



BASIC SCIENCE ARTICLE

Musashi1 enhances chemotherapy resistance of pediatric glioblastoma cells in vitro

Rebecca Pötschke^{1,2}, Gerrit Gielen³, Torsten Pietsch³, Christof Kramm⁴, Jan-Henning Klusmann², Stefan Hüttelmaier¹ and Caspar D. Kühnöl²

BACKGROUND: Glioblastoma (GBM) is the most aggressive form of glioma in adults and children and is associated with very poor prognosis. Pediatric tumors are biologically distinct from adult GBM and differ in response to current GBM treatment protocols. Regarding pediatric GBM, new drug combinations and the molecular background of chemotherapy effects need to be investigated, in order to increase patient survival outcome.

METHODS: The expression of the RNA-binding protein Musashi1 (MSI1) in pediatric glioma samples of different WHO tumor grades was investigated on the protein (immunohistochemistry) and on the RNA level (publicly accessible RNA sequencing dataset). The impact of the chemotherapeutic temozolomide (TMZ) in combination with valproic acid (VPA) was tested in two pediatric glioblastoma-derived cell lines. The supportive effect of MSI1 expression against this treatment was investigated via transient knockdown and protein overexpression.

RESULTS: MSI1 expression correlates with pediatric high-grade glioma (HGG). The combination of TMZ with VPA significantly increases the impact of drug treatment on cell viability in vitro. MSI1 was found to promote drug resistance to the combined treatment with TMZ and VPA.

CONCLUSION: MSI1 expression is a potential marker for pediatric HGG and increases chemoresistance. Inhibition of MSI1 might lead to an improved patient outcome and therapy response.

Pediatric Research (2020) 87:669–676; <https://doi.org/10.1038/s41390-019-0628-9>

INTRODUCTION

Glioblastoma (GBM) is a World Health Organization (WHO) classified grade IV tumor and the most aggressive form of glioma with a median survival time after diagnosis of approximately 15 months. It is the most common primary central nervous system (CNS) tumor in adults.^{1,2} Pediatric GBM is a rare disease that includes children and young adults up to the age of 18 years, but similar to adult GBM, it carries a poor prognosis.^{3,4} It is clinically and biologically distinct from the adult disease, resulting in different capabilities of adjuvant therapies.⁵ However, the prognosis of high-grade pediatric glioma may vary significantly depending on the histological subtype, age at diagnosis, and tumor localization.^{6,7} The standard therapy includes surgical resection, followed by radiation and usually chemotherapy with an alkylating agent temozolomide (TMZ).⁸ New therapy strategies have evolved to increase chemotherapy response. For example, various histone deacetylase (HDAC) inhibitors (HDACi), which are claimed to induce apoptosis, cell cycle arrest, and DNA damage repair in cells, are tested in clinical studies.⁹ Thus one current treatment option employs a combination of radiochemotherapy and TMZ with either valproic acid (VPA) or chloroquine (clinical trial HIT-HGG-2013, (ClinicalTrials.gov/NCT03243461)). VPA is a Food and Drug Administration-approved drug; HDACi is primarily applied for seizure disorders and is widely used in children. It is involved

in modulating chromatin structure via histone acetylation, thereby increasing DNA accessibility and cytotoxicity of drugs targeting DNA.¹⁰ In vitro studies showed increased sensitivity for TMZ and radiotherapy after VPA treatment.^{11,12} In several reports, the combination of VPA with radiochemotherapy and TMZ showed a longer survival time in adult and pediatric patients compared to radiochemotherapy used with TMZ alone.¹³

Patients commonly suffer from tumor recurrence, indicating that a percentage of tumor cells are radiochemotherapy resistant and survive the aggressive treatment. These resistant cells are likely GBM-cancer stem cells (GBM-CSCs), which share common stem cell features and can resemble the primary tumor.¹⁴ One typical marker of stem cells is the neural RNA-binding protein (RBP) Musashi1 (MSI1).^{15,16} It is highly expressed during embryogenesis and its expression declines during development.¹⁷ Interestingly, it is re-expressed in various tumors, including GBM, suggesting an oncofetal pattern of expression.^{18–21} Accordingly, MSI1 can be used as a prognostic marker in tumor progression and is associated with poor outcome for patients.²² MSI1 controls the balance between self-renewal and differentiation by controlling target mRNA translation and potentially turnover.^{23–25} Thus MSI1 promotes sustaining of the stem cell state, which is an essential feature for tumorigenesis as well as for tumor recurrence.^{26,27}

¹Molecular Cell Biology, Institute of Molecular Medicine, Martin-Luther-University, Halle (Saale), Germany; ²Department of Pediatric Hematology/Oncology, University Hospital, Halle (Saale), Germany; ³Institute of Neuropathology, University Hospital, Bonn, Germany and ⁴Division of Pediatric Hematology and Oncology, University Medical Center, Göttingen, Germany

Correspondence: Stefan Hüttelmaier (stefan.huettelmaier@medizin.uni-halle.de) or Caspar D. Kühnöl (caspar.kuehnoel@uk-halle.de)

Received: 21 August 2019 Accepted: 25 September 2019

Published online: 22 November 2019

Although the understanding of the origin and biological features of GBM through the application of genome- and epigenome-wide molecular profiling techniques has greatly improved, there have been no significant gains made in patient survival in the past decades. Thus it is crucial to investigate key mechanisms involved in drug resistance in order to find novel, effective GBM targeting strategies. Most studies focus exclusively on adult GBM and translate these findings to pediatric GBM, even though differences in treatment efficacy are well known.²⁸ In this study, we analyze MSI1 expression in pediatric GBM and investigate its role in chemoresistance toward TMZ/VPA combination treatment.

MATERIAL AND METHODS

MSI1 expression in pediatric brain tumor dataset

MSI1 expression in pediatric brain tumors was evaluated with the aid of public pediatric brain tumor dataset published by Jones et al.²⁹ Fresh frozen tumor samples were subjected to the Trizol RNA isolation protocol. RNA was then analyzed using Illumina HumanHT-12 v3 Expression BeadChip. The analyzed tumor cohort comprises samples from 64 children between 1 and 16 years and 8 adult brain controls.

Immunohistochemistry

The neuropathological analysis of pediatric glioma samples (diffuse astrocytoma (WHO grade II, IDH-mutant); anaplastic astrocytoma (WHO grade III, IDH-mutant), and glioblastoma (WHO grade IV, IDH-wild type) were diagnosed according the 2016 WHO classification of tumors of the CNS; control tissue origins from cerebellum of a 5-year-old girl (cause of death not neoplastic) retrieved from the archive of the Institute of Neuropathology, University of Bonn Medical Centre comprised of hematoxylin and eosin staining as well as immunohistochemical (IHC) analysis. IHC was performed on a Ventana Benchmark XT Immunostainer (Roche Ventana, Darmstadt, Germany) with a monoclonal antibody directed against Musashi-1 (clone 7B11.1; Merck/Millipore, MABE268, dil. 1/400). Antibody labeling was assessed microscopically by an experienced neuropathologist. Specific cytoplasmic and/or nuclear staining was considered as positive staining. Human cerebellar tissue served as a positive control. Our analysis was approved by the Institutional Review Board (IRB) of the University clinic Goettingen (Germany), as part of the HIT-HGG-2013 clinical trial (for HGG) and the IRB of the Ludwig-Maximilian-University Munich, as part of the clinical trial SIOP LGG 2004 (for LGG). Respective parents of the patients gave their informed consent for storage and use of tumor material for future research like the present one.

Cell culture

The pediatric GBM cell line KNS42 was obtained from Japan Cancer Research Resources. The pediatric GBM cell line SF188 was kindly provided by Chris Jones (Paediatric Oncology, The Institute of Cancer Research, Sutton, UK). Cells were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher) supplemented with 10% fetal serum albumin and 1% GlutaMAX (Thermo Fisher) at 5% CO₂ and 37°C. Transient knockdown (KD) via Lipofectamine RNAiMAX (Thermo Fisher) was performed according to the manufacturer's protocol. KD was stable for 96 h. For stable overexpression, the MSI1 coding sequence was cloned into the pLVX-TO(zeo)-GFP plasmid (Invitrogen).

Western blotting

Western blotting was performed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and MOPS running buffer (ThermoFisher Scientific, NuPAGE system) and transferred with Trans-Blot Turbo Transfer System (BioRad) and SDS-PAGE was done with Life Technologies NuPAGE MOPS

Electrophoresis System. MSI1 was detected via anti-MSI1 (Abcam, ab154497). Anti-VCL (Sigma, V9131) was used as a loading control. Secondary antibodies were purchased from Licor (rabbit/mouse-IRDye680/800). Blots were analyzed with the Odyssey Infrared Scanner (LiCOR).

Quantitative real-time polymerase chain reaction (RT-PCR)

RNA was isolated with TRIZOL/Phenol extraction. cDNA was transcribed with M-MLV Reverse Transcriptase (Promega). Per well cDNA was added to ORA qPCR-Green ROX L Mix 2× (HighQu) with specific primers, and the reaction was measured with the Roche Light Cycler 480 II. Resulting C_q-values (cycle of quantification) from first point of inflection of second deviation (second derivative maximum) were used for calculation of changes in mRNA abundance compared to control with the $\Delta\Delta C_q$ -method.

Immunofluorescence

For detection of endogenous MSI1 in KNS42, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% (v/v) Triton X-100 and blocked with 1% bovine serum albumin (all were in phosphate-buffered saline). Nuclei were stained with 4,6-diamidino-2-phenylindole. Actin filaments were stained with Phalloidin. MSI1 was stained with primary antibody anti-MSI1 (Abcam, ab154497) and secondary antibody anti-rabbit-FITC (Dianova). Images were taken with Leica SP5X.

Cell proliferation assay

KNS42 cells or SF188 cells were plated onto a 96-well plate in 200 μ l complete culture media (KNS42 5×10^3 cells/well, SF188 1×10^3 cells/well). Cells were treated with varying concentrations of VPA (diluted in H₂O) and/or TMZ (diluted in dimethyl sulfoxide) or vehicle control. Cells were treated for 96 h at 5% CO₂ and 37°C. To assess cell viability in response to TMZ and VPA treatment, alamarBlue (ThermoFisher Scientific) was used, according to the manufacturer's protocol. alamarBlue reagent (1:10) was added to each well and incubated for 3 h. The color change and fluorescence increase indicate cell viability. Fluorescence (excitation 560 nm, emission 590 nm) was determined on an Infinite[®] 200 Pro plate reader (Tecan).

Statistical analysis

Prism software (Prism, 7.0, GraphPad), Sigma Plot, Student's *t* test, and Mann–Whitney test were used to analyze the data. *P* values <0.05 were considered to be statistically significant. To analyze the effects of the drug combination, the co-efficient of drug interaction (CDI) was used.³⁰ CDI is calculated as follows: $CDI = AB/(A \times B)$, where *AB* is the measured value for combined treatment/value for the control and *A* and *B* are the values for the single treatment/value for the control. Thus $CDI < 1$, $= 1$, or > 1 indicates that the combination treatments are synergistic, additive, or antagonistic, respectively. $CDI < 0.7$ indicates that the drug combination is significantly synergistic.

RESULTS

MSI1 expression is substantially enhanced in pediatric GBM. Expression of MSI1 is identified to be enriched in adult GBM, where it is proposed to drive tumor malignancy. However, it is not sufficiently investigated whether MSI1's expression persists or is re-expressed in pediatric GBM. Thus MSI1 expression was initially analyzed in pediatric brain tumor samples by IHC in diffuse astrocytoma (WHO II), anaplastic astrocytoma (WHO III), and GBM (WHO IV) and fetal brain serving as control tissue. Consistent with the reported expression of MSI1 in proliferative tissue, moderate expression was observed in the cerebellum of a fetal brain sample (Fig. 1a). Predominantly, granule cells have no MSI1 staining. MSI1 expression can be detected in proliferating glial cells (i.e., Bergmann gliosis) as well as in Purkinje cells

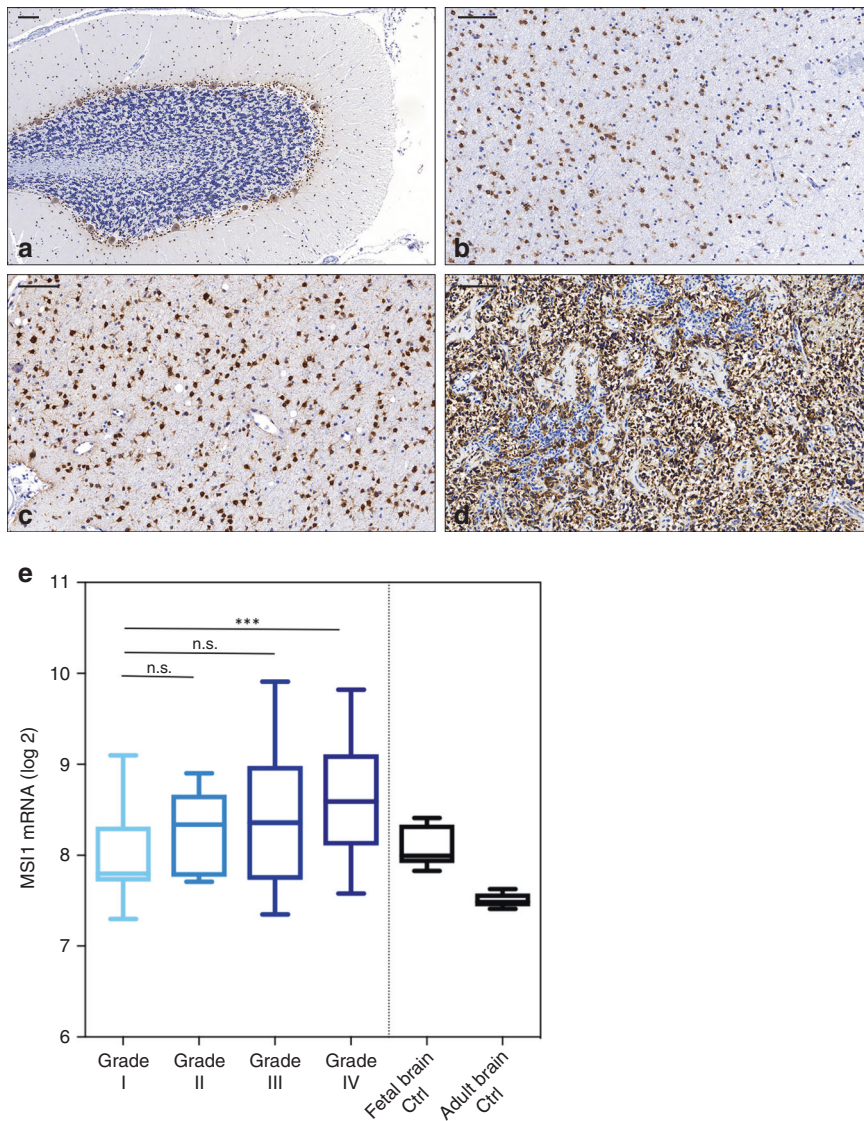


Fig. 1 MS11 expression in fetal brain control (a), diffuse astrocytoma (WHO II) (b), anaplastic astrocytoma (WHO III) (c) and GBM (WHO IV) (d). Size bar, 100 μ m. e MS11 RNA expression increases over pediatric brain tumor grades. Fetal brain control shows a higher MS11 expression compared to adult brain control (pediatric brain tumor dataset, ref. ²⁹). Statistical significance was determined by Mann–Whitney test; *** $p < 0.001$.

(Supplementary Fig. S1A). MS11 expression was also observed in diffuse and anaplastic astrocytoma but was markedly higher in GBM tumor tissue (Fig. 1b–d). To substantiate the IHC finding, which shows an increasing MS11 expression with increasing WHO tumor grades, the expression of MS11 mRNA across a tumor cohort was examined, which includes pediatric brain tumor as well as fetal and adult control samples (using an RNA-sequencing dataset).²⁹ The tumor cohort comprised brain tumors from 64 children (1–16 years of age): 19 WHO grade I tumors, 9 WHO grade II tumors, 9 WHO grade III tumors, 19 WHO grade IV tumors, and 8 fetal brain controls. In addition, eight adult brain controls were analyzed. In the fetal brain controls, MS11 expression was higher compared to adult brain tumor controls, likely to persisting neurogenesis that declines during adulthood (Fig. 1e). In all four WHO tumor grades, MS11 expression was detectable to some extent. MS11 was the least expressed in WHO grade I tumors, and this expression was at a comparable level with WHO grade II and WHO grade III tumors. In contrast, MS11 expression was significantly higher in the WHO grade IV tumors

compared to the WHO grade I tumors. Thus, although it is expressed in non-malignant pediatric brain tissue, MS11 expression is markedly higher with increasing malignancy of brain tumors and shows the highest expression in GBM.

MS11 expression in pediatric GBM cell lines KNS42 and SF188
This study aims to investigate MS11's influence on the combined drug treatment of VPA and TMZ. For comparative cell culture experiments, pediatric GBM cell lines KNS42 and SF188 were used. KNS42 was derived from a 16-year-old male patient³¹ (RRID: CVCL_0378). SF188 was derived from an 8-year-old male patient (RRID: CVCL_6948). Both cell lines are well characterized.^{32,33} For MS11 expression analysis, both protein and mRNA levels were detected. KNS42 cells show a higher MS11 expression compared to SF188 cells. In contrast, MS11 expression was barely detectable at the mRNA level in SF188 cells (Fig. 2a). Therefore, KNS42 cells were used for detection of endogenous MS11 expression upon drug treatment as well as for transient KD studies. KNS42 cells show a prominently cytoplasmic localization of MS11 with a minor portion

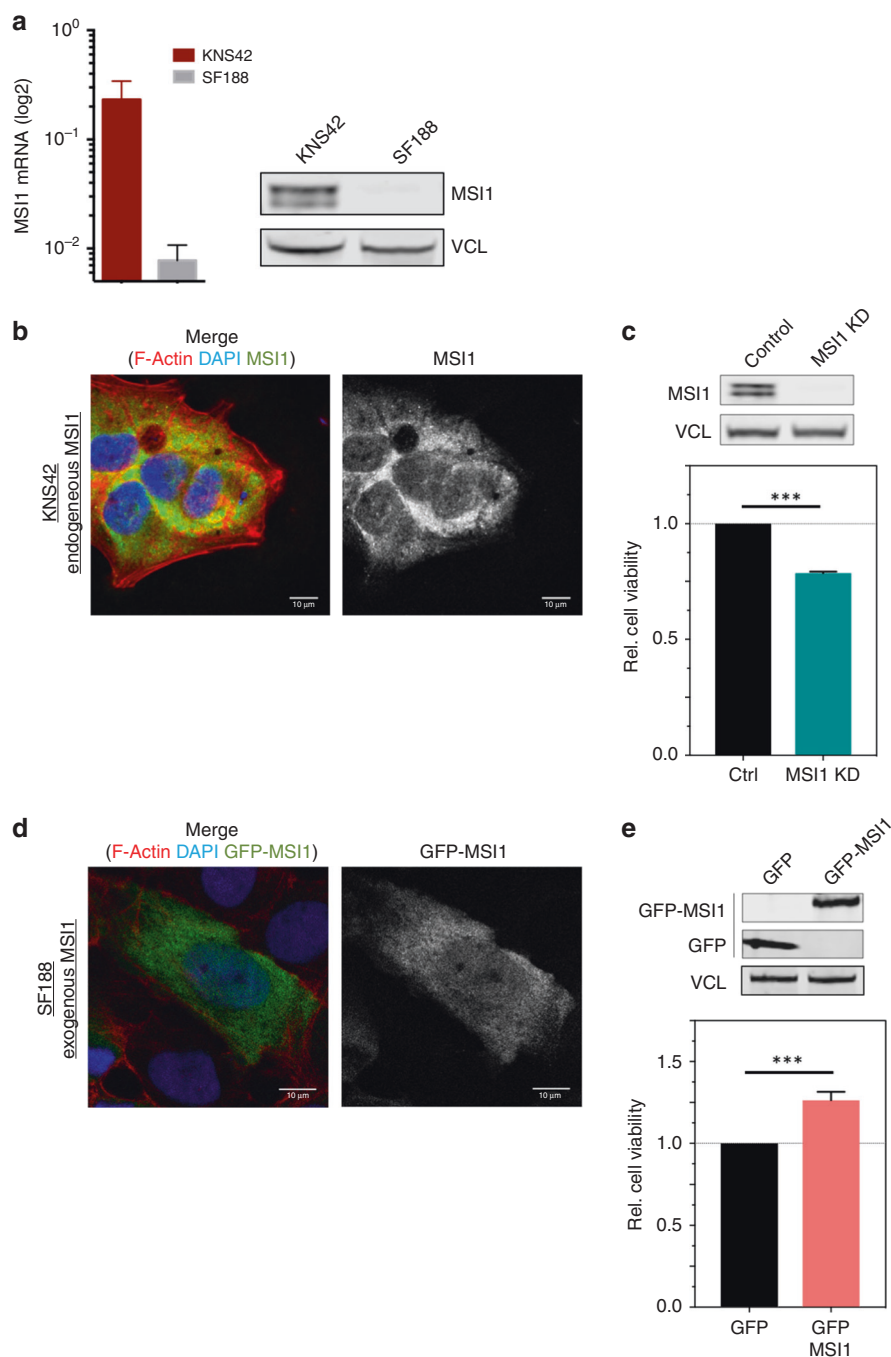


Fig. 2 **a** Expression of MSI1 in pediatric glioblastoma cell lines. KNS42 cells express higher MSI1 (RNA and protein level) compared to SF188 cells. MSI1 mRNA was normalized to GAPDH and VCL. **b** Immunofluorescence of endogenous MSI1 expression in KNS42 cells. MSI1 protein is prominently located in the cytoplasm with a lower concentration observed in the nucleus. Size bar, 10 μ m. **c** Verification of MSI1 KD at the protein level. VCL served as a loading control. Cell viability in MSI1 KD cells was decreased down to 80% after 72 h. **d** Immunofluorescence of GFP-MSI1 expression in SF188 cells. MSI1 protein is prominently located in the cytoplasm but is also expressed in the nucleus. Size bar, 10 μ m. **e** Verification of GFP-MSI1 overexpression in SF188 cells at the protein level. Cell viability of GFP-MSI1-expressing cells is 25% higher compared to GFP-expressing cells. Statistical significance was determined by Student's *t* test; ****P* < 0.001.

also expressed in the nucleus (Fig. 2b). MSI1 KD was verified at the protein (Fig. 2c) and RNA level (data not shown) and significantly decreased cell viability (Fig. 2c). SF188 cells were used for stable MSI1 overexpression. Comparable to endogenous MSI1, exogenous MSI1 localizes predominantly not only within the cytoplasm but also within the nucleus (Fig. 2d). MSI1 overexpression was verified at the protein level and it significantly increased the number of viable cells (Fig. 2e). In conclusion, these findings

indicate that MSI1 promotes the growth of tumor cells derived from pediatric GBM patients.

KNS42 cell viability and endogenous MSI1 expression upon drug treatment

The current standard therapy of GBM consists of TMZ as single-agent chemotherapy. Recent studies show that a combination of different chemotherapeutics can increase their cytotoxic effect.

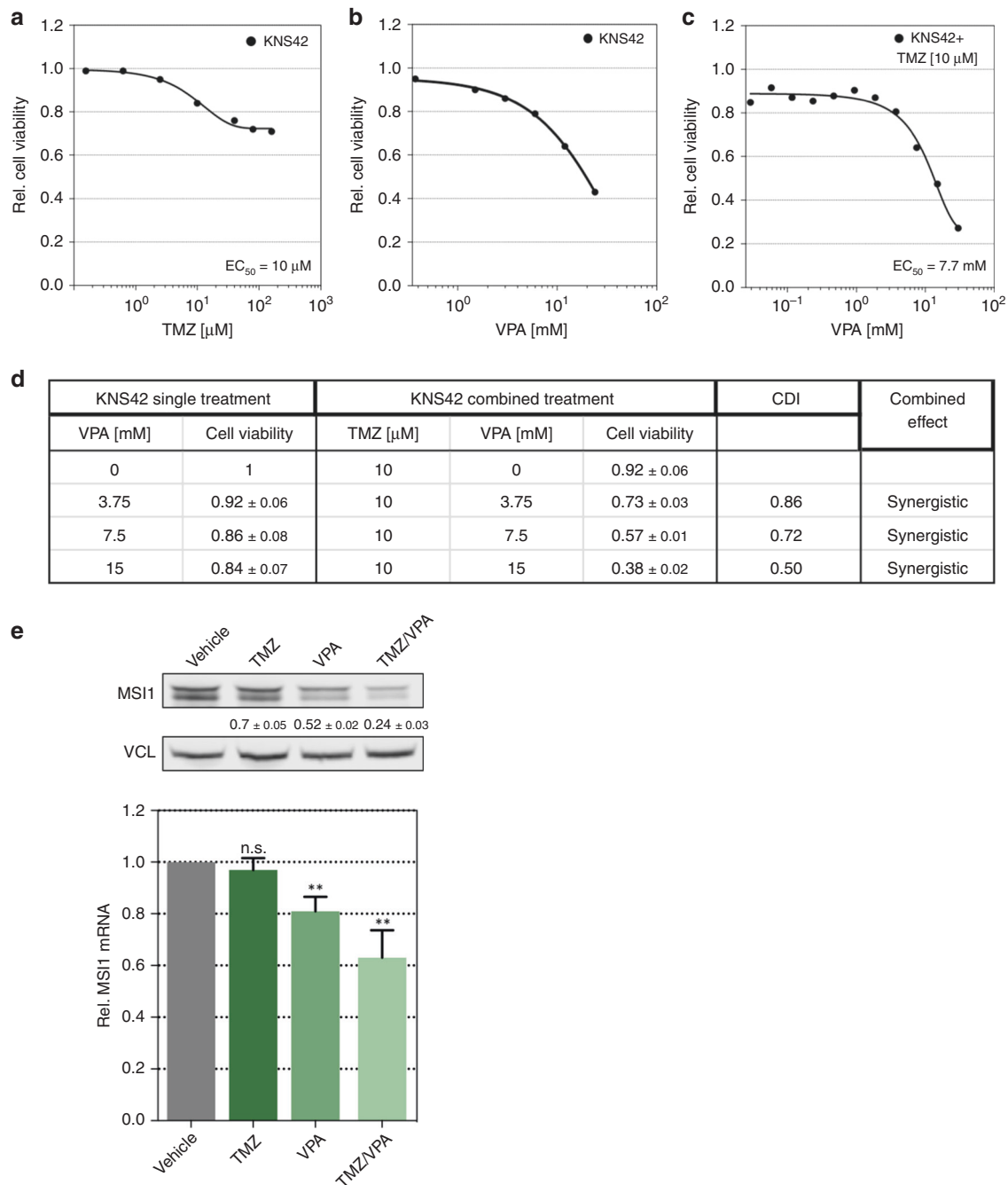


Fig. 3 **a** Cell viability of KNS42 upon TMZ treatment. $EC_{50} = 10 \mu\text{M}$. **b** Cell viability of KNS42 upon VPA treatment. **c** Cell viability of KNS42 cells upon combined treatment with TMZ and VPA. Therefore, KNS42 cells were treated with a stable TMZ concentration ($10 \mu\text{M}$) and variable VPA doses. Resulting EC_{50} for VPA due to this treatment condition is 7.7 mM . **d** Combined treatment of TMZ and VPA on KNS42 cells show synergistic effect (CDI < 1, synergistic; CDI = 1, additive; CDI > 1 antagonistic). **e** Effects of MSI1 on the protein and RNA levels after application of TMZ, VPA, and combination of TMZ and VPA to wild-type KNS42 cells. MSI1 mRNA expression was normalized to GAPDH, VCL, and vehicle. Statistical significance was determined by Student's *t* test; ** $P < 0.001$.

MSI1 was proposed to promote the survival of GBM-CSC and, as demonstrated here, promotes the viability of pediatric GBM-derived cancer cells. Thus a reduction of MSI1 expression could serve as a marker of therapy efficacy and reduced risk of recurrence.

The effect of TMZ, VPA, and the combination of both chemotherapeutics was evaluated in KNS42 cells (Fig. 3). For studies on MSI1 expression upon drug treatment in KNS42, EC_{50} (effective concentration) concentrations for both drugs were

determined. These analyses revealed that KNS42 cells show a higher resistance to both drugs. Even at $300 \mu\text{M}$, TMZ treatment resulted in <40% reduction of cell viability and cells apparently tolerated these high doses of the drug. The determined EC_{50} value was $10 \mu\text{M}$ for TMZ (Fig. 3a). VPA was used at concentrations up to 30 mM without achieving a plateau for the cell viability curve as for TMZ treatment. This suggested that the cells did not respond to the treatment. Therefore, a precise EC_{50} value could not be determined. Comparable studies and the applied concentrations

of both drugs reported in GBM treatment were inspected.^{11,12} From these studies, we decided to use 5 mM VPA in further in vitro experiments with the KNS42 cells. VPA as single drug treatment in wild-type KNS42 cells showed a higher effect on the cell viability than TMZ alone (Fig. 3b). For combined drug treatment, a stable concentration of 10 μ M TMZ (EC₅₀ value) was applied to KNS42 cells, while VPA concentration was variable. The combined drug treatment led to reduced cell viability compared to both single drug treatments and resulted in an EC₅₀ of 7.7 mM for VPA (Fig. 3c). To evaluate the nature of interaction of both drugs in KNS42 cells, the CDI was determined (Fig. 3d). Treatment with the combination of both drugs indicates a synergistic effect (CDI < 0.7 significantly synergistic; CDI < 1, synergistic; CDI = 1, additive; CDI > 1 antagonistic). Aiming to evaluate MSI1 expression as a therapeutic marker, KNS42 cells were treated with TMZ (10 μ M), VPA (5 mM), and combination of both drugs (TMZ (10 μ M), VPA (5 mM)) (Fig. 3e). TMZ showed no significant effect on MSI1 expression at the used concentration. VPA treatment showed a significant reduction of MSI1 at the protein and mRNA level. This downregulation was further pronounced by the combined treatment with TMZ and VPA. Thus, in agreement with the enhanced efficacy, the combined treatment with TMZ and VPA resulted in a significantly pronounced downregulation of MSI1 expression.

Effects of MSI1 on cell viability upon drug treatment

The poor prognosis and substantial therapy resistance of GBMs is thought to rely on the resistance of GBM-CSCs largely. In view of MSI1's expression in neural stem cells and upregulated synthesis in GBMs, it remained to be determined whether the protein contributes to therapy resistance. This was tested by depleting MSI1 in KNS42 cells to mimic a putative therapeutic benefit by the pharmacological targeting of the protein in the future. In addition, MSI1 overexpression in SF188 was evaluated to test whether elevated MSI1 abundance confers higher therapy resistance. In both single drug treatments, no effect on cell viability was observed upon MSI1 KD (Supplementary Fig. S2A). For combined drug treatment, control cells and MSI1 KD cells were supplied with 10 μ M TMZ and varying VPA concentrations of 1.8, 3.75, and 7.5 mM. Since MSI1 KD cells show a proliferation effect (Fig. 2c), normalization was performed to the vehicle of control cells and MSI1 KD cells, respectively. For all three conditions, control cells showed significantly higher cell viability compared to MSI1 KD cells (Fig. 4a). For a VPA dose of 3.75 mM in combination with the 10 μ M of TMZ, a difference of 27% in cell viability between control cells and MSI1 KD was the highest observed impact of the combined drug treatment.

In SF188 cells stably expressing GFP-MSI1, EC₅₀ concentrations of 80 μ M for TMZ and 4 mM for VPA were determined (Supplementary Fig. S2B). Thus, for combined drug treatment experiments, control cells, as well as GFP-MSI1-expressing cells, were exposed to 80 μ M TMZ and varying doses of VPA (1.8, 3.75, and 7.5 mM). Since MSI1-overexpressing cells show a proliferation effect as well (Fig. 2c), normalization was performed to GFP- and GFP-MSI1 cells treated with vehicle. For all three conditions, GFP-MSI1 cells showed significantly higher cell viability compared to GFP cells (Fig. 4b). Here the highest effect on cell viability (22% difference between GFP vs. GFP-MSI1) was achieved at the lowest applied VPA concentration of 1.8 mM and 80 μ M of TMZ.

These findings provide strong evidence that MSI1-expressing cells show a higher resistance to a combination of TMZ and VPA drug treatment.

DISCUSSION

RBPs are known to be re-expressed in several cancer types.³⁴ Owing to their versatile roles in posttranscriptional gene regulation, they influence tumorigenesis in several ways.³⁵ MSI1 has

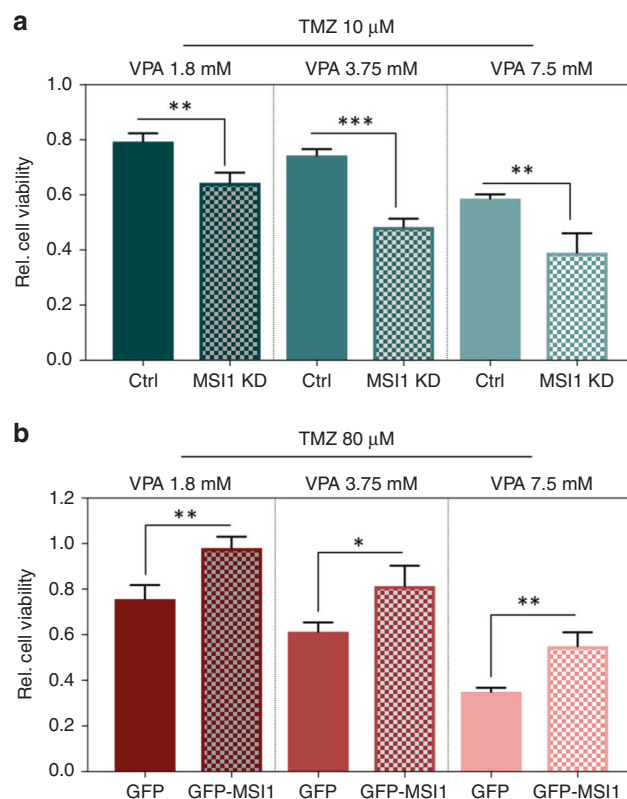


Fig. 4 **a** Impact of combined TMZ and VPA drug treatment on cell viability upon transient MSI1 KD. For all three conditions, MSI1 KD shows a significantly reduced cell viability compared to the control cells. **b** Impact of combined TMZ and VPA drug treatment on cell viability upon MSI1 overexpression. For all three conditions, GFP-MSI1-overexpressing cells show a significant higher cell viability compared to the GFP-overexpressing cells. Statistical significance was determined by Student's *t* test; *****P* < 0.001, ****P* < 0.01, **P* < 0.05.

been reported as an oncogenic RBP and re-expressed in multiple tumors,³⁶ which is described to be expressed in adult GBM. Its expression as well as its role in pediatric GBM remained to be investigated. Only one study described a weak MSI1 expression in a pediatric tumor, a moderate abundance in undifferentiated neurospheres, and claimed the absence of MSI1 in differentiated neurospheres in one pediatric glioblastoma tumor sample.¹⁵ The study did not include IHC staining, and MSI1 expression was determined via semiquantitative RT-PCR. Nakano et al. investigated MSI1 expression via IHC in the primary pediatric brain tumors medulloblastoma (six samples) and ependymoma (five samples) but did not include pediatric glioblastoma.²¹ We report enhanced MSI1 expression in pediatric GBM at the mRNA and protein level and demonstrate that MSI1 expression is associated with increasing tumor grade. The findings suggest MSI1 as a marker of pediatric GBM and that MSI1 expression indicates poor prognosis.

Two studies reported the involvement of MSI1 in promoting therapy resistance in adult GBM. In GBM-derived cells treated with cisplatin, it was proposed that MSI1 inhibits drug-induced apoptosis via an AKT/interleukin-6 regulatory pathway.³⁷ De Araujo et al. described the impact of MSI1 on DNA damage repair and radio-resistance by controlling DNA-protein-kinase catalytic subunit.³⁸ Both studies used adult GBM cell lines. Here we investigated MSI1 in pediatric GBM cells. The two cell lines used, KNS42 and SF188, show a high drug resistance, which was already documented by other studies.^{32,33} KNS42 cells are TMZ resistant,

although their MGMT promoter is hypermethylated suggesting MGMT-independent mechanisms of TMZ resistance. SF188 cells are TMZ resistant, which is potentially mediated by MGMT expression.³³ In both cell lines, MSI1 expression was clearly associated with enhanced cell survival.

Nifterik et al. investigated the interaction of VPA on TMZ and radiation. They show that a combination of VPA and TMZ increases radiation response compared to TMZ and radiation only (independent of MGMT protein). They assume that the synergistic effect of VPA and TMZ resulted from a loosening of chromatin structure through VPA and subsequent increase of DNA methylation as well as damage through TMZ.¹²

Our studies reveal that MSI1 is a bona fide marker of the combined treatment of TMZ and VPA. Although MSI1 expression remained unaffected by TMZ, the combined treatment led to a significantly pronounced downregulation of MSI1 when compared to VPA treatment alone. This supports the conclusion that combined treatments are generally more effective than single agents used alone.

Hosein et al. showed that VPA is effective as a monotherapy in primary GBM lines (at clinically achievable concentrations).¹¹ There are different clinical studies that investigate a combination of VPA and TMZ with radiation or VPA treatment with radiation alone.^{13,39}

Our work supports these findings, because, in KNS42 cells, VPA as monotherapy had already a significant effect on MSI1 expression and cell viability.

In addition to revealing MSI1 as a therapeutic marker, we evaluated its potential in modulating therapy resistance. Consistent with its proposed expression and role in GBM-CSCs,¹⁵ we observed that MSI1 promotes therapy resistance to combined TMZ/VPA treatment. Thus targeting of MSI1 is expected to impair therapy resistance and improve the efficacy of combined TMZ/VPA treatment. Therapeutic targeting of MSI1 could be achieved with an MSI1 inhibitor. Yi et al. described that Luteolin impairs MSI1-mRNA association and disrupts MSI1-directed cancer phenotypes in glioma cells.⁴⁰ Although it has to be clarified whether Luteolin promotes the efficacy of combined TMZ/VPA treatment in pediatric GBM via MSI1 inhibition, the presented findings strongly suggest that future experiments need to investigate MSI1 chemical inhibition in the context. This study suggests that MSI1 could be a promising marker and potential drug target to improve therapeutic efficacy in pediatric GBM treatment.

ACKNOWLEDGEMENTS

We thank Chris Jones for kindly providing the cell line SF188. This work was supported by the Verein zur Förderung krebskranker Kinder Halle (Saale) e. V. (to C.D.K.) and the Deutsche Forschungsgemeinschaft RTG1591 (to S.H.) grants.

AUTHOR CONTRIBUTIONS

Conception and design: R.P., C.D.K. Administrative support: All authors. Provision of study materials: All authors. Collection and assembly of data: R.P., G.G. Data analysis and interpretation: All authors. Manuscript writing: R.P., C.D.K. Final approval of manuscript: All authors.

ADDITIONAL INFORMATION

The online version of this article (<https://doi.org/10.1038/s41390-019-0628-9>) contains supplementary material, which is available to authorized users.

Competing interests: The authors declare no competing interests.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

REFERENCES

- Johnson, D. R. & O'Neill, B. P. Glioblastoma survival in the United States before and during the temozolomide era. *J. Neurooncol.* **107**, 359–364 (2012).
- Wen, P. Y. & Kesari, S. Malignant gliomas in adults. *N. Engl. J. Med.* **359**, 492–507 (2008).
- Wolff, J. E. et al. Measuring performance status in pediatric patients with brain tumors—experience of the HIT-GBM-C protocol. *Pediatr. Blood Cancer* **55**, 520–524 (2010).
- Cohen, K. J. et al. Temozolomide in the treatment of high-grade gliomas in children: a report from the Children's Oncology Group. *Neuro Oncol.* **13**, 317–323 (2011).
- Das, K. K. et al. Pediatric glioblastoma: clinico-radiological profile and factors affecting the outcome. *Childs Nerv. Syst.* **28**, 2055–2062 (2012).
- Wolff, J. E. et al. Subpopulations of malignant gliomas in pediatric patients: analysis of the HIT-GBM database. *J. Neurooncol.* **87**, 155–164 (2008).
- Suri, V. et al. Pediatric glioblastomas: a histopathological and molecular genetic study. *Neuro Oncol.* **11**, 274–280 (2009).
- Kramm, C. M. et al. Improved survival after gross total resection of malignant gliomas in pediatric patients from the HIT-GBM studies. *Anticancer Res.* **26**(5B), 3773–3779 (2006).
- Mottamal, M. et al. Histone deacetylase inhibitors in clinical studies as templates for new anticancer agents. *Molecules* **20**, 3898–3941 (2015).
- Barker, C. A. et al. Valproic acid use during radiation therapy for glioblastoma associated with improved survival. *Int. J. Radiat. Oncol. Biol. Phys.* **86**, 504–509 (2013).
- Hosein, A. N. et al. The effect of valproic acid in combination with irradiation and temozolomide on primary human glioblastoma cells. *J. Neurooncol.* **122**, 263–271 (2015).
- Van Nifterik, K. A. et al. Valproic acid sensitizes human glioma cells for temozolomide and gamma-radiation. *J. Neurooncol.* **107**, 61–67 (2012).
- Weller, M. et al. Prolonged survival with valproic acid use in the EORTC/NCIC temozolomide trial for glioblastoma. *Neurology* **77**, 1156–1164 (2011).
- Singh, S. K. et al. Identification of a cancer stem cell in human brain tumors. *Cancer Res.* **63**, 5821–5828 (2003).
- Hemmati, H. D. et al. Cancerous stem cells can arise from pediatric brain tumors. *Proc. Natl Acad. Sci. USA* **100**, 15178–15183 (2003).
- Kaneko, Y. et al. Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. *Dev. Neurosci.* **22**, 139–153 (2000).
- Sakakibara, S. et al. Mouse-Musashi-1, a neural RNA-binding protein highly enriched in the mammalian CNS stem cell. *Dev. Biol.* **176**, 230–242 (1996).
- Wang, X. Y. et al. Musashi1 regulates breast tumor cell proliferation and is a prognostic indicator of poor survival. *Mol. Cancer* **9**, 221 (2010).
- Wang, X. Y. et al. Musashi1 as a potential therapeutic target and diagnostic marker for lung cancer. *Oncotarget* **4**, 739–750 (2013).
- Kanemura, Y. et al. Musashi1, an evolutionarily conserved neural RNA-binding protein, is a versatile marker of human glioma cells in determining their cellular origin, malignancy, and proliferative activity. *Differentiation* **68**, 141–152 (2001).
- Nakano, A. et al. Expression of the neural RNA-binding protein Musashi1 in pediatric brain tumors. *Pediatr. Neurosurg.* **43**, 279–284 (2007).
- Dahlrot, R. H. et al. Prognostic value of Musashi-1 in gliomas. *J. Neurooncol.* **115**, 453–461 (2013).
- Imai, T. et al. The neural RNA-binding protein Musashi1 translationally regulates mammalian numb gene expression by interacting with its mRNA. *Mol. Cell. Biol.* **21**, 3888–3900 (2001).
- Battelli, C. et al. The RNA-binding protein Musashi-1 regulates neural development through the translational repression of p21WAF-1. *Mol. Cell. Neurosci.* **31**, 85–96 (2006).
- Cambuli, F. M. et al. A mouse model of targeted musashi1 expression in whole intestinal epithelium suggests regulatory roles in cell cycle and stemness. *Stem Cells* **33**, 3621–3634 (2015).
- Glazer, R. I., Vo, D. T. & Penalva, L. O. Musashi1: an RBP with versatile functions in normal and cancer stem cells. *Front. Biosci. (Landmark Ed.)* **17**, 54–64 (2012).
- Siddall, N. A. et al. The RNA-binding protein Musashi is required intrinsically to maintain stem cell identity. *Proc. Natl Acad. Sci. USA* **103**, 8402–8407 (2006).
- Jones, C., Perryman, L. & Hargrave, D. Paediatric and adult malignant glioma: close relatives or distant cousins? *Nat. Rev. Clin. Oncol.* **9**, 400–413 (2012).
- Jones, T. A. et al. Molecular analysis of pediatric brain tumors identifies microRNAs in pilocytic astrocytomas that target the MAPK and NF-kappaB pathways. *Acta Neuropathol. Commun.* **3**, 86 (2015).
- Zhao, Y. et al. Cytotoxicity enhancement in MDA-MB-231 cells by the combination treatment of tetrahydropalmatine and berberine derived from *Corydalis yanhusuo* W. T. Wang. *J. Interact. Ethnopharmacol.* **3**, 68–72 (2014).
- Takeshita, I. et al. Characteristics of an established human glioma cell line, KNS-42. *Neurol. Med. Chir. (Tokyo)* **27**, 581–587 (1987).

32. Bjerke, L. et al. Histone H3.3. mutations drive pediatric glioblastoma through upregulation of MYCN. *Cancer Discov.* **3**, 512–519 (2013).
33. Gaspar, N. et al. MGMT-independent temozolomide resistance in pediatric glioblastoma cells associated with a PI3-kinase-mediated HOX/stem cell gene signature. *Cancer Res.* **70**, 9243–9252 (2010).
34. Kechavarzi, B. & Janga, S. C. Dissecting the expression landscape of RNA-binding proteins in human cancers. *Genome Biol.* **15**, R14 (2014).
35. Pereira, B., Billaud, M. & Almeida, R. RNA-binding proteins in cancer: old players and new actors. *Trends Cancer* **3**, 506–528 (2017).
36. Kudinov, A. E. et al. Musashi RNA-binding proteins as cancer drivers and novel therapeutic targets. *Clin. Cancer Res.* **23**, 2143–2153 (2017).
37. Chen, H. Y. et al. Musashi-1 regulates AKT-derived IL-6 autocrinal/paracrine malignancy and chemoresistance in glioblastoma. *Oncotarget* **7**, 42485–42501 (2016).
38. de Araujo, P. R. et al. Musashi1 impacts radio-resistance in glioblastoma by controlling DNA-protein kinase catalytic subunit. *Am. J. Pathol.* **186**, 2271–2278 (2016).
39. Felix, F. H. et al. Potential role for valproate in the treatment of high-risk brain tumors of childhood—results from a retrospective observational cohort study. *Pediatr. Hematol. Oncol.* **28**, 556–570 (2011).
40. Yi, C. et al. Luteolin inhibits Musashi1 binding to RNA and disrupts cancer phenotypes in glioblastoma cells. *RNA Biol.* **15**, 1420–1432 (2018).