Dec1 expression predicts prognosis and the response to temozolomide chemotherapy in patients with glioma

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Abstract. Differentiated embryo chondrocyte expressed gene 1 (Dec1), a crucial cell differentiation mediator and apoptosis inhibitor, is abundantly expressed in various types of human cancer and is associated with malignant tumor progression. As poor differentiation and low apoptosis are closely associated with poor survival rates and a poor response to radio/chemotherapy in patients with cancer, the prognostic value of Dec1 expression was examined in the present study and its correlation with response to temozolomide (TMZ) chemotherapy was analyzed in patients with glioma. Dec1 expression was analyzed by immunohistochemistry in 157 samples of newly diagnosed glioma and 63 recurrent glioblastoma cases that relapsed during TMZ chemotherapy. Correlations with clinical variables, prognosis and the response to TMZ chemotherapy were analyzed in the newly diagnosed gliomas. Dec1 expression was also compared with the apoptosis index determined by TdT-mediated dUTP nick ending-labeling assay in recurrent glioblastomas. The antiglioma effect of TMZ in nude mice xenografts with Dec1 expression was examined *in vivo*. High expression of Dec1, which was significantly associated with high pathological tumor grade and poor response to TMZ chemotherapy, was demonstrated to be an unfavorable independent prognostic factor and predicted poor survival in patients with newly diagnosed glioma. In patients with recurrent glioblastoma, there was a negative correlation between Dec1 expression and the apoptotic index. In nude mice treated with TMZ, Dec1 overexpression potentiated proliferation, but attenuated TMZ-induced apoptosis. In conclusion, Dec1 is a prognostic factor for the clinical outcome and a predictive factor for the response to TMZ chemotherapy in patients with glioma.

Introduction

Gliomas are the most common primary malignant tumor in the central nervous system and account for >70% of all brain tumors (1). The 2007 World Health Organization (WHO) classification criteria distinguishes gliomas into four pathological grades (WHO I to IV) (2). Among them, glioblastoma multiforme (GBM, WHO IV) accounts for approximately half of all gliomas and carries the worst prognosis (3).

Despite the rapid development of multimodal therapeutic strategies over recent years, including surgical resection, local radiotherapy and systemic chemotherapy, the prognosis of gliomas remains dismal. For example, temozolomide (TMZ) is a new generation alkylating agent that has been demonstrated to be one of the most effective chemotherapeutic agents for treating glioma. However, TMZ only extends the overall survival from 12 to 15 months (4), due to intrinsic or acquired chemoresistance to alkylating agents (5). However, the majority of patients with GBM are currently treated with a uniform standardized regimen, regardless of the individual molecular characteristics of each tumor, which may be important for the clinical outcome. Indeed, recent large-scale genomic analyses have demonstrated that remarkable molecular heterogeneity exists in GBMs (6). The molecular information in each tumor tissue is extremely valuable for establishing individualized treatment for gliomas. Thus, the identification of a key molecule, not only for clarifying glioma mechanisms, but also to predict prognosis and the response to treatment in human gliomas has become an increasingly urgent issue.

Differentiated embryo chondrocyte expressed gene 1 (Dec1), also termed split and hairy related protein 2, basic helix-loop-helix binding protein 2 (BHLHB2) or stimulated with retinoic acid 13 (Stra13), is a member of the basic helix-loop-helix (bHLH) family of transcriptional factors. Dec1 has critical functions in various cellular events, including cell differentiation and proliferation (7-9), cell-cycle arrest (10,11),

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inhibition of apoptosis (12-15), immunoregulation (16-18), cellular metabolism (19-21) and circadian rhythms (22-24). Accumulating evidence has identified overexpression of Dec1 in a variety of human tumors and highlighted its contribution to oncogenesis. For example, Stra13 expression was significantly increased during the progression from normal to *in situ* and invasive breast carcinoma, and positively correlated with the tumor grade (25). Zheng *et al* (26) identified that Dec1 expression was significantly increased during the tumor progression from well-differentiated to moderately-differentiated and poorly-differentiated gastric cancer tissues (26). Additionally, Stra13 was reported to be abundantly expressed in colon cancers, but not in adjacent normal tissues (12). In renal carcinoma, Stra13 acted as a target of von Hippel-Lindau tumor suppressor protein (pVHL), and its downregulated expression by pVHL indicated its effect in renal carcinogenesis (27). There was a close association between Dec1 overexpression and hypoxia inducible factor 1-α, and carbonic anhydrase-9 in non-small cell lung cancer (28). Together this evidence suggests crucial roles for Dec1 in malignancy progression. However, the profile of Dec1 expression, or its prognostic and therapeutic significance in human gliomas has not been systematically analyzed.

The present study investigated Dec1 expression by immunohistochemistry (IHC) and further analyzed the correlations between Dec1 expression and clinical variables, prognosis, response to TMZ chemotherapy and TMZ-induced apoptosis in human glioma specimens. Furthermore, the contribution of Dec1 overexpression to the antiglioma effect of TMZ *in vivo* was evaluated.

Materials and methods

Patients and specimens. A total of 157 resected glioma specimens obtained from the Department of Neurosurgery, Xi'Jing Hospital, Fourth Military Medical University (Xi'an, China) from 2007 to 2010, were analyzed in this study. Slides were carefully evaluated and categorized by histological subtype and pathological grade (according to the WHO classifications in 2007) by two pathologists as follows: 45 cases of low-grade glioma (LGG), including grade I (10 pilocytic astrocytomas and 4 myxopapillary ependymomas) and grade II (16 diffuse astrocytomas, 9 oligoastrocytomas and 6 oligodendrogliomas); 112 cases of high-grade glioma (HGG), including grade III (7 anaplastic astrocytomas, 8 anaplastic oligodendrogliomas and 7 anaplastic oligoastrocytomas) and grade IV (90 GBMs). All patients were histologically diagnosed with gliomas, which were disassociated tissues during the initial surgery and did not receive radio- or chemotherapy prior to surgery. Among the newly diagnosed gliomas, 86 patients with HGG were treated with oral TMZ following surgery. The clinical information is presented in Table I. Patients were followed-up for 5 years by telephone or questionnaire letters.

Progression-free survival (PFS) was defined as the time from the date of the initial surgery to the first recurrence (confirmed by MRI) or death. Overall survival (OS) was calculated from the date of the initial surgery until death or the last follow-up. TMZ resistance was standardized as PFS of >1 year. Patients who succumbed to diseases not directly associated with glioma or due to unexpected events were excluded from the study. The use of samples in the current study was approved by the Research Ethics Committee of Neurosurgery Department, Xi'Jing Hospital, Fourth Military Medical University (Xi'an, China). Informed consent was obtained from all patients.

IHC and evaluation of staining. IHC assays were performed by the polymer-peroxidase method as follows. Briefly, the 3-μm thick slides were deparaffinized and rehydrated, then the sections were rinsed in distilled water for 1 min. Antigen retrieval was performed by boiling in 0.01 M citrate buffer (pH 6.0) in a pressure cooker for 86 sec. Following cooling, 0.3% hydrogen peroxide was used to block nonspecific sites for 30 min, and sections were then incubated with Triton-X for 20 min. Following a wash with PBS and incubation with goat serum (1:20 dilution; OriGene Technologies, Inc.) at room temperature for 1 h, sections were incubated with rabbit polyclonal anti-Dec1 antibody (1:250 dilution; cat. no. ab90594; Abcam, Cambridge, MA, USA) or rabbit monoclonal Ki-67 antibody (1:500-1,000 dilution; cat. no. ab16667; Abcam) at 4˚C overnight. The specimens were rinsed briefly in PBS and incubated at room temperature first with horseradish peroxidase-conjugated anti-rabbit antibody (cat. no. TA130023; OriGene Technologies, Inc., Beijing, China) for 30 min, and then avidin-biotin peroxidase (OriGene Technologies, Inc.) and 20 min. The slides were stained with diaminobenzidine (OriGene Technologies, Inc.) and water was added to terminate the staining. Slides were counterstained with Meyer's hematoxylin (Baso Diagnostics, Inc., Zhuhai, China).

Dec1 immunostaining was evaluated independently and blindly by two pathologists. The results were compared and re-examined until a consensus was reached for each score. The immunoreactivity score (IRS) of Dec1 was evaluated according to the percentage of positive cells and the staining intensity. IRS was determined by multiplying the two factors: i) Percentage of positive cells (<5%, scored 0; 6-25%, scored 1; 26-50%, scored 2; 51-75%, scored 3; >75%, scored 4); and ii) staining intensity (negative staining, 0; weak staining, 1; moderate staining, 2; strong staining, 3). The proliferative index was calculated by determining the percentage of KI-67-positive cells.

TdT-mediated dUTP nick ending-labeling (TUNEL) assay. To investigate TMZ-induced apoptosis of tumor cells in glioma patients, an additional 63 patients with recurrent GBM were selected from those that had been histologically documented with malignant glioma and suffered a relapse following the postoperative TMZ chemotherapy. Specimens of these recurrent GBM patients were isolated from the second surgery and used for the TUNEL assay with a DeadEnd™ Fluorometric TUNEL system (Promega Corporation, Madison, WI, USA), which was performed according the manufacturer's instructions. Tissues were counterstained with DAPI. Images were captured using a fluorescent microscope (Olympus BX51; Olympus Corporation, Tokyo, Japan) using an RGB filter. To evaluate the percentage of apoptotic cells (apoptotic index) in human glioma specimens, TIFF (RGB) images were transferred into the RGB channel in Adobe Photoshop CS5 software (Adobe System Inc., San Jose, CA, USA). Identical

adjustment of contrast and brightness was performed within for all images presented.

Cell culture, plasmid construction and stable gene transfection. The U87 GBM cell line (American Type Culture Collection, Manassas, VA, USA) was maintained as an adherent monolayer culture in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) in a humidified incubator containing 5% CO_2 at 37°C.

Plasmids containing full-length Dec1 were generated by reverse transcription-polymerase chain reaction and subcloned into the pENTR™3C vector (Invitrogen; Thermo Fisher Scientific, Inc.). Primers for Dec1 were as follows: 5'-ggaagg agttcgaaccatggagcggatccccagcgcgca-3' (forward), and 5'-tgc ggccgcactcgagctagtctttggtttctaagtttaaag-3' (reverse). Dec1 pLenti-6.3 expression vectors were obtained using the Gateway™ technology (Invitrogen; Thermo Fisher Scientific, Inc.). The pLenti-6.3-cherry vector was used as a negative control. The recombinant expression vector and control vector were packaged in 293T cells (American Type Culture Collection) with the plasmids psPAX2 and pMD2.G (Invitrogen; Thermo Fisher Scientific, Inc.) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. U87 stable cell lines overexpressing Dec1 and cherry were selected with 8 mg/ml blasticidin (Invitrogen; Thermo Fisher Scientific, Inc.) and maintained in medium containing 4 mg/ml blasticidin.

Tumor generation and drug treatment in a mouse model. Nude female BALB/c mice (18-20 g) used for all *in vivo* studies were obtained from the Laboratory Animal Center of the Fourth Military Medical University (Xi'an, China), and experiments were performed in accordance with guidelines of the Fourth Military Medical University Animal Care and Use Committee. The mice were maintained under pathogen-free conditions in an air flow cabinet at 23°C, with 12 h/12 h day/night cycle, and water and food *ad libitum*. When mice were six weeks old, tumor generation was initiated by subcutaneous injection of $1x10^6$ stable U87 cells overexpressing Dec1 or cherry (n=5 per group) in each flank of a nude mouse. Palpable tumors were generated ~5-7 days postinoculation, and TMZ was added to the diet at 50 mg/kg for 5 consecutive days. The tumor size was measured with a slide caliper and the tumor volume was recorded using the following formula: Volume = a x b^2/ 2 (a = the larger dimension; b = the smaller dimension). Following 30 days of inoculation, the mice were sacrificed by decapitation. Tumors were removed, weighed and paraffin-embedded. Pathological sections (3-μm) were prepared for TUNEL and Ki-67 immunostaining analysis.

Statistical analysis. All statistical analyses were performed using the SPSS 13.0 package (SPSS Inc., Chicago, IL, USA). Values are presented as the mean ± standard deviation. Relationships between Dec1 expression levels and clinical variables were analyzed by the Mann-Whitney test or the Kruskal-Wallis test. The Kaplan-Meier method was used to plot survival curves and the prognostic differences in groups with different Dec1 expression were tested by log-rank analysis. Hazard ratios

(HR) of different prognostic factors were evaluated by Cox multivariate analysis. Differences of Dec1 IRS and the apoptotic index (AI) between the low Dec1 expression group and the high Dec1 expression group or between the TMZ-resistant group and the TMZ-sensitive group were compared using the Student's *t*-test. The nonparametric Spearman's rank correlation coefficient was performed to assess the correlation between AI and Dec1 IRS. P<0.05 was considered to indicate a statistically significant difference.

Results

Correlation between Dec1 expression with clinical variables in glioma patients. Correlations between Dec1 expression levels with clinical variables were analyzed in 157 cases of newly diagnosed gliomas (Table I). Dec1 expression levels were evaluated by IHC, and patients were stratified as low Dec1 expression (IRS <8) or high Dec1 expression (IRS ≥8), according to the mean IRS of Dec1 (6.44±3.73). Among the total 157 cases, 82 patients exhibited low Dec1 expression, whereas 75 cases exhibited high Dec1 expression. Representative staining images are presented in Fig. 1. In LGG, a lower number of Dec1 positive cells was observed exhibiting a weak staining intensity, whereas in HGG, nearly all tumor cells were strongly stained. Dec1 expression increased with the progression of pathologic grades (P=0.023). In addition, Dec1 expression was also significantly correlated with the Karnofsky performance status (KPS; P=0.016). No statistically significance was detected between the expression levels of Dec1 and age, gender, tumor location, tumor size or surgical resection.

High Dec1 expression is associated with poor prognosis in patients with glioma. The Kaplan-Meier analyses for PFS and OS were performed to evaluate the prognostic differences between Dec1 expression groups in patients with glioma. The postoperative mean PFS and OS of all eligible patients with glioma were 18.1 and 23.1 months, respectively. The postoperative mean PFS and OS of patients with low Dec1 expression were 22 and 27 months, respectively, whereas those of patients with high Dec1 expression were 13.8 and 18.8 months, respectively. Patients with low Dec1 expression exhibited longer PFS and OS compared to those with high Dec1 expression (log-rank test, P=0.0001 for both; Fig. 2A). In addition, the considered variables, including age, KPS and WHO grade, also exhibited statistically significance at log-rank test in all cases of glioma for PFS and OS (data not shown). However, gender, tumor location, tumor size or surgery resection had no prognostic value for PFS and OS in the present cohort (data not shown).

Kaplan-Meier analyses were also performed in different subgroups stratified according to the malignant degree. As high pathological grade of gliomas is considered a marker of high risk, the patients were classified into two subgroups: LGG and HGG. In LGG, the postoperative mean PFS and OS were 34.5 and 42 months, respectively. The postoperative mean PFS and OS of patients with low Dec1 expression were 39.2 and 45.3 months, respectively, whereas those of patients with high Dec1 expression were 26.8 and 36.6 months, respectively. High Dec1 expression was significantly correlated with shorter PFS and OS in LGG (log-rank test, P=0.0085 and P=0.0135, respectively; Fig. 2B). Similarly in HGG, the postoperative

Table I. Correlations between Dec1 expression and the clinical variables in glioma patients.

Variable	Description	n	Dec1-low expression	Dec1-high expression	P-value
Age (years)	<50	76	36	40	0.239[a]
	≥50	81	46	35	
Gender	Male	75	45	30	0.063[a]
	Female	82	37	45	
KPS	<80	56	22	34	0.016[a]
	≥80	101	60	41	
Grade	I	14	12	2	0.023[b]
	II	31	16	15	
	III	22	14	8	
	IV	90	40	50	
Tumor location	Supratentorial	94	50	44	0.769[a]
	Infratentorial	63	32	31	
Tumor size	<3 cm	78	38	40	0.383[a]
	≥3 cm	79	44	35	
Surgery resection[c]	Total	91	48	43	0.879[a]
	Subtotal	66	34	32	
TMZ chemotherapy[d]	Resistant	46	16	30	0.020[a]
	Sensitive	40	24	16	

P-values were estimated using the [a]Mann-Whitney test and the [b]Kruskal-Wallis test. [c]Surgery resection was evaluated by postoperative MRI within 72 h after operation. Complete resolution on the postoperative MRI (T1 low-intensity lesion for grade I, II and III; T1 gadolinium-enhanced lesion for grade IV) was considered as total resection. [d]TMZ chemotherapy; 86 cases of high-grade glioma patients were treated with oral TMZ after surgery. TMZ resistance was standardized as the progression-free survival of <1 year. Dec1, differentiated embryo chondrocyte expressed gene 1; MRI, magnetic resonance imaging; TMZ, temozolomide.
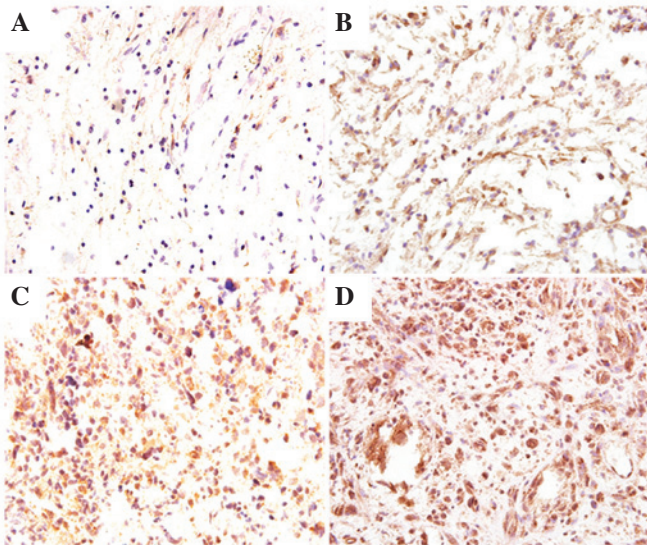

Figure 1. Representative images of differentiated embryo chondrocyte expressed gene 1 immunostaining in human glioma tissues. (A) Pilocytic astrocytoma, WHO I; (B) astrocytoma, WHO II; (C) anaplastic astrocytoma, WHO III; and (D) glioblastoma, WHO IV. WHO, World Health Organization grade.

mean PFS and OS were 11.5 and 15.5 months, respectively. The postoperative mean PFS and OS of patients with low Dec1 expression were 13.1 and 17.6 months, respectively, while those of patients with high Dec1 expression were 10 and 13.6 months, respectively. Dec1 expression in HGG also proved to be a prognostic factor for both PFS and OS (log-rank test, P=0.0175 and P=0.0070, respectively; Fig. 2C). In addition, age and KPS had prognostic value for PFS and OS in LGG, while radiotherapy postsurgery did not contribute to long-term benefits in the current cohort (data not shown). In HGG, age, KPS and standard treatment also significantly predicted PFS and OS (data not shown).

Using the Cox proportional hazards model, univariate and multivariate analyses were performed in patients with glioma (Tables II and III). In LGG, low Dec1 expression was identified as a favorable prognostic indicator for PFS (P=0.009; HR, 0.226) and OS (P=0.010; HR, 0.246). Similarly, age and KPS were also identified as independent prognostic factors for PFS and OS. However, for PFS and OS, no statistically significance was found among other variables, including gender, tumor location, tumor size, surgery resection or radiotherapy (Table II). The Cox proportional hazards model in HGG also revealed low Dec1 expression as a favorable predictor of PFS (P=0.015; HR, 0.581) and OS (P=0.009; HR, 0.569). In addition, KPS and standard treatment were also demonstrated to be independent prognostic factors for PFS and OS in HGG, whereas other variables, including age, gender, tumor location, tumor size and surgery resection were not prognostic factors (Table III).
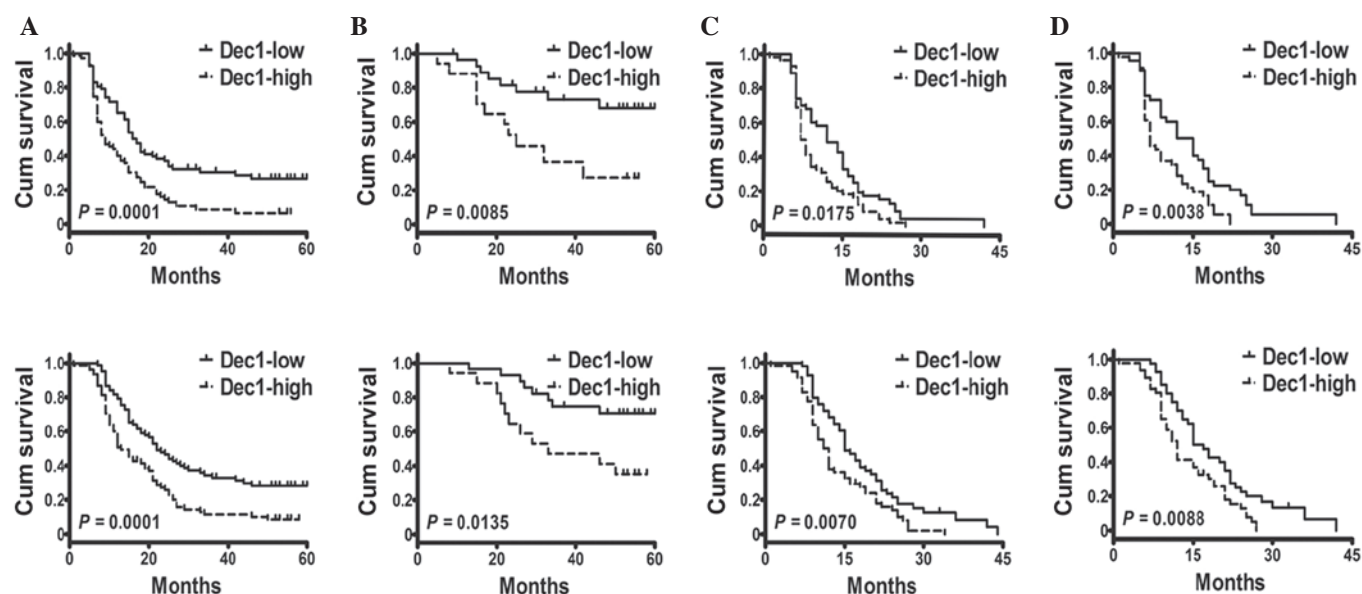
Figure 2. Correlation of Dec1 expression with progression-free survival (upper) and overall survival (lower) in (A) all cases, (B) low-grade gliomas, (C) high-grade gliomas and (D) temozolomide-treated gliomas. Cum, cumulative; Dec1, differentiated embryo chondrocyte expressed gene 1.

High Dec1 expression predicts poor responses to TMZ chemotherapy in HGG patients. Among the patients with HGG, 86 cases were treated with TMZ following surgery. The mean Dec1 IRS in all TMZ-treated patients with HGG was 7.14 ± 3.87, and the mean Dec1 IRS in the TMZ-resistant group (8.39 ± 3.44) was significantly higher than that of the TMZ-sensitive group (5.7 ± 3.86, $t=3.389$; $P=0.001$). In addition, 30 out of 46 (65.2%) patients with high Dec1 expression exhibited resistance to TMZ chemotherapy, compared with 16 out of 40 (40%) patients with low Dec1 expression. A statistically significant correlation was observed between Dec1 expression level and the response to TMZ chemotherapy ($P=0.020$; Table I). The mean postoperative PFS and OS of all TMZ-treated patients with HGG were 11.9 and 15.9 months, respectively. The mean PFS and OS of patients with low Dec1 expression were 14.3 and 18.3 months, respectively, whereas those of patients with high Dec1 expression were 9.7 and 13.7 months, respectively. HGG patients with low Dec1 expression exhibited better survival than those with high Dec1 expression when they received TMZ chemotherapy (log-rank test, $P=0.0038$ and $P=0.0088$, respectively; Fig. 2D).

Correlation of Dec1 expression with apoptosis in TMZ-treated recurrent GBMs. All 63 cases of TMZ-treated recurrent GBM specimens were examined by Dec1 IHC and the TUNEL assay, and representative images of both are presented in Fig. 3A. TMZ-induced apoptotic cells were detected in all these specimens. The AI was expressed as the percentage of the apoptotic cells and ranged from 0.31-5.81% (mean, $2.18\pm1.35\%$). The AI in low Dec1 expression group ($2.70\pm1.42\%$) was significantly higher compared with the high Dec1 expression group ($1.76\pm1.15\%$; $t=2.822$, $P=0.007$; Fig. 3B). Furthermore, the increase of Dec1 IRS in the recurrent GBM specimens was significantly correlated with a decrease of AI ($r=-0.341$, $P=0.006$; Fig. 3C). In addition, a significant difference in AI was observed between the TMZ-resistant group and

TMZ-sensitive group ($1.81\pm1.20\%$ and $2.67\pm1.40\%$, respectively; $t=-2.539$, $P=0.014$; Fig. 3D).

Dec1 overexpression significantly promotes the tumorigenicity of U87 cells xenografts in TMZ-treated nude mice. To confirm the correlation between overexpression of Dec1 and TMZ resistance in patients with GBM, the effects of Dec1 overexpression on TMZ resistance were investigated *in vivo*. U87 GBM cells stably expressing Dec1 or cherry (control) were established and transplanted into nude mice to generate tumor xenograft models. At ~30 days post-implantation, tumors were removed. A significant TMZ-resistant effect was observed in the U87-Dec1 tumors compared with the U87-cherry tumors (Fig. 4A). Following treatment with TMZ, the mean tumor volume and weight in U87-Dec1 tumors (1257 ± 382.7 mm^3 and 114 ± 12.99 mg, respectively) was significantly higher compared with the U87-cherry tumors (468.8 ± 193.4 mm^3 and 44 ± 2.63 mg, respectively; $P<0.001$ for both; Fig. 4B and C). In U87-Dec1 and U87-cherry tumors treated with TMZ, Dec1 levels were assessed by IHC and proliferation and apoptosis was detected by Ki-67 immunostaining and TUNEL, respectively (Fig. 4D). The proliferative index in U87-Dec1 tumors ($51.6\pm3.74\%$) was significantly higher compared with the U87-cherry tumors ($38.20\pm3.55\%$; $t=2.600$, $P=0.032$; Fig. 4E), and the AI was significantly lower in U87-Dec1 tumors ($15.26\pm2.20\%$) compared with U87-cherry tumors ($44.10\pm2.69\%$; $t=8.296$, $P<0.0001$; Fig. 4F).

Discussion

The present study investigated the expression status of Dec1 in 157 cases of newly diagnosed glioma and 63 cases of TMZ-treated recurrent GBM. High Dec1 expression, which was closely associated with pathological grade, KPS and poor response to TMZ chemotherapy, was a significant risk factor for PFS and OS in the patients with newly diagnosed glioma. In addition, a negative correlation between Dec1 expression

Table II. Univariate and multivariate analyses of progression-free survival and overall survival in low-grade glioma.

Clinical variables	Unadjusted HR[a] (95% CI)	P-value	Adjusted HR[b] (95% CI)	P-value
Progression-free survival				
Age				
<50 vs. ≥50	0.363 (0.141-0.930)	0.035	0.273 (0.089-0.836)	0.023
Gender				
Male vs. female	1.329 (0.534-3.307)	0.540	1.350 (0.490-3.720)	0.562
KPS				
≥80 vs. <80	0.198 (0.076-0.513)	0.001	0.207 (0.059-0.725)	0.014
Dec1 expression				
Low vs. high	0.312 (0.124-0.786)	0.013	0.226 (0.074-0.693)	0.009
Tumor location				
Sup. vs. inf.	1.451 (0.550-3.830)	0.452	0.720 (0.237-2.186)	0.562
Tumor size				
≥3 cm vs. <3 cm	2.814 (1.105-7.165)	0.030	1.409 (0.423-4.691)	0.576
Surgery resection				
Total vs. subtotal	0.820 (0.311-2.159)	0.688	0.435 (0.101-1.874)	0.264
Radiotherapy				
No vs. yes	1.213 (0.353-4.165)	0.759	2.418 (0.388-15.06)	0.344
Overall survival				
Age				
<50 vs. ≥50	0.362 (0.141-0.930)	0.035	0.283 (0.092-0.873)	0.028
Gender				
Male vs. female	1.425 (0.570-3.559)	0.448	1.055 (0.385-2.889)	0.917
KPS				
≥80 vs. <80	0.242 (0.095-0.618)	0.003	0.257 (0.080-0.828)	0.023
Dec1 expression				
Low vs. high	0.337 (0.135-0.840)	0.020	0.246 (0.085-0.710)	0.010
Tumor location				
Sup. vs. inf.	1.318 (0.501-3.468)	0.576	0.585 (0.196-1.748)	0.337
Tumor size				
≥3 cm vs. <3 cm	3.007 (1.178-7.678)	0.021	1.772 (0.554-5.664)	0.334
Surgery resection				
Total vs. subtotal	0.890 (0.337-2.350)	0.814	0.521 (0.120-2.266)	0.385
Radiotherapy				
No vs. yes	1.309 (0.381-4.501)	0.669	1.996 (0.312-12.77)	0.466

[a]HR in univariate models. [b]HR in multivariable models. HR, hazard ratio; KPS, Karnofsky performance status; Dec1, differentiated embryo chondrocyte expressed gene 1; sup, supratentorial; inf, infratentorial.

with the AI was detected in the TMZ-treated recurrent GBMs. In *in vivo* experiments, Dec1 overexpression promoted the tumorigenicity of U87 xenografts in nude mice treated with TMZ and significantly attenuated the antiglioma effect of TMZ. Together, the results demonstrated that Dec1 may be a valuable prognostic indicator for clinical outcome, and notably, a predictive factor for the response to TMZ chemotherapy in patients with glioma.

Various studies have investigated Dec1 to clarify its role in oncogenesis in multiple types of human tumor (12,14,25-30).

However, its value as a prognostic marker has not been previously determined. In the current study, in LGG and HGG, high Dec1 expression was correlated with unfavorable outcomes and was identified as an independent prognostic factor (Fig. 2; Tables II and III). In gliomas, complete resection is nearly impossible for the primary tumor due to its infiltrative nature. Therefore, radiotherapy- or chemotherapy-induced apoptosis of the residual glioma cells will dramatically impact the clinical outcome (Fig. 3D). The anti-apoptotic effect of Dec1 has been previously confirmed by other

Table III. Univariate and multivariate analyses of progression-free survival and overall survival in high-grade glioma.

Clinical variables	Unadjusted HR[a] (95% CI)	P-value	Adjusted HR[b] (95% CI)	P-value
Progression-free survival				
Age				
<50 vs. ≥50	0.628 (0.420-0.938)	0.023	0.768 (0.493-1.196)	0.243
Gender				
Male vs. female	0.860 (0.584-1.267)	0.445	0.945 (0.620-1.440)	0.793
KPS				
≥80 vs. <80	0.363 (0.234-0.562)	0.000	0.570 (0.351-0.925)	0.023
Dec1 expression				
Low vs. high	0.643 (0.434-0.954)	0.028	0.581 (0.375-0.900)	0.015
Tumor location				
Sup. vs. inf.	0.941 (0.631-1.401)	0.763	1.052 (0.697-1.587)	0.809
Tumor size				
≥3 cm vs. <3 cm	0.879 (0.594-1.299)	0.516	0.994 (0.643-1.537)	0.978
Surgery resection				
Total vs. subtotal	1.055 (0.711-1.565)	0.790	0.849 (0.564-1.277)	0.432
Standard treatment[c]				
No vs. yes	3.081 (2.031-4.673)	0.000	2.823 (1.736-4.591)	0.000
Overall survival				
Age				
<50 vs. ≥50	0.641 (0.429-0.956)	0.029	0.861 (0.554-1.339)	0.506
Gender				
Male vs. female	0.827 (0.561-1.218)	0.336	0.877 (0.577-1.334)	0.541
KPS				
≥80 vs. <80	0.347 (0.226-0.535)	0.000	0.549 (0.340-0.887)	0.014
Dec1 expression				
Low vs. high	0.598 (0.402-0.889)	0.011	0.569 (0.374-0.866)	0.009
Tumor location				
Sup. vs. inf.	0.849 (0.570-1.266)	0.422	0.761 (0.501-1.157)	0.202
Tumor size				
≥3 cm vs. <3 cm	0.815 (0.552-1.205)	0.306	0.978 (0.634-1.509)	0.920
Surgery resection				
Total vs. subtotal	1.169 (0.788-1.734)	0.437	0.974 (0.651-1.455)	0.896
Standard treatment[c]				
No vs. yes	3.531 (2.340-5.329)	0.000	3.124 (1.941-5.027)	0.000

[a]HR in univariate models. [b]HR in multivariable models. [c]Standard treatment; radiotherapy + temozolomide chemotherapy in patients with high-grade glioma. HR, hazard ratio; KPS, Karnofsky performance status; Dec1, differentiated embryo chondrocyte expressed gene 1; sup, supratentorial; inf, infratentorial.

studies (12-14,31-33). Indeed the results of the present study indicated that Dec1 overexpression significantly decreased apoptosis *in vivo* (Figs. 3 and 4). Based on this evidence, we speculate that the correlation between high Dec1 expression and the poor outcome may be explained by the ability of Dec1 to confer partial resistance to chemotherapy-induced apoptosis. In addition, HGGs were either poorly differentiated or undifferentiated, and consequently carried a dismal prognosis. The current study observed that Dec1 expression

was increased with increasing WHO grade, which was consistent with its capacity to antagonize differentiation in various types of human tumor (25,26). Furthermore, several previous studies provided evidence of Dec1 involvement in multiple cellular pathways, including p53, VHL/hypoxia and Janus kinase/Signal Transducer and Activator of Transcription signaling, which are crucial for tumor malignancy (27,31,32,34-36). Poor differentiation and high malignancy were closely associated with unfavorable clinical
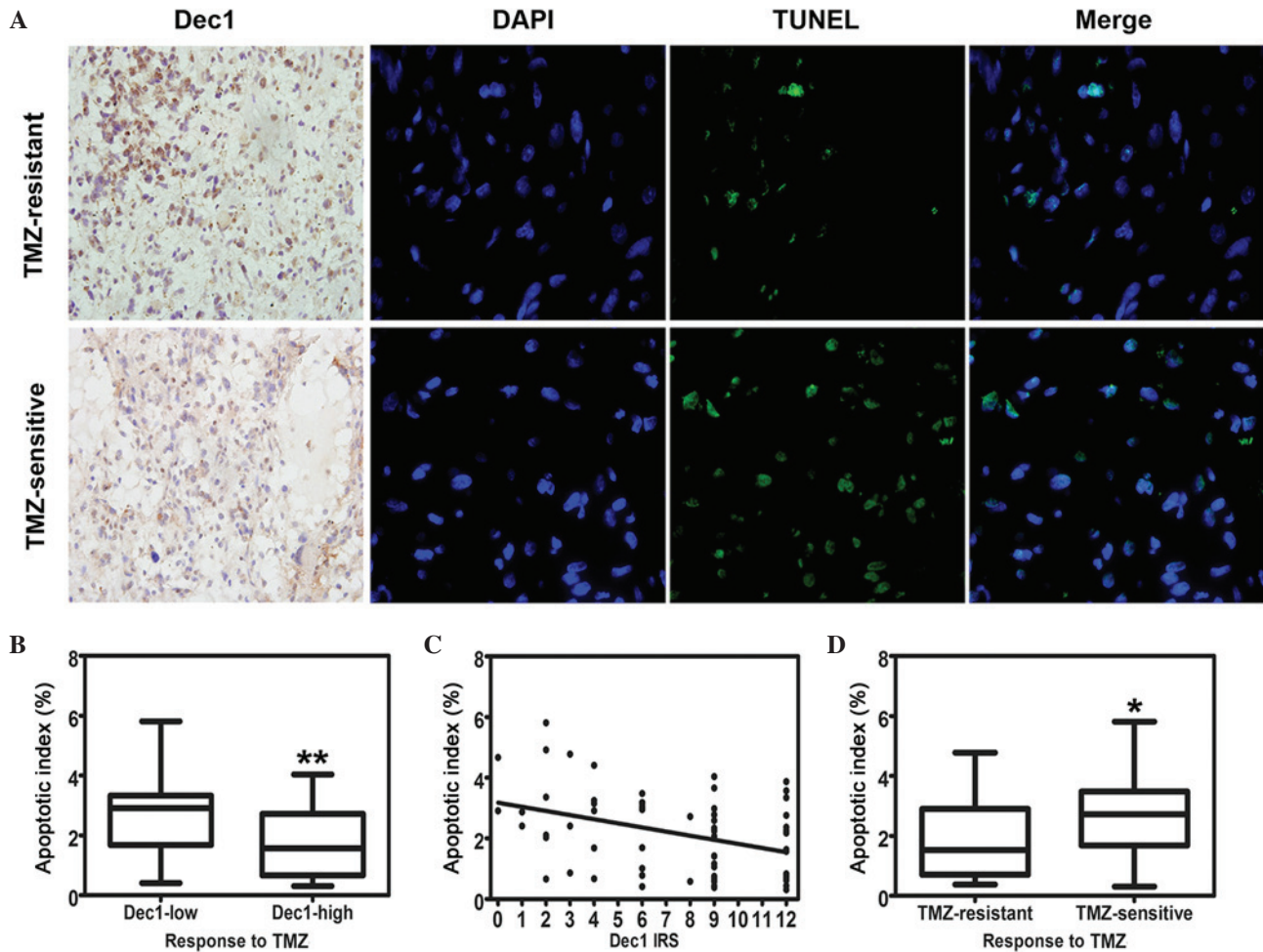
Figure 3. Correlation of Dec1 expression with TMZ-induced apoptosis in TMZ-treated patients with recurrent GBMs. (A) Representative photographs of Dec1 immunostaining (magnification, x400) and apoptosis detection by TUNEL assay (magnification, x1,000). Upper row: recurrent GBM patients resistant to TMZ chemotherapy exhibited high expression of Dec1 and fewer apoptotic tumor cells. Lower row: recurrent GBM patients sensitive to TMZ chemotherapy exhibited low expression of Dec1 and more apoptotic tumor cells. Arrowheads indicate apoptotic cells in recurrent GBM tissues. (B) Box-whisker plot presenting the correlation of Dec1 expression levels with apoptotic index ($^{**}P=0.006$ vs. Dec-low). (C) Scatterplot of the correlation of Dec1 IRS with apoptotic index. With the increase in Dec1 IRS, the apoptotic index decreased markedly (r=-0.341). By linear regression, a trend line representing the best fit indicated on the scatterplot. (D) Box-whisker plot presenting the correlation of the response to TMZ chemotherapy with apoptotic index ($^{*}P<0.05$ vs. TMZ-resistant). The values represent the mean ± standard deviation of 63 fields. GBM, glioblastoma multiforme; Dec1, differentiated embryo chondrocyte expressed gene 1; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; TMZ, temozolomide; IRS, immunoreactivity score.

outcome. Other reasons for the poor prognosis of gliomas may be the functions of Dec1 in cell differentiation and malignancy. Therefore, inhibition of Dec1 expression may contribute to the long-term benefits for patients with glioma, suggesting that Dec1 might be a novel potential molecular target for glioma biological therapy.

By contrast with the role of Dec1 as a significant risk factor for the poor outcome of gliomas described above, an opposing prognostic effect of Dec1 had been previously reported (37), demonstrating that low BHLHB2 (Dec1) expression predicted shorter survival in pancreatic ductal adenocarcinoma. Notably, the correlation of Dec1 expression levels with differentiation or malignant progression in various types of human cancer is not unique. In a previous study, Dec1 expression was increased during the progression from well differentiated to poorly differentiated tumor tissues in gastric cancer, which was consistent with findings in breast cancer and the present study (25,26). Shi *et al* (30) reported that low Dec1 expression was associated

with poor histological differentiation and malignant progression in hepatocellular carcinoma. These findings indicate that the expression of Dec1 might be a context-dependent prognostic factor. Similar to Dec1, Kruppel like factor 5 (KLF5) has also been previously reported to exert opposing prognostic roles in different types of cancer. For instance, patients with non-small cell lung cancer with high levels of KLF5 expression had a significantly better disease-specific survival compared with intermediate to no KLF5 expression (38). Patients with nuclear KLF5 staining had a significantly lower disease-free survival rate compared with patients with negative nuclear staining in gastric cancer (39). Clarifying the different prognostic effects of Dec1 in various types of human tumor warrants further investigation.

To date, the most important breakthrough and most commonly used strategy in HGG chemotherapy was the introduction of the alkylating agent, TMZ (40). Thus, the response to TMZ chemotherapy significantly affects the prognosis of
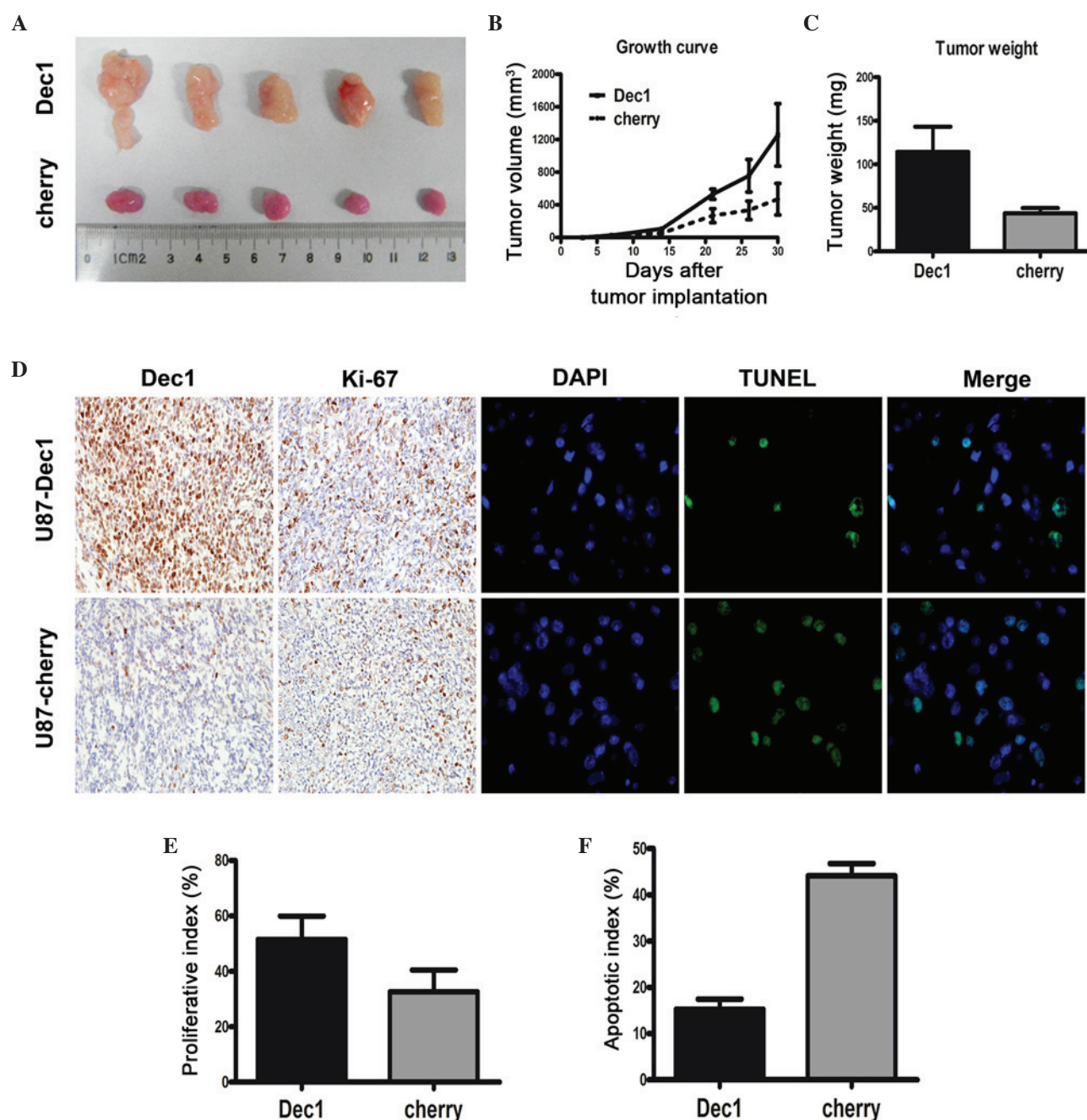
Figure 4. U87 glioblastoma multiforme cells with stable Dec1 expression exhibit a significant TMZ-resistance effect in xenografts of nude mice treated with TMZ. (A) *In vivo* study: U87-Dec1 and U87-cherry glioma xenografts were generated (n=5). After xenografts reached a palpable size, mice were treated with TMZ for 30 days, and glioma xenografts were weighed. (B) A growth curve was plotted by measuring the tumor volume. (C) Tumor weight was measured 30 days after the inoculation. (D) Representative images of Dec1 and Ki-67 immunostaining (magnification, x400) and apoptosis detection by TUNEL (magnification, x1,000) in U87-Dec1 and cherry tumors. (E) Box-whisker plot presenting the difference in proliferative index between U87-Dec1 and cherry tumors. (F) Box-whisker plot presenting the difference of apoptotic index between U87-Dec1 and cherry tumors. TMZ, temozolomide; Dec1, differentiated embryo chondrocyte expressed gene 1; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

gliomas. The present study provided additional evidence that high Dec1 expression negatively affected the response to TMZ chemotherapy in patients with HGG, which indicated that Dec1 was a prognostic factor and predictive factor for the response to TMZ chemotherapy in glioma patients. Given this evidence, high Dec1 expression may be correlated with poor prognosis in patients with glioma.

Traditionally, prognostic factors intend to objectively predict patient clinical outcome independent of treatment, while predictive factors aim to foretell the response of a patient to a specific therapeutic intervention and are associated with tumor sensitivity or resistance to the therapy. However, various factors exhibit both prognostic and predictive significance. For instance, human epidermal growth factor receptor 2 (HER2) has been validated as a prognostic factor and predictor of response to HER2-targeting therapy (41).

The original aim of the current study was to investigate the correlation of Dec1 expression with tumor malignancy and

determine whether Dec1 expression may predict the outcome in 157 cases of newly diagnosed glioma. Surprisingly, the 86 cases of the TMZ-treated glioma patients were classified into resistant- and sensitive-groups according to the duration of PFS, a statistical significance between Dec1 expression levels and the response to TMZ chemotherapy was detected (P=0.020; Table I). Furthermore, the low Dec1 expression group exhibited longer PFS and OS compared with the high Dec1 expression group in the TMZ-treated patients with HGG (Fig. 2D). Together this evidence revealed that, similar to HER2, Dec1 displayed a strong prognostic effect, and also appeared to predict a poor response to TMZ treatment.

Standard therapeutic strategies of maximal tumor resection and postoperative radiation combined with TMZ chemotherapy are almost never curative for GBMs, due to the infiltrative nature of GBMs, and the intrinsic or acquired resistance to radiation and chemotherapy (42). Therefore, identifying mechanisms of resistance to TMZ chemotherapy in GBMs may provide useful information for further individualized therapeutic strategies. The results of the current study indicated that Dec1 overexpression significantly attenuated the antiglioma effect of TMZ *in vivo* (Fig. 4). In addition, several previous studies provide evidence of the involvement of Dec1 in TMZ resistance. One report observed that Dec1 and Dec2 decreased mutL homolog 1 (MLH1) expression via binding to the E-box motifs in the MLH1 promoter region (43). MLH1 is considered to be an important factor in the DNA mismatch repair (MMR) pathway, and defects in this pathway are critical for mediating the tolerance of the cytotoxic effect of alkylating agents (44). Furthermore, the cytotoxicity of TMZ was low in colon cancer cells lines with hMLH1 mutation, and all five cell lines in the National Cancer Institute tumor panel that were deficient in hMLH1 activity were resistant to TMZ (45,46). Therefore, as a MLH1 repressor, Dec1 overexpression may consequently decrease the cytotoxicity of TMZ. Additionally, it is well established that via the MMR system, TMZ induces double-strand breaks and triggers p53-dependent cell cycle arrest and apoptosis (47). As a novel target gene of the p53 family, Dec1 inhibited DNA damage-induced cell death by attenuating p53 induction of macrophage inhibitory cytokine-1. Furthermore, as a hypoxia-regulated transcription factor, Dec1 is upregulated by hypoxia in cancer, and the hypoxia-adaptation involves decreased leakage of reactive oxygen species (ROS) (48,49). TMZ-induced DNA damage caused enhanced ROS generation and decreased ROS production-induced acquisition of chemoresistance (50). Generally, chemoresistance in gliomas may be influenced by the dysregulation of apoptosis-regulating genes and proteins. The results of the present study demonstrated an antagonizing role of high Dec1 expression in TMZ-induced apoptosis of the tumor cells in TMZ-treated recurrent GBMs, which was consistent with previous studies indicating that Dec1 is an anti-apoptotic transcription factor (12-15,31-33). Together this evidence suggests that Dec1 is involved in TMZ-resistance and suggests that inhibition of Dec1 expression may enhance the cytotoxicity of TMZ. The underlying mechanisms involved require further investigation.

In conclusion, the data of the present study provided evidence that Dec1 expression is closely associated with the malignant grade and TMZ-induced apoptosis of human glioma. It was additionally demonstrated that Dec1 is a prognostic indicator to evaluate the outcome of patients with glioma. Furthermore, Dec1 expression negatively affected the response to TMZ chemotherapy in patients with HGG. This valuable prognostic indicator for clinical outcome and predictive factor of the response to TMZ chemotherapy may be a potential novel molecular target for glioma biological therapy, particularly in patients with TMZ-resistance.

Acknowledgements

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