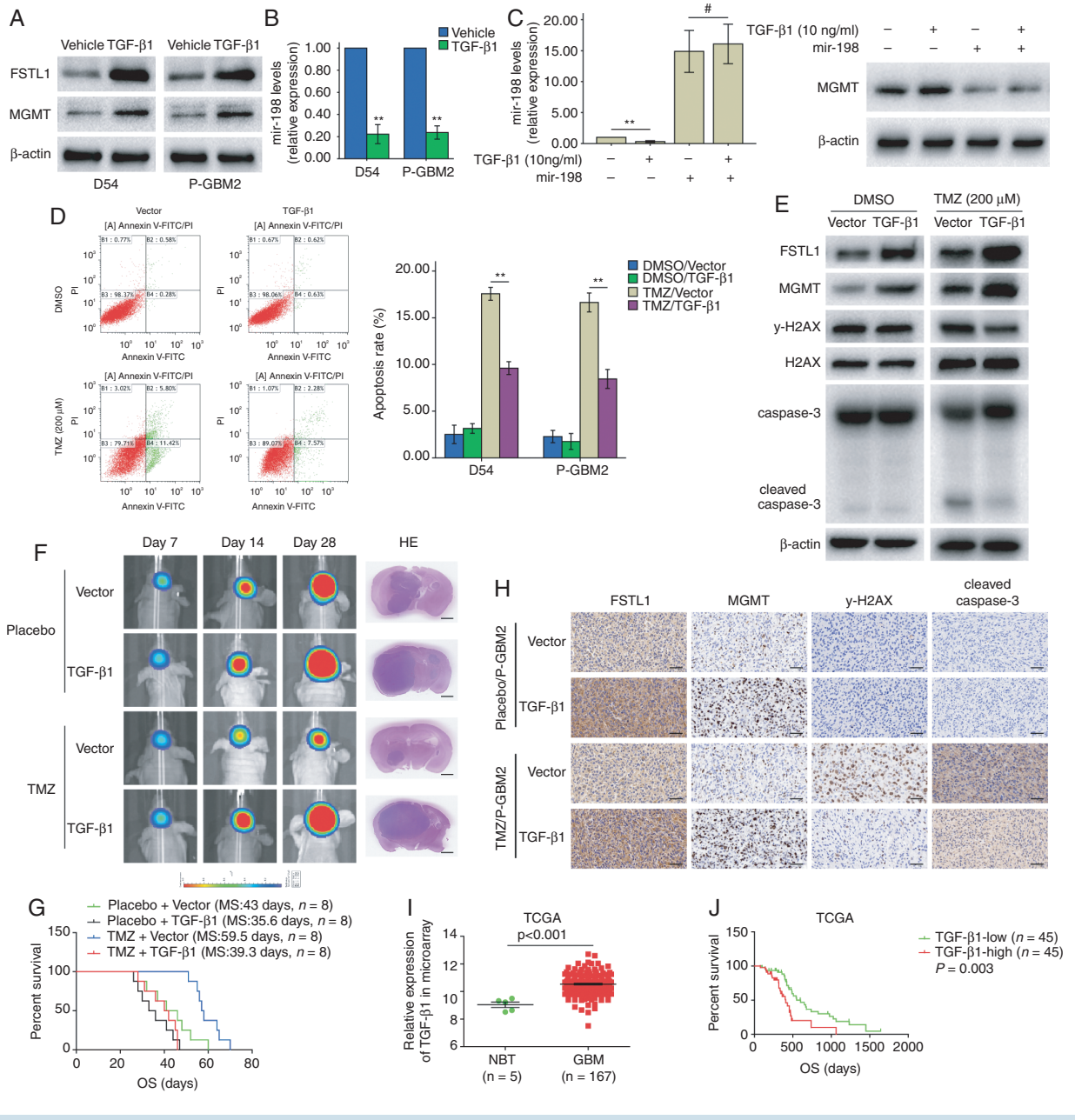

Fig. 1 TGF-β1 confers TMZ resistance. (A, B) GBM cells were incubated with vehicle or TGF-β1 (10 ng/mL) for 48 h. The levels of FSTL1, MGMT, and miR-198 were examined by WB or qRT-PCR. (C) D54 cells stably expressing miR-198 were treated with or without TGF-β1 for 48 h. QRT-PCR analysis for miR-198. WB analysis for MGMT. (D, E) GBM cells stably expressing TGF-β1 were exposed to DMSO or 200 μM TMZ for 48 hours. Flow cytometry was used to measure cells apoptosis, western blot analysis for the indicated proteins. (F) Representative pseudocolor bioluminescence images of intracranial xenografts bearing TGF-β1–overexpressing P-GBM2 or vector control cells in the absence or presence of TMZ on the days as indicated. Representative hematoxylin and eosin (H&E) staining for tumor cytostructure. Scale bar = 2 mm. (G) Survival curve of TGF-β1–overexpressing P-GBM2 or vector control cell-derived intracranial xenografts, the *P*-values for the corresponding Kaplan–Meier (KM) survival curves were listed on Supplementary Table 3. (H) IHC analysis of FSTL1, MGMT, γ-H2AX and cleaved caspase-3 expression in intracranial xenografts, scale bar =50 μm. (I) Levels of TGF-β1 were analyzed in GBM and NBT of TCGA database. (J) KM curves showing the overall survival of patients with high or low expression of TGF-β1 in GBM patients receiving TMZ therapy using the database of TCGA. Data are presented as mean ± SEM (**$P < 0.01$, #$P > 0.05$).

and HOXD-AS2 (Supplementary Figure 7A–C). Moreover, KSRP-interacting H19 and HOXD-AS2 were significantly enriched following TGF-β1 treatment (Supplementary Figure 7D). HOXD-AS2 or H19 overexpression significantly weakened the association between KSRP and Drosha/Dicer complexes (Fig. 3F). Additionally, H19 upregulation impaired the KSRP association with FSTL1 and favored KSRP binding to H19 (Fig. 3G). Notably, the binding of KSRP

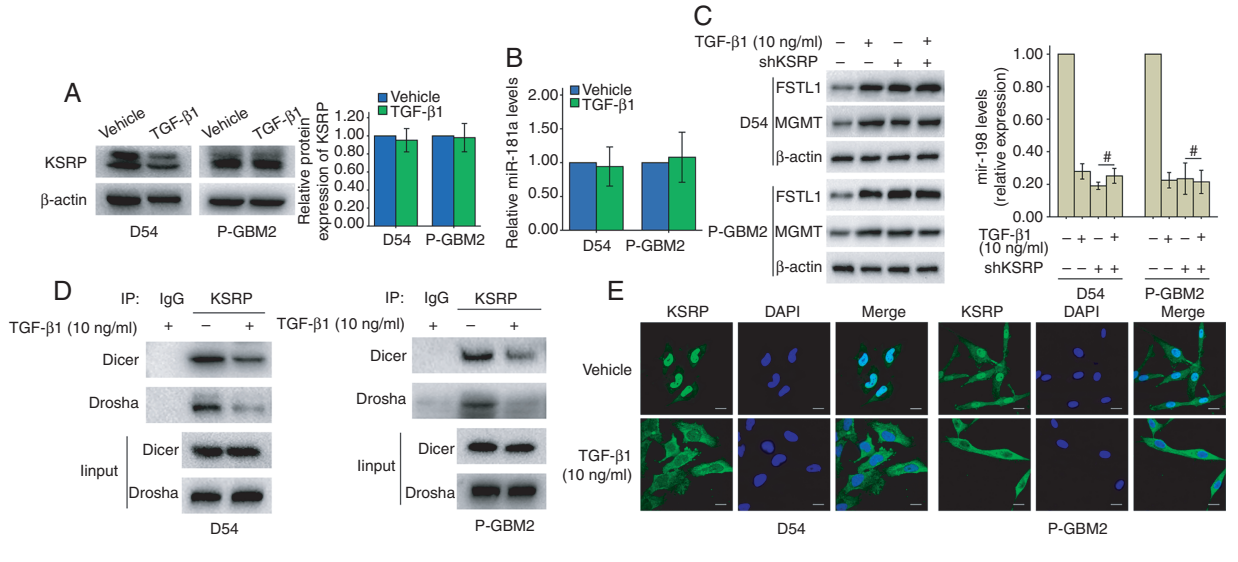

Fig. 2 TGF-β1 regulates miR-198 switching and MGMT expression through KSRP. (A, B) The levels of KSRP and miR-181a were examined by WB or qRT-PCR in GBM cells treated with vehicle or TGF-β1 (10 ng/mL) for 48 h. (C) GBM cells transfected with lentivirus-mediated KSRP shRNA (shKSRP) were treated with TGF-β1 (10 ng/mL) for 48 h. Western blot analysis for the indicated proteins. QRT-PCR analysis for miR-198. (D) Co-IP was performed using lysates prepared from GBM cells treated with TGF-β1 (10 ng/mL) using anti-KSRP antibody. WB analysis was performed with indicated antibodies. (E) Immunofluorescence assay was performed on GBM cells treated with or without TGF-β1 (10 ng/mL, 48 h), using anti-KSRP antibody (green). Merge: Green+Blue. Scale bar: 20 μm. Data are presented as mean ± SEM (**$P < 0.01$, #$P > 0.05$).

with FSTL1 was also weakened by HOXD-AS2 (Fig. 3H). TGF-β1 treatment prevented the nuclear translocation of KSRP while concomitantly arresting it in the cytoplasm. To uncover the mechanism of TGF-β1 affecting the distribution of KSRP, GBM cells were transfected with lncRNAs or an empty vector. Western blotting showed an increase in cytoplasmic KSRP levels, whereas nuclear KSRP levels were reduced significantly (Supplementary Figure 8). Immunofluorescence verified the above results, namely, that H19 or HOXD-AS2 exhibited competitive binding to KSRP and blocked the nuclear translocation of KSRP (Fig. 3I). These data imply that H19 and HOXD-AS2 may represent key regulators of the TGF-β1–mediated distribution of KSRP.

H19 and HOXD-AS2 Regulate MGMT Expression Through Competitively Binding KSRP

H19 and HOXD-AS2 exhibited competitive binding to KSRP and blocked the binding of KSRP with primary miR-198. D54 cells overexpressing H19 or HOXD-AS2 showed a significant increase in FSTL1 and MGMT, and a decrease in miR-198 (Fig. 4A, B). Next, we investigated the effects of KSRP on lncRNA-induced MGMT expression. D54 cells were co-transfected with KSRP and H19 or HOXD-AS2 plasmids. As shown in Fig. 4C and Supplementary Figure 9A, transfection of KSRP plasmids at a low dose (500 ng) did not influence the level of FSTL1, whereas transfection at high doses (1000 and 2000 ng) was sufficient to rescue lncRNA-enhanced MGMT expression. KSRP knockdown blocked the functions of lncRNAs on MGMT, FSTL1, and miR-198 expression (Fig. 4D, E). These data imply that KSRP is a key regulator that mediates lncRNA-mediated FSTL1/miR-198 switching and MGMT expression.

KSRP binds to its RNA targets through 4 K-homology domains (KH1, KH2, KH3, and KH4).[9] Each domain recognizes single-stranded RNA sequences with different specificities and affinities.[22–24] To investigate the domains of KSRP that interact with lncRNAs or FSTL1, 4 KSRP mutants (mut) (KH1GDDG, KH2GDDG, KH3GDDG, and KH4GDDG) without RNA-binding capacities were constructed. Flag-KSRP mut or Flag-KSRP-wildtype (wt) were transfected into D54 cells (Supplementary Figure 10). H19 and HOXD-AS2 were co-immunoprecipitated in cells transfected with KSRP-wt and KSRP-mut-KH4 but not KSRP-mut-KH1, KSRP-mut-KH2, or KSRP-mut-KH3 (Fig. 4F, G). KH1 and KH3 domains of KSRP recognize G-rich and G/U-rich sequences. KH2 prefers AU-rich sequences, while KH4 favors GA-rich sequences[23]. There is a GUG motif within the 3′-UTR of the FSTL1 transcript, and RIP revealed that KSRP bound to the G-rich motif of FSTL1 mRNA. As shown in Fig. 4H, FSTL1 mRNA was immunoprecipitated from cells transfected with Flag-KSRP-wt and Flag-KSRP-mut-KH4 but not Flag-KSRP-mut-KH1, -KH2, or -KH3. Furthermore, mutations in KH1, KH2, and KH3 domains attenuated the regulation of KSRP in FSTL1/miR-198 switching and MGMT expression (Fig. 4I, J). This observation revealed a competitive relationship between lncRNAs and FSTL1 mRNA.

HOXD-AS2 and H19 Confer TMZ Resistance Through KSRP

Among the possible TMZ-resistance mechanisms, MGMT is the most clinically relevant.[2] We found that ectopic

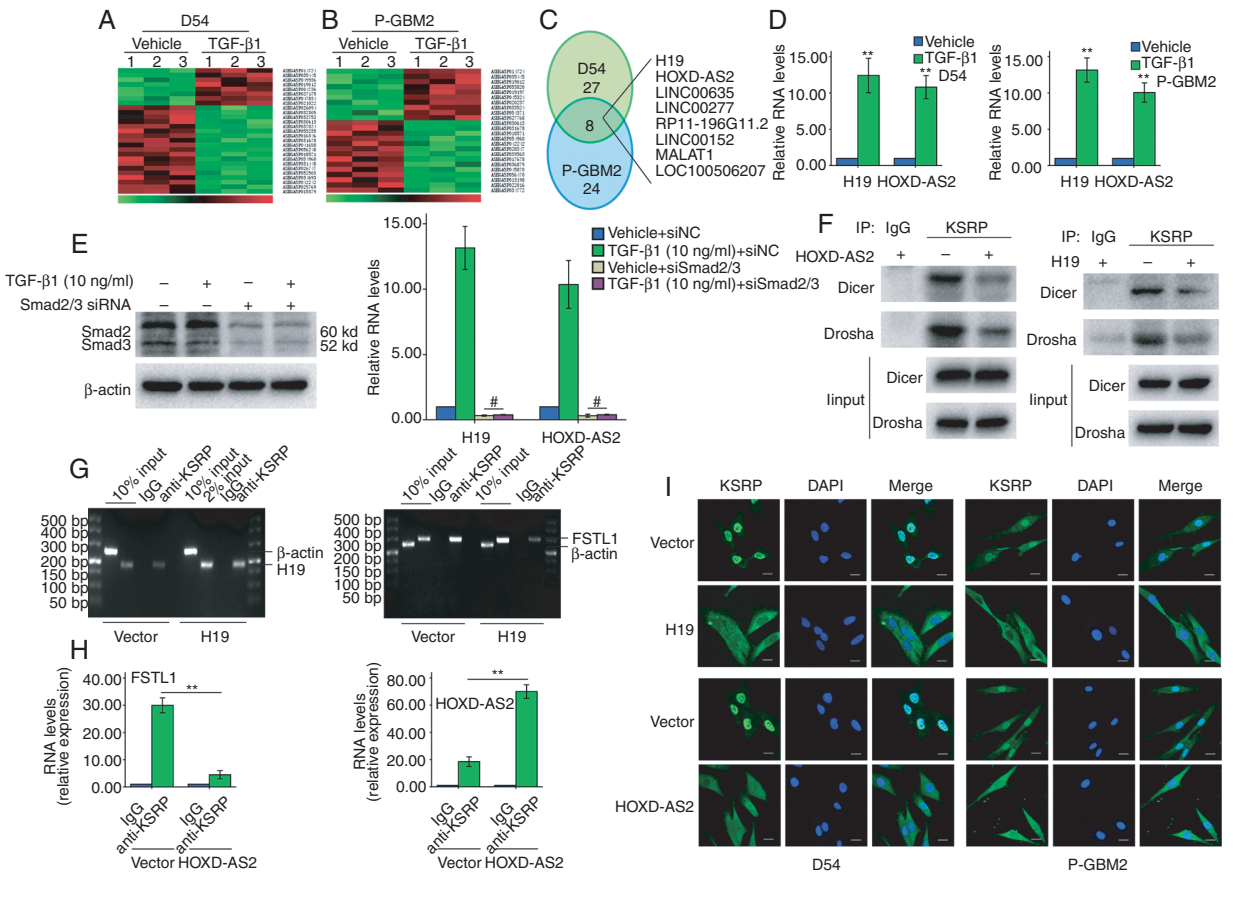

Fig. 3 TGF-β1 influenced the combination among KSRP, FSTL1, and lncRNAs. (A, B) GBM cells were treated with or without recombinant TGF-β1 (10 ng/mL). RIP assays were performed using anti-KSRP antibody. Microarray analysis was used to compare lncRNA expression levels. Hierarchical clustering analysis of lncRNAs that were differentially expressed in cells samples. (C) Overlap of lncRNAs in D54 and P-GBM2 cells. (D) QRT-PCR analysis of H19 and HOXD-AS2 expression in GBM cells. (E) WB analyses for Smad2/3 and qRT-PCR analysis for H19 and HOXD-AS2. (F) D54 cells were subjected to co-IP analysis using anti-KSRP antibody for IP and blotting. (G, H) RIP assays were performed using anti-KSRP antibody. QRT-PCR showing fold enrichment of FSTL1, H19, and HOXD-AS2. (I) Immunofluorescence assay was performed on GBM cells using anti-KSRP antibody (green). Merge: Green+Blue. Scale bar: 20 μm. Data are presented as mean ± SEM (**$P < 0.01$, #$P > 0.05$).

expression of H19 or HOXD-AS2 elevated MGMT levels. In addition, knockdown of KSRP abolished the lncRNA-induced enhancement of MGMT. The homeobox (HOX) family plays important roles in positioning bodily structures, and is also crucial for oncogenesis.[25] As a member of the HOX family, HOXD-AS2 is upregulated in gliomas, and its expression positively correlates with glioma grades.[26] GBM patients with high HOXD-AS2 levels experience significantly shorter overall survival times.[27] Using catRAPID,[28] we revealed the predicted binding between KSRP and the 3′ end of HOXD-AS2 with a high discriminative power (Supplementary Figure 11). Ectopic expression of HOXD-AS2 rescued TMZ-induced increases in apoptosis (Supplementary Figure 12A), whereas it decreased γ-H2AX and cleaved caspase-3 levels (Supplementary Figure 12B). Furthermore, the growth rate of tumors in the HOXD-AS2 group was significantly faster than that of tumors in control mice (Supplementary Figure 12C). Additionally, our in vivo experiments showed that ectopic expression of HOXD-AS2 significantly decreased the expression of γ-H2AX and cleaved caspase-3 in the presence of TMZ (Supplementary Figure 12D). Compared with those of the vector control group, mice injected with HOXD-AS2 cells experienced significantly shorter overall survival times after TMZ treatment (Supplementary Figure 12E and Supplementary Table 3). In addition, mice subcutaneously injected with D54/HOXD-AS2–overexpressing cells showed a significant increase in tumor growth compared with that in mice implanted with vector control-infected cells (Supplementary Figure 12F–H). Furthermore, the levels of HOXD-AS2 in GBMs were higher than those in NBTs (Supplementary Figure 12I). Further studies using the database of TCGA indicated that low HOXD-AS2 levels exhibited a favorable prognosis and improved responses to TMZ therapy (Supplementary Figure 12J).

The KSRP-binding domain of H19 is located at its first exon in murine mesenchymal C2C12 cells.[21] This site is well conserved among mammalian species. To determine whether the KSRP-binding domain of H19 in D54 cells is similar to that in mice, MS2-tagged wt H19 and MS2-tagged

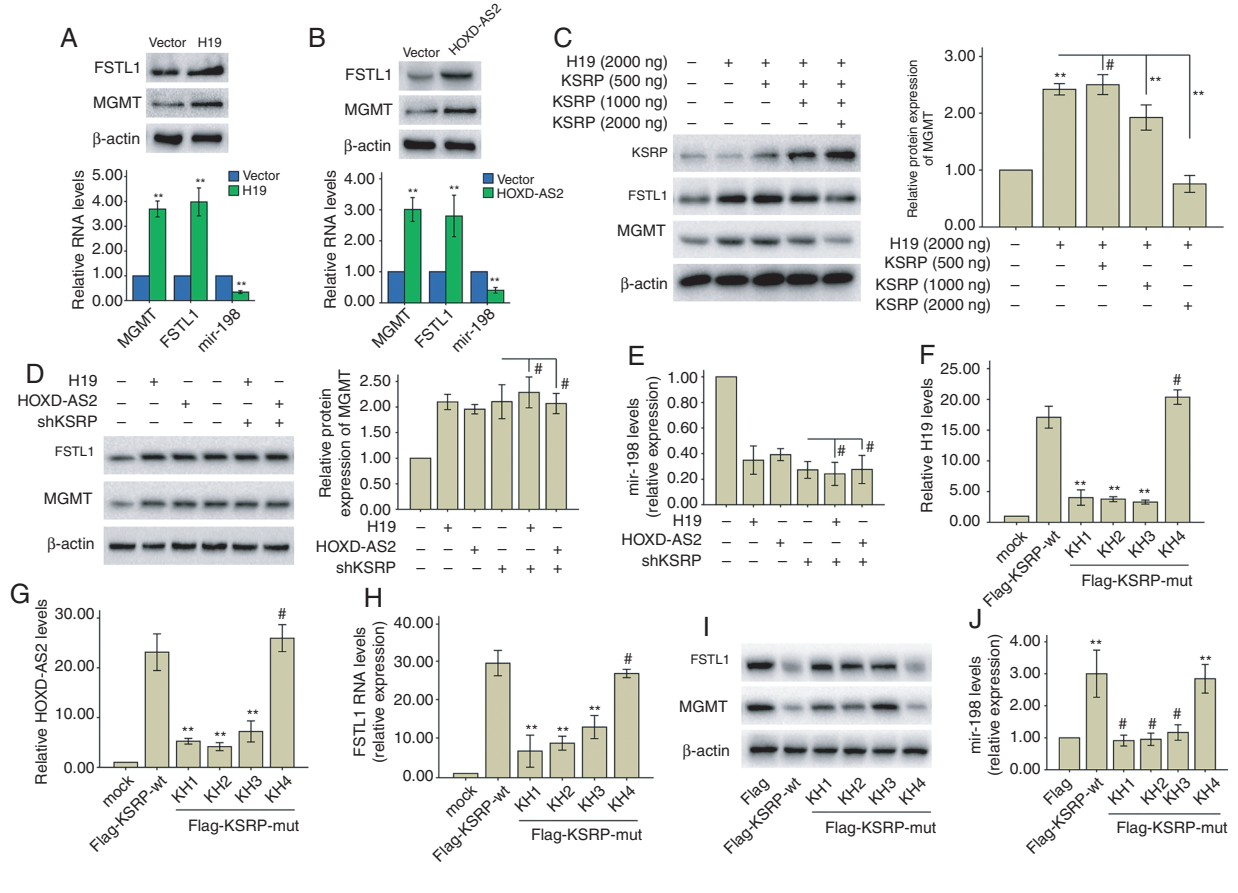

Fig. 4 H19 and HOXD-AS2 competitive combination KSRP. (A, B) Histograms show relative levels of FSTL1, MGMT, and miR-198 in D54 cells. (C) H19 and KSRP plasmids were co-transfected into D54 cells with indicated dosage. WB was performed using indicated antibodies. (D, E) D54 cells stably expressing shKSRP were transfected with H19 or HOXD-AS2 plasmid. The levels of FSTL1, MGMT, and miR-198 were detected by WB or qRT-PCR. (F–H) D54 cells separately transfected with Flag-KSRP-wt or Flag-KSRP-mut(KH1,KH2,KH3,KH4) were subjected to RIP analysis using anti-Flag antibody. The levels of H19, HOXD-AS2 and FSTL1 were analyzed by qRT-PCR. (I, J) QRT-PCR analyses for miR-198 and WB analysis for FSTL1 and MGMT. Data are presented as mean ± SEM (** $P < 0.01$, # $P > 0.05$).

mut H19 were transfected into D54 cells. Mutating the KSRP-binding domain weakened the coupling between H19 and KSRP (Fig. 5A, B). Compared with the effects in the H19-wt group, the regulatory effects of H19-mut on FSTL1/miR-198 switching and MGMT expression were significantly weakened (Fig. 5C, D). Of note, overexpression of miR-198 reversed H19-induced enhancement of MGMT (Fig. 5E). To explore the potential role of H19 in TMZ resistance, GBM cells stably expressing H19-wt, H19-mut, or an empty vector were treated with dimethyl sulfoxide (DMSO) or TMZ (200 μM), and then cellular viability was assayed by CCK8 at various time points (Supplementary Figure 13A). The results showed that overexpression of H19-wt significantly promoted cellular survival. However, mutating the KSRP-binding domain of H19 weakened the TMZ-resistance function of H19. Moreover, the TMZ-induced increases in cleaved caspase-3 levels and tumor-cell apoptosis were rescued by H19-wt, but not H19-mut (Fig. 5F, G). Orthotopic and subcutaneous GBM murine xenograft models showed that upregulation of H19-wt resulted in faster growth and larger tumors than in the vector control group (Fig. 5H and Supplementary Figure 13B–D). However, disrupting the KSRP-binding domain weakened the chemoresistant function of H19. Moreover, ectopic expression of H19-wt, but not H19-mut, elevated the levels of cleaved caspase-3 and γ-H2AX upon TMZ treatment in vivo (Fig. 5I). Survival curves showed that mice injected with H19-wt cells had a poor prognosis compared with that of H19-mut and negative-inhibitor groups (Fig. 5J and Supplementary Table 3). Analysis of the database of TCGA revealed that patients with low-level H19 expression benefited from TMZ therapy (Fig. 5L). This diminished TMZ-resistance function of H19-mut suggests a link between H19 and KSRP-mediated chemoresistance. Taken together, these results demonstrated that the KSRP-MGMT signaling pathway may be one of the mechanisms by which H19 confers TMZ resistance.

TGF-β1 Expression Correlates with H19, HOXD-AS2, miR-198, and FSTL1 Levels

To verify the associations between TGF-β1 and H19, HOXD-AS2, miR-198, and FSTL1 in GBM patients, two glioblastoma tissue samples with high or low TGF-β1

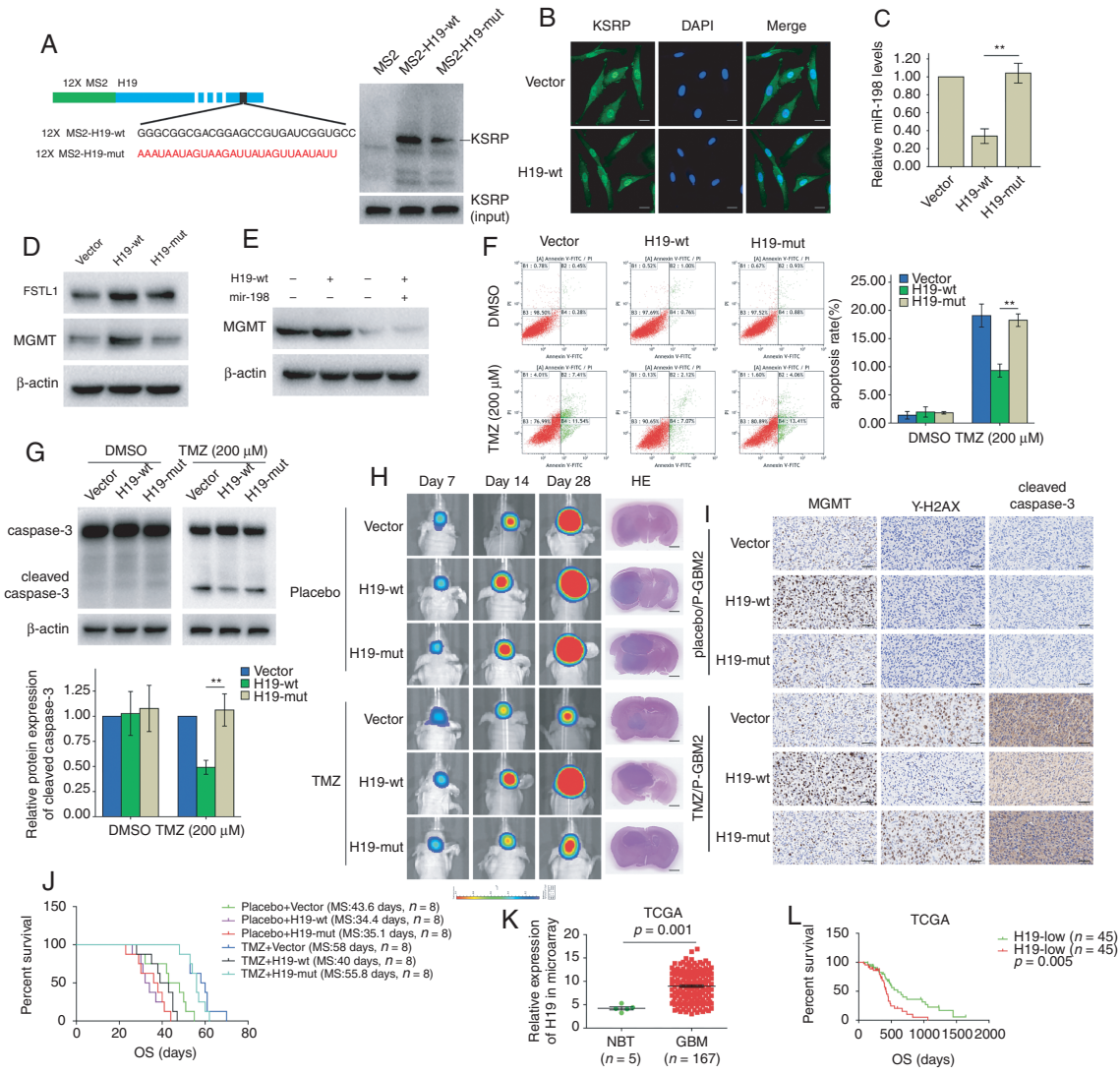

Fig. 5 Link between H19 and chemoresistance mediated by KSRP. (A) D54 cells were transfected with FLAG-fused MS2 binding protein (FLAGMS2BP) together with pCDNA3.1(-)-H19wt-12xMS2, pCDNA3.1(-)-H19mut-12xMS2, or pCDNA3.1(-)-12xMS2. Total cell extracts were precipitated by anti-FLAG M2 Magnetic Beads. Protein was purified and analyzed by WB to detect KSRP. (B) Immunofluorescence assay was performed on D54 cells transfected with vector or H19-mut, using anti-KSRP antibody (green). Merge: Green+Blue. Scale bar: 20 μm. (C, D) QRT-PCR analysis for miR-198, and WB analyses for MGMT and FSTL1. (E) WB analyses for MGMT using D54 cells. (F, G) GBM cells transfected with vector, H19-wt, or H19-mut were treated with DMSO or 200 μM TMZ for 48 h. Flow cytometry was used to measure cell apoptosis, western blot analysis for cleaved caspase-3. (H) Representative pseudocolor bioluminescence images of intracranial xenografts bearing P-GBM2 cells stably expressing vector, H19-wt, or H19-mut in the absence or presence of TMZ on the days as indicated. Representative H&E staining for tumor cytostructure. scale bar = 2 mm. (I) IHC analysis of the indicated proteins expression. Scale bar: 50 μm. (J) Survival curve of P-GBM2 cells-derived intracranial xenografts treated with placebo or TMZ, the *P*-values for the corresponding Kaplan–Meier (KM) survival curves were listed on Supplementary Table 3. (K) Levels of H19 were analyzed in GBM and NBT of TCGA database. (L) KM curves showing the overall survival of patients with high or low expression of H19 in GBM patients receiving TMZ therapy using the database of TCGA . Data are presented as mean ± SEM (** $P < 0.01$, # $P > 0.05$).

expression were chosen to analyze expression levels of these five molecules by immunohistochemistry and FISH. As shown in Fig. 6A, tumors with high expression of TGF-β1 had high levels of H19, HOXD-AS2, and FSTL1, and a low level of miR-198, whereas tumors with low expression of TGF-β1 had low levels of H19, HOXD-AS2, and FSTL1, and a high level of miR-198. Our previous study showed that FSTL1 exhibits higher expression in high-grade glioma tissues compared with that in nontumor brain tissues and low-grade glioma samples[8]. Next, we detected the expression levels of H19, HOXD-AS2, and miR-198 in clinical glioma samples. Increased H19 and HOXD-AS2 expression levels were correlated with the glioma grade (Fig. 6B, C). Compared with those in NBT and low-grade glioma samples, high-grade glioma tissues expressed lower levels of miR-198 (Fig. 6D). Pearson correlation analysis showed that the levels of H19 and HOXD-AS2 were positively correlated with FSTL1 and were negatively correlated with miR-198

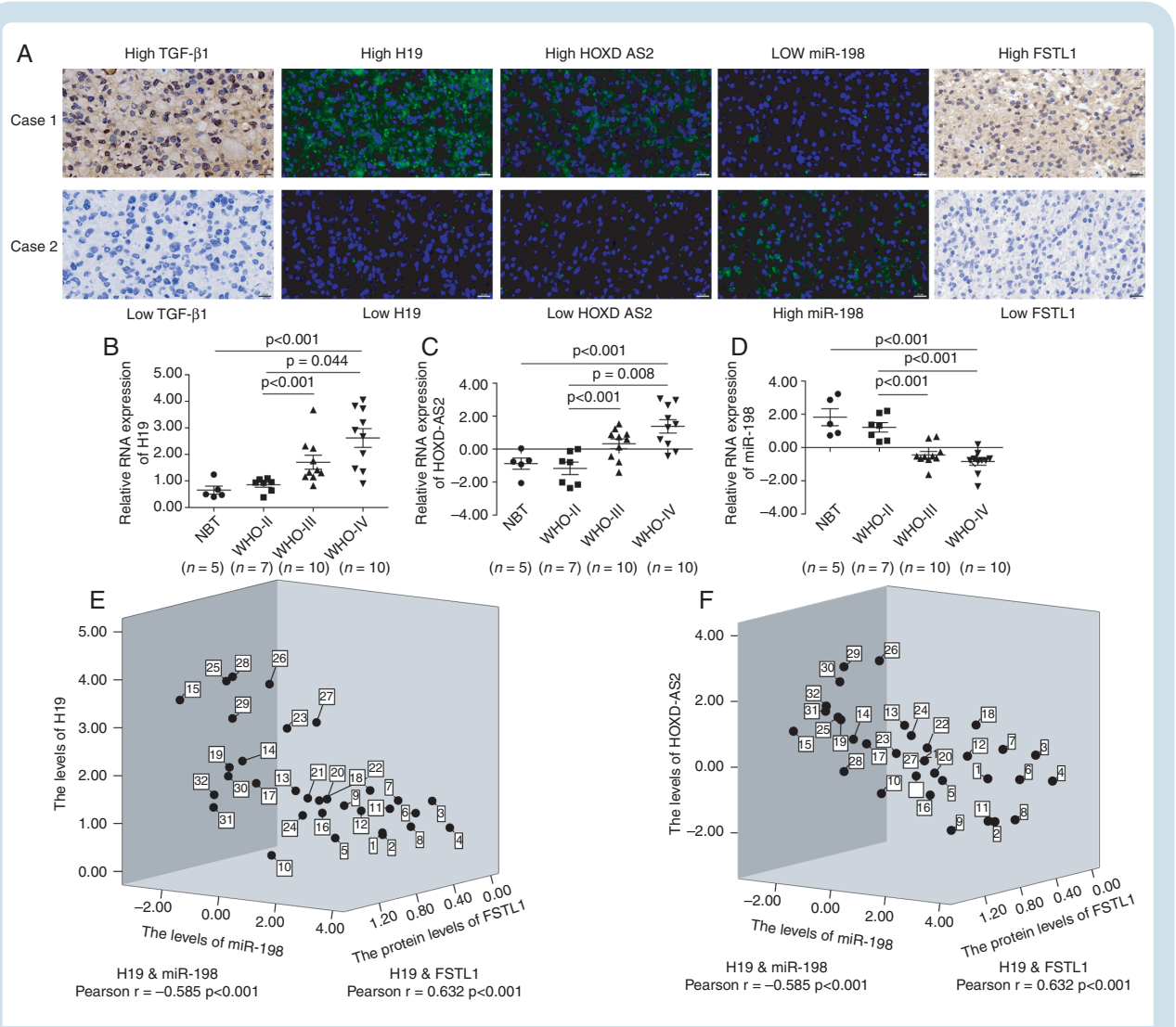

Fig. 6 H19 and HOXD-AS2 overexpression correlates with miR-198 and FSTL1 levels. (A) IHC or FISH analysis of TGF-β1, H19, HOXD-AS2, miR-198 and FSTL1 in 2 representative GBM specimens. scale bar =20 μm. (B–D) the levels of H19, HOXD-AS2 and miR-198 in nontumor brain tissues (NBT) and gliomas were examined by qRT-PCR. (E, F) the relationship among miR-198, FSTL1, H19, and HOXD-AS2. One-way ANOVAs were performed. Data are presented as mean ± SEM (**$P < 0.01$).

(H19 and FSTL1, $r = 0.632$, $P < 0.001$; HOXD-AS2 and FSTL1, $r = 0.681$, $P < 0.001$; H19 and miR-198, $r = –0.585$, $P < 0.001$; HOXD-AS2 and miR-198, $r = –0.573$, $P < 0.001$; Fig. 6E, F). These findings elucidate significant correlations of these molecules in GBMs.

Discussion

In the present study, our in vitro and in vivo experiments showed that TGF-β1 contributed to TMZ resistance in MGMT-promoter hypomethylated glioblastoma cells. Ectopic expression of TGF-β1 in MGMT-promoter hypomethylated glioblastoma cells rescued TMZ-induced increases in apoptosis. Additionally, TGF-β1 depletion sensitized P-GBM2-R (P-GBM2 TMZ-resistant) cells to TMZ (Supplementary Figure 14). However, TMZ sensitivity in MGMT-promoter methylated glioblastoma cells was not influenced by ectopically expressed TGF-β1. Mice injected with TGF-β1–overexpression cells exhibited a poor prognosis compared with those in the negative-inhibitor group when treated with TMZ. Furthermore, TGF-β1 overexpression increased—whereas TGF-β1 silencing decreased—MGMT levels. The dataset of TCGA implied that patients with low levels of TGF-β1 benefited from TMZ therapy. TGF-β1 is a multifunctional cytokine that regulates glioma cell proliferation, invasion, angiogenesis, immunosuppression, and radiotherapeutic resistance by activating Smad proteins. Many small-molecule inhibitors have been designed against TGF-β or TGF-β receptor kinase. Such compounds abolish TGF-β signaling and inhibit the proliferation and motility of human glioma cells.[29] Our present study showed that TGF-β1 conferred TMZ resistance through MGMT. Hence, a novel combination of TMZ with a TGF-β inhibitor may represent an effective therapy for GBMs.

Previous studies have revealed that MGMT expression is upregulated by FSTL1 and downregulated by miR-198.[7,8] KSRP binds to the 3′-UTR of FSTL1 mRNA and favors FSTL1 mRNA processing to miR-198[5]. KSRP depletion abolished the effects of TGF-β1 on MGMT expression. Sundaram et al reported that TGF-β1 represses KSRP production through miR-181a that binds to the 3′-UTR of KSRP mRNA. However, in our present study, we did not detect a significant change in miR-181a in glioma cells after TGF-β1 treatment. Our previous study indicated that hsa-miR-181a expression negatively correlates with glioma grade and acts as a tumor suppressor.[30] The expression of hsa-miR-181a is controlled by multiple mechanisms, such as promoter methylation, histone acetylation, and histone methylation. Histone modifications play an important role in the regulation of miRNAs. Methylation of histone H3 at promoter regions blocks transcription factor binding and represses transcription.

Polycomb group proteins, including polycomb repressive complex (PRC) 1 and PRC2, are chromatin-modifying complexes. PRC2 epigenetically represses miR-181a expression by facilitating H3K27me3 trimethylation.[31] The histone methyltransferase enhancer of zeste homolog 2 (EZH2), a key component of the PRC2 complex, is elevated in gliomas. Overexpression of EZH2 markedly increases trimethylation of H3K27 and decreases the miR-181a level in gliomas.[32] Furthermore, ectopic expression of EZH2ΔSET, which is missing its catalytic SET domain, does not affect the levels of H3K27me3 or miR-181a. The trimethylation of H3K27 prohibits Smad4 binding to the promoter region of miR-181a and prevents TGF-β1 from activating miR-181a expression in gliomas. Here, we investigated the level of H3K27me3 in the miR-181a promoter region using ChIP followed by qRT-PCR. We found high levels of H3K27me3 in both GBM samples and GBM cell lines (Supplementary Figure 15).

Inconsistent with the finding by Sundaram et al that TGF-β1 affects KSRP expression through miR-181a,[5] we identified a novel pathway by which TGF-β1 regulates KSRP functions in gliomas. We identified and characterized 2 lncRNAs (H19 and HOXD-AS2) that were upregulated by TGF-β1 through SMAD signaling. RNA immunoprecipitation assays showed that H19 and HOXD-AS2 bound to the KH1/KH2/KH3 domains of KSRP. Upregulation of H19 or HOXD-AS2 resulted in competitive binding to KSRP and prevented KSRP from participating in the FSTL1/miR-198 process. Ectopic expression of H19 or HOXD-AS2 led to a significant decrease in miR-198 and increase in MGMT expression. HOXD-AS2 or H19 upregulation contributed to TMZ resistance through KSRP/mir-198/MGMT signaling. Additionally, mutating the KSRP-binding domain weakened H19-induced TMZ resistance. TGF-β1 stimulation upregulated lncRNAs levels and favored the interaction between lncRNAs and KSRP. Moreover, KSRP depletion abolished the effects of TGF-β1, H19, and HOXD-AS2 on miR-198 and MGMT expression. Hence, TGF-β1 regulated FSTL1/miR-198 switching and MGMT expression independently of miR-181a in gliomas.

In summary, our study revealed a novel pathway through which TGF-β1 decreased miR-198 levels and increased MGMT accumulation to confer TMZ resistance. This study complements the known mechanisms of TGF-β1 regulation of miR-198/FSTL1 switching and MGMT expression in gliomas. Taken together, we propose that TGF-β signaling may represent a target for development of therapeutic strategies to treat gliomas with improved patient outcomes.

Supplementary Material

Supplementary data are available at *Neuro-Oncology* online.

Keywords

glioblastoma | KSRP | MGMT | temozolomide resistance | TGF-β1

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Conflict of interest statement. The authors declare that they have no conflict of interest.

Authorship statement. EN, JZ, MX, and YY designed the study. EN, XJ, FM, TY, and TZ performed the experimental work. EN, XJ, JZ, MX, YW, and ZS performed data analyses. EN, XJ, YW, and JZ produced the main draft of the text and the figures. All authors have seen, corrected, and approved the final manuscript.

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