Bioscience Reports (2020) **40** BSR20193515
https://doi.org/10.1042/BSR20193515

PORTLAND PRESS

Research Article

The role of DOT1L in the proliferation and prognosis of gastric cancer

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Background: Disruptor of telomeric silencing-1-like (DOT1L), a methyltransferase of H3K79, was observed to be amplified and overexpressed in certain malignancies. This work was aimed at investigating the differences in DOT1L expression and its regulatory mechanism in gastric cancer (GC) and healthy samples.
Methods: Immunohistochemistry was used to detect DOT1L levels in 101 cases of GC and marching adjacent normal tissues. DOT1L was inhibited by small interfering RNA (siRNA) and EPZ5676; a targeting drug. The ability of cells to proliferate were checked by cell counting kit-8 (CCK-8) and clone formation assays, with flow cytometry for observing the cell cycle. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blot revealed the gene and protein profiles. Finally, the outcome of EPZ5676 administration was checked on a murine model.
Results: The expression of DOT1L is significantly increased in gastric malignant tumors that is related to the degree of differentiation, lymph node metastasis and TNM staging. DOT1L serves as an independent marker for the prognosis of overall survival (OS) with high levels implying worse prognosis. In addition, DOT1L regulates cyclin-dependent kinase (CDK) 4 (CDK4) and CDK6 through H3K79me2, which leads to a change in the cell cycle at G_1, thereby affecting the proliferation of tumors *in vitro* and *in vivo*.
Conclusions: This is a first clinical demonstration of the applicability of DOT1L overexpression in gastric tumors. The work is suggestive of altered proliferation of cells by DOT1L via regulating cyclins and H3K79 methylation. This indicates the role of DOT1L in the prognosis and possible medical intervention of GC.

Introduction

Among the most ubiquitous malignancies is gastric cancer (GC), globally occupying the fifth rank in incidence and third in terms of mortality [1]. The number of new diagnoses was 1033701 with 782685 loss of lives in 2018, according to estimates [2]. Although the current comprehensive treatment based on surgery has made great progress, its clinical efficacy in patients is not significant, and the 5-year survival rate even now is below 30% [3]. Due to the lack of obvious clinical symptoms in early GC, most patients manifest advanced stages when diagnosed. Thus, it has been found that tumor markers with high sensitivity and specificity can improve the early discovery rate and improve the prognosis of GC.

With the rapid development of epigenetics, more and more small molecule drugs targeting histones and related regulatory proteins are being used for targeted experimental treatment in various tumors [4]. Disruptor of telomeric silencing-1-like (DOT1L) is an evolutionarily conserved protein, and its homolog DOT1 was first discovered in yeast through genetic screening. It is the only methyltransferase specific for the Lys^{79} of histone H3 ($H3K79me_x$, x = 1, 2, or 3) [5]. According to recent reports, dot1l-mediated

Received: 10 October 2019
Revised: 12 December 2019
Accepted: 10 January 2020

Accepted Manuscript online: 13 January 2020
Version of Record published: 31 January 2020

Bioscience Reports (2020) **40** BSR20193515
https://doi.org/10.1042/BSR20193515

H3K79 methylation not only enables the transcriptionally active gene with the close association of its upstream and downstream regulatory genes along with regulating the cell cycle, chromatin silencing, and DNA damage repair [6–7]. In addition, the vital functioning of DOT1L in the mixed lineage leukemia (MLL)-rearranged leukemia has been shown [8], and thus DOT1L-targeted inhibitors have been used in rearranged leukemia [9]. In solid tumors, DOT1L is also involved in its progression, such as breast cancer [10], ovarian cancer [11], and lung cancer [12]. However, to date, the therapeutic value and the prognostic impact of DOT1L in GC has not been evaluated. This work involved a clinicopathological analysis and correlation with patient prognosis by examining the expression of DOT1L in GC with the observations showing that the molecule may serve as a new target for GC therapy.

Material and methods

Patients and tissue samples

All patients (n=101, age in the range 30–89 years, a mean age of 60.33 years) underwent subtotal gastrectomy between December 2012 and December 2013 in the First Affiliated Hospital of Bengbu Medical College. Their GC and corresponding adjacent normal tissues (distance > 4 cm) were taken from the Department of Pathology. The patients showed primary GC with no exposure to preoperative chemotherapy or radiotherapy. The study involved patient consent and received approval from the Ethics Committee of the above-mentioned institution.

Immunohistochemical studies

Immunohistochemical studies (IHC) employed tissue sections fixed and embedded in formalin and paraffin, sequentially. In short, the slices were submerged in 0.01 mol citrate buffer and subjected to boiling for 10 min to repair the antigens. Normal goat serum (10%) was added for 30 min to block non-specific binding. This was followed by overnight incubation with DOT1L polyclonal antibody, H3K79me2 polyclonal antibody, cyclin-dependent kinase (CDK) 4 (CDK4) polyclonal antibody, and CDK6 polyclonal antibody (1:200; all antibodies were from Abcam; U.S.A.) at 4°C. This was followed by 30 min of incubation with goat anti-rabbit IgG (1:500; Abbkine; U.S.A.) at room temperature. 3,3′-diaminobenzidine tetrahydrochloride solution (DAB Solution; Solarbio, Beijing, China) was used for chemical staining, while the re-dyeing of sections was done with Hematoxylin. After the use of an alcohol gradient for dehydration and xylene, neutral resin was used to seal the slices.

Immunohistochemical scoring criteria [13] that was used incorporated both staining intensity with the staining gradation as (0 = none, 1 = weak, 2 = moderate, 3 = strong) and positive tumor cells represented in terms of % as (0 = none, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, 4 = 76–100%). The total score used in the analysis was calculated by multiplying the staining intensity and positive cell score, and according to the total score, division was done into a high-expression group ($\geq$4) and a low-expression group (<4). All sections were evaluated by two senior pathologists.

Cell culture and transfection

Shanghai Cell Bank of the Chinese Academy of Sciences was the source of the human GC cell lines sgc7901, bgc823 and mgc803. Dulbecco's modified Eagle's medium (DMEM, HyClone, U.S.A.) plus 10% fetal bovine serum (FBS, Gibco, U.S.A.), 50 U/ml penicillin, and 50 μg/ml streptomycin was utilized for culture of cells in a humidified incubator containing 95% air and 5% CO_2 at 37°C. The cells were transduced with pLKO.1 puro lentiviral particles (GeneChem, Shanghai, China) while DOT1L was knocked down by shDOT1L (shRNA; same brand): 5′-CCGGGCCCGCAAGAAGAAGCTAAACCT CGAGGTTTAGCTTCTTCTTGCGGGCTTTTTG-3′. Lentiviral particles of shDOT1L or shCON (control) were utilized to transfect SGC7901 and MGC803 with puromycin utilized for selection.

EPZ5676

EPZ-5676 (Selleckchem; U.S.A.) (Pinometostat; (2R,3R,4S,5R)-2-(6-amino-9H-purin-9-yl)-5-((((1r,3S)-3-(2-(5-(tert-butyl)-1H-benzo[d]im-idazol-2-yl)ethyl)cyclobutyl)(isopropyl)amino)methyl)tetra hydrofuran-3,4-diol), is a potent and selective inhibitor of DOT1L with a Ki of ≤80 pM and >37000-fold selectivity over a panel of other histone methyltransferases (HMTs). In this experiment, GC cells were cultured with different concentrations of EPZ5676 to observe the changes in cell proliferation. In addition, the effect of EPZ5676 on the growth inhibition of GC cells *in vivo* was observed after administering the drug in nude mice.

Western blotting

A 1:100 of RIPA lysis buffer (Sigma, U.S.A.) with proteinase inhibitor was utilized for extracting the total protein whose concentration was quantified using the BCA kit (Thermo Scientific, U.S.A.). Protein extracts (50 μg) were

electrophoresed on 8% polyacrylamide gel and then transferred to a PVDF membrane. This was followed by 120 min of soaking in 5% skim milk at room temperature, followed by an overnight treatment with 1:1000 of rabbit anti-human monoclonal antibodies against DOT1L, H3K79me2, CDK4 and CDK6 (Abcam; U.S.A.) at 4°C along with 1:2000 of mouse anti-human monoclonal antibody H3 and β-actin (Abbkine, U.S.A.). The next step was incubation of 1:5000 of the corresponding goat anti-rabbit IgG and goat anti-mouse IgG (Abbkine, U.S.A.) at room temperature for 2 h. Finally, the images were developed using the ECL chemiluminescence kit and analyzed using the Ge Gene gel imaging system (Syngene, Cambridge, U.K.).

Quantitative reverse transcription polymerase chain reaction

GC cell total RNA was isolated for Trizol™ Plus RNA Purfication kit (Invitrogen, Thermo Scientific, U.S.A.), followed by reverse transcription to cDNA with Revert Aid First Strand cDNA short kit (Thermo Scientific, U.S.A.). The primers were supplied by Shanghai GeneChem for PCR and their sequences were as follows: DOT1L-F 5′-CATCACTATGGCGTCGAGAAA-3′; DOT1L-R 5′-CGCCTCTCTCCAATGTGTATT-3′; β-actin-F 5′-CAGGAAGGAAGGCTGGAAG-3′; β-actin-R 5′-CGGGAAATCGTGCGTGAC-3′. SYBR Premix Ex Taq II (Takara Biotechnology Co., Dalian, China) was employed to perform triplicate quantitative reverse transcription polymerase chain reaction (qRT-PCR) using Fast Real-time PCR 7500 system (Applied Biosystems, Foster City, CA, U.S.A.) in accordance with the prescribed protocols of the manufacturer. The PCR conditions were as follows: (a) denaturation at 95°C for 15 s, (b) annealing at 52.4°C for 30 s, (c) extension at 72°C for 34 s, for a total of 40 cycles. The internal reference was β-actin. The $2^{-\Delta\Delta C_t}$ approach was employed to assess gene levels.

Colony formation assay

Each group of cells in log growth phase was digested with trypsin, and 1000 cells/well were spread on a six-well plate. After 10 days of culturing in the incubator, methanol was utilized to fix the cells for 20 min, stained with Crystal Violet (Sigma, U.S.A.) for 5 min followed by washing. Microscopy was employed for enumeration of cell colonies that were >50 per well.

Assays for cell proliferation

This was checked by cell counting kit-8 (CCK-8, Abbkine, U.S.A.); 96-well plates were used to seed 1×10^3 GC cells per 100 μl and culture conditions as previously mentioned for 24–96 h. After the fresh medium was changed, CCK-8 reagent was added followed by 60 min of incubation, and spectrophotometry to assess the OD_{450} value.

Analyzing the cell cycle

Each cell was fixed in ice-cold 70% ethanol in 4°C o/n followed by suspension in PBS with 0.1 mg/ml RNase A and 5 mg/ml propidium iodide (Sigma, U.S.A.). According to the cell DNA content, flow cytometry (BD Accuri™ C6-plus, U.S.A.) was utilized for analyzing the cell cycle. The percentages of G_1, S, G_2/M phase cells were ascertained by ModFit LT 5.0.

Tumor xenografts

Twelve 4-week-old NOD-SCID male mice were purchased from the Cavens Laboratory Animal Center (Changzhou, China) and housed in the SPF Animal Center in Bengbu Medical College. Random assignment of the animals was done into one of two groups: a control group and EPZ5676 group. Resuspension of log-phase SGC7901 cells in PBS was done at 1×10^7/ml, of which 0.2 ml was injected into the underarm of each mouse. EPZ5676 at 5 mg/kg was administered intraperitoneally on a weekly basis in the test group for 4 weeks. The control group was injected with an equal volume of solvent. After anesthetizing the mice with 3% pentobarbital sodium, tumors were isolated for studies. The experiments were in accordance with the Ethics Committee of the Institute.

Statistical analysis

The application of Chi-square test was done to check the association of DOT1L expression with that of GC clinico-pathological factors. Overall survival (OS) curves were analyzed by Kaplan–Meier, and then the bilateral log-rank test was performed. Univariate and multivariate Cox proportional hazard models were made for assessing the prognostic significance.

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https://doi.org/10.1042/BSR20193515

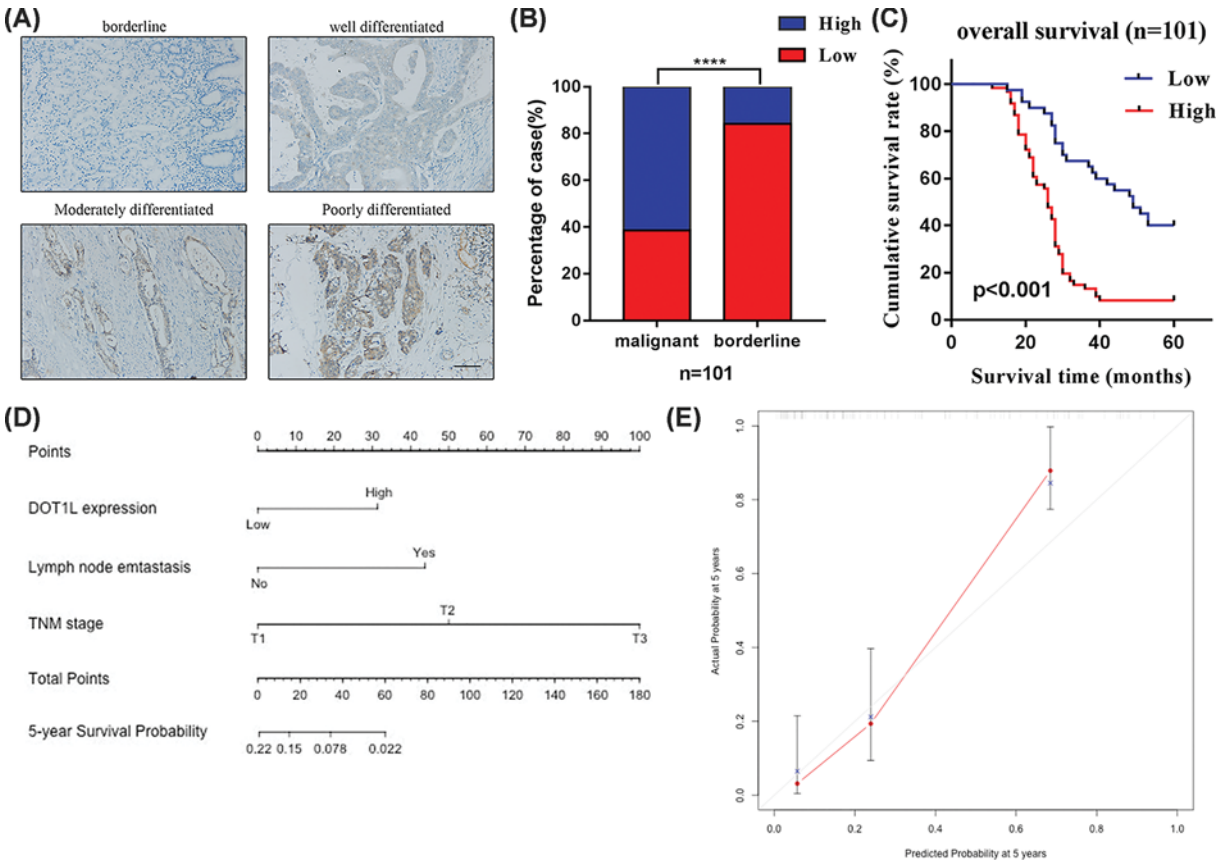

Figure 1. Expression of DOT1L protein in GC tissue and Kaplan–Meier plots with nomogram

(**A**) Immunohistochemical staining of DOT1L protein in human GC tissues. DOT1L protein was expressed in normal gastric tissues and GC tissues at different degrees of differentiation. The brown color in the image indicates positive staining. Magnification 400×. The scale is 25 μm. (**B**) The K-M curve showed that the OS rate of patients with high DOT1L expression was significantly lower than that of patients with low expression. (**C**) The difference in DOT1L expression between GC tissues and adjacent normal tissues was compared by χ^2 test. (**D**) Nomogram to predict 5-year survival rate in patients with GC. (**E**) The calibration plots for predicting OS at 5 years. ****$P<0.0001$

A nomogram was built based on multivariable Cox regression results, using the R version of 3.3.01 RMS package. Using the Akaike information criterion, backward stepdown decline was applied to select the final model. The accuracy of the model was assessed using concordance index (C-index). By drawing the corresponding nomogram to predict survival probability, the 5-year OS was assessed after calculation using the nomogram of the calibration curve.

For basic experiments, the Student's *t* test was utilized to compare the mean ± standard deviation (SD) data with Chi-square test to assess categorical data, while analysis of variance (ANOVA) was applied for three or more groups.

Results

Expression variation of DOT1L between GC and paracarcinoma tissues

IHC of 101 GC cases and its surrounding tissues (Figure 1A) showed high DOT1L expression in 62 (61.4%) GC samples and in 16 (15.8%) cases of paracancer tissues (malignant vs borderline, $P<0.0001$) (Figure 1B).

After 5 years of follow-up, 80 (79.2%) of the 101 patients had died, with a median OS of 28 months (95% CI: 26.4–29.6 months) and an average survival time of 34.3 months (range: 11–60 months).

Relationship between clinicopathological parameters of GC and expression of DOT1L

To clarify the prognostic function of DOT1L in GC pathology, we analyzed the DOT1L levels in 101 GC patients. DOT1L expression correlated with differentiation degree ($P=0.04$), lymph node metastasis ($P<0.01$) and TNM stage

Table 1 Association of DOT1L expressions with clinical characteristics of GC patients

Clinical characteristics (*n*=101)	DOT1L expression		χ^2	*P*-value
	Low	High		
Age (years)			0.122	0.727
<60	19	28		
≥60	20	34		
Gender			1.732	0.188
Male	30	40		
Female	9	22		
Tumor size (cm)			1.927	0.165
<5	25	31		
≥5	14	31		
Tumor location			2.603	0.452
Fundus	8	12		
Body	14	25		
Antrum	16	19		
Whole	1	6		
Degree of differentiation			6.444	**0.040**
Complete	2	1		
Moderate	19	17		
Poor	18	44		
Lymph node metastasis			22.432	<**0.001**
No	25	11		
Yes	14	51		
TNM stage			16.744	<**0.001**
I	8	1		
II	14	12		
III	17	49		
Depth of invasion			3.399	0.065
T1–T2	9	6		
T3–T4	30	56		

The bold value indicated a signficant results with a $P<0.05$.

($P<0.01$), but had no significant relationship with age ($P=0.727$), gender ($P=0.188$), tumor size ($P=0.165$), tumor location ($P=0.452$), depth of invasion ($P=0.065$), invading vessel ($P=0.924$) and invading nerve ($P=0.748$) (Table 1).

OS analyses

Kaplan–Meier plots show that the high level of DOT1L in GC patients was associated with lower OS against those with lesser DOT1L expression (Figure 1C). In addition, Cox regression was followed to assess the prognosis of OS. Univariate analysis showed the following prognostic factors: DOT1L expression ($P<0.001$), invasion depth ($P=0.005$), lymph node metastasis ($P<0.001$), TNM stage ($P<0.001$) and the degree of differentiation ($P=0.01$). Meanwhile, the result of multivariate analysis points out at high DOT1L expression ($P=0.014$), lymph node metastasis ($P=0.023$) and TNM stage ($P=0.030$) as independent prognostic factors of OS in GC (Table 2). In summary, the involvement of DOT1L to function as a patient prognostic marker with proto-oncogenes is strongly indicated.

The establishment of a nomogram

A nomogram model was established using regression analysis (Figure 1D). The nomogram C-index was 0.8 and suggested that the TNM stage made the largest contribution to prognosis, followed by DOT1L expression and lymph node metastasis. Assignment of score on the point scale was done for these independent prognostic variables. The scores of all indicators were added to obtain the total score, and the total score corresponded to the predicted value of risk. Here, a calibration plot of 5-year survival shows an ideal consistency between prediction and observation of nomogram (Figure 1E).

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Table 2 Association of DOT1L expressions and clinical characteristics with the survival rates of GC patients

Clinical characteristics	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	*P*-value	Hazard ratio	95% CI	*P*-value
DOT1L expression						
Low vs High expression	0.381	0.248–0.586	<**0.001**	0.523	0.312–0.877	**0.014**
Age (years)						
<60 vs ≥60	1.018	0.682–1.521	0.929			
Gender						
Male vs Female	1.205	0.657–1.600	0.913			
Tumor size (cm)						
<5 vs ≥5	0.672	0.449–1.006	0.054			
Depth of invasion						
T1–T2 vs T3–T4	0.424	0.235–0.767	**0.005**	0.764	0.294–1.986	0.580
Lymph node metastasis						
No vs Yes	0.284	0.183–0.443	<**0.001**	0.520	0.296–0.913	**0.023**
TNM stage			<**0.001**			**0.030**
I vs II	0.181	0.086–0.380	<**0.001**	0.439	0.117–1.640	0.221
I vs III	0.257	0.153–0.430	<**0.001**	0.390	0.193–0.791	**0.009**
Degree of differentiation						
Complete+Moderate vs Poor	0.581	0.384–0.879	**0.010**	0.799	0.485–1.314	0.376

The bold value indicated a signficant results with a $P<0.05$.

Knockdown of DOT1L inhibits GC cell proliferation and induces cell-cycle arrest at G_1

According to the above studies, the high expression of DOT1L is closely associated with GC malignancy. Therefore, we used a loss-of-function method to study the function of DOT1L. The DOT1L expression in different GC cell lines was evaluated by Western blot (Figure 2A). Compared with other cell lines, SGC7901 and MGC803 had higher DOT1L expression. Thus, we selected these two cell lines, for knocking down DOT1L by shRNA lentiviral particles and EPZ5676. Lowered expression was observed by qRT-PCR and Western blot (Figure 2B,C). The clone formation assay was used to observe the effect of DOT1L on GC proliferation. The results showed that in comparison with controls, the clone formation ability of transfected SGC7901 and MGC803 was significantly reduced. Consistent with knockdown of DOT1L expression using shRNA, the GC proliferation also reduced because of inhibition of DOT1L enzymatic activity pharmacologically with EPZ5676 treatment (Figure 2D). Intriguingly, through the CCK-8 experiment, it was found that the inhibition of proliferation of SGC7901 and MGC803 gradually increased with time and the concentration of EPZ5676 (Figure 2E). To clarify the cause of this slowdown in GC cell proliferation caused by DOT1L inhibition, we evaluated cell cycle by flow cytometry. Compared with the empty vector control group, the number of transfected SGC7901 and MGC803 G_1 phase cells increased, and the S and G_2/M phase cells decreased (Figure 2F). Similarly, we found the same results with EPZ5676 drug treatment of SGC7901 and MGC803. These results indicated that inhibition of DOT1L in GC may cause arrest at G_1 and block cell proliferation *in vitro*.

DOT1L regulates CDK4 and CDK6 to change cell cycle by H3K79 dimethylation

According to inhibition of DOT1L, GC cell proliferation was slowed down and G_1 arrest was induced. The role of H3K79 is suggested on account of DOT1L functioning as a specific methyltransferase of H3K79, suggestive of its regulation of the cell cycle by CDKs [14,18]. CDK4/CDK6 complex can promote cell cycle, from G_1 to S phase [15]. This led us to estimate its levels by Western blot (Figure 3A). Transfected SGC7901 and MGC803 cells were significantly reduced in H3K79me2 compared with the empty vector control group, CDK4 and CDK6 were significantly decreased (Figure 3B,C). These results suggest that DOT1L modulates CDK4 and CDK6 by altering H3K79me2, leading to G_1 phase stagnation of GC cells.

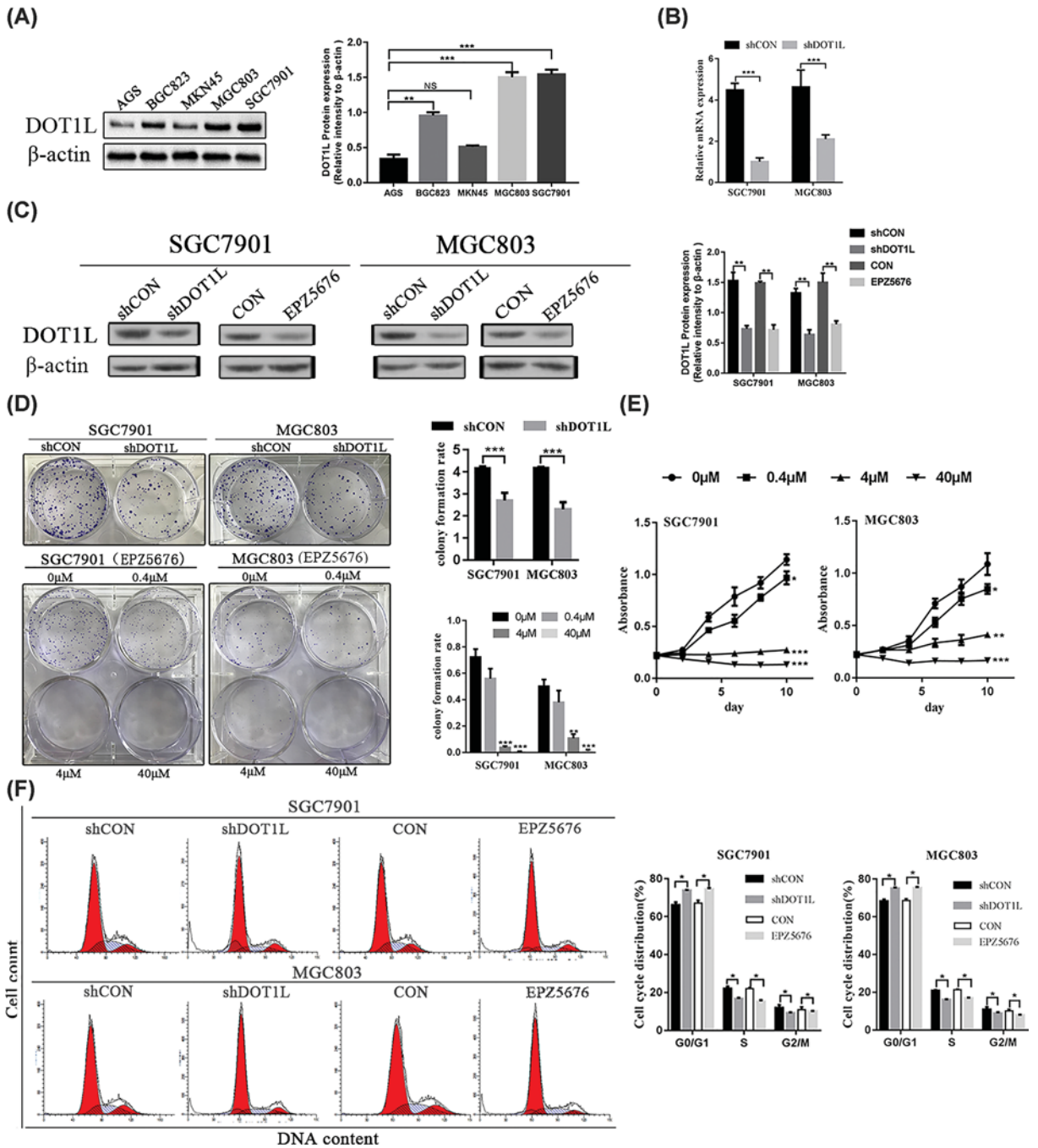

Figure 2. Knockdown of DOT1L inhibits GC cell proliferation and cell cycle G1 arrest

(**A**) Western blot was used to detect the expression of DOT1L in various cell lines of GC. (**B**) The expression level of DOT1L mRNA in SGC7901 and MGC803 cells was knocked down by shRNA lentiviral transfection and detected by qRT-PCR. (**C**) SGC7901 and MGC803 cells were transfected with shRNA to knock down DOT1L and cultured with 5 μM of EPZ5676 for 10 days to inhibit DOT1L protein, and DOT1L expression was detected by Western blot. β-actin was used as an internal reference. (**D**) The proliferation of SGC7901 and MGC803 cells transfected with shDOT1L shRNA and the proliferation of SGC7901 and MGC803 cells after inhibition of DOT1L by different concentrations of EPZ5676 (0, 0.4, 4, or 40 μM) were detected by colony cloning assay. (**E**) CCK-8 assay measures SGC7901 and MGC803 cell culture for 10 days at different concentrations (0, 0.4, 4, or 40 μM) of EPZ5676. The OD450 values were measured at 0, 2, 4, 6, 8, and 10 days of culture. (**F**) Flow cytometry was used to detect shDOT1L shRNA transfected SGC7901 and MGC803 and cells were cultured with EPZ5676 to observe cell cycle changes. **P*<0.05, ***P*<0.01 and ****P*<0.001.

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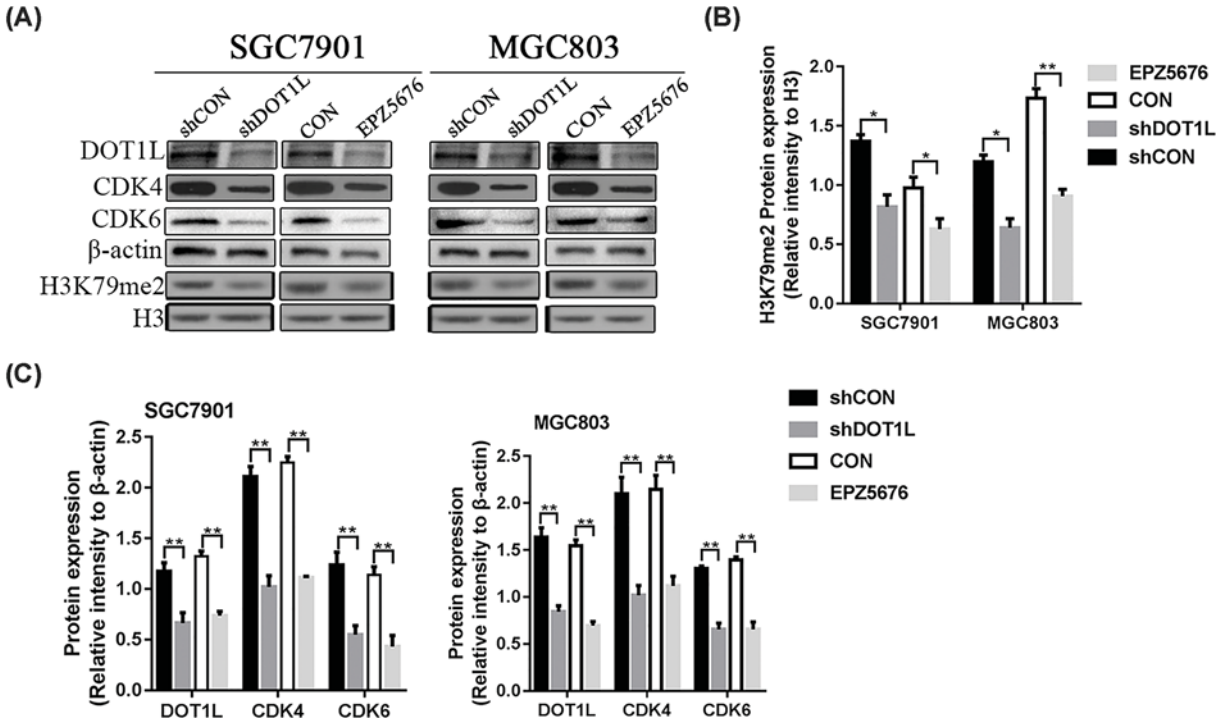

Figure 3. Knockdown of DOT1L can inhibit the expression of H3k79me2 and decrease the expression of cyclin CDK6

(**A**) Western blot confirmed that the expression of H3K79me2 and the corresponding G_1 arrest protein CDK4 and CDK6 in SGC7901 and MGC803 cells was detected by knocking down DOT1L with shDOT1L shRNA and DOT1L inhibitor EPZ5676. (**B**) The expression level of H3K79me2 was determined by optical density analysis of Western blot using H3 as a control. (**C**) The expression levels of DOT1L, CDK4 and CDK6 were determined by optical density analysis of Western blot using β-actin as a control. $^{*}P<0.05$, and $^{**}P<0.01$.

In vivo inhibition of DOT1L and tumor by EPZ5676

To further investigate whether the DOT1L gene can also inhibit GC growth *in vivo*, we injected SGC7901 cells into the armpit of nude mice to construct an ectopic tumor formation model. After 28 days, it was found that mice treated with EPZ5676 grew more slowly than mice that did not receive any treatment (Figure 4A). The isolated tumors were analyzed, and it was evident that the size and weight of the untreated group were larger than those of the treatment group (Figure 4B,C). Furthermore, immunohistochemical images showed that mice treated with EPZ5676 had reduced expression of H3K79me2, CDK4 and CDK6 in mouse tumors compared with the control group (Figure 4D). These results indicate that EPZ5676 could inhibit tumor growth in GC.

Discussion

We found that the expression of DOT1L is related to the development and progression of GC. In humans, DOT1L by MLL fusion partner proteins such as ENL, AF4, AF9 or AF10 binding, initiation and maintenance of participation rearrangement leukemia resulting in abnormal H3K79 methylation [16–17]. In malignant tumors of solid organs, Oktyabri et al. [10] found that increasing the expression of DOT1L may increase the expression of BCAT1, leading to changes in the sphere formation and cell migration activity of breast cancer cells. In kidney cancer, Yang et al. [18] suggested a higher level of DOT1L in patients with renal clear cell carcinoma with worse survival prognosis post-surgery than those with a low DOT1L expression. It can be seen that DOT1L is involved crucially to control gene transcription.

To confirm the expression of DOT1L in GC, we collected tissue wax blocks for immunohistochemical staining and found that DOT1L levels were conspicuously elevated in GC compared with the neighboring healthy tissues. In addition, these increased levels were connected with poor differentiation, low TNM stage and lymphatic metastasis. Additionally, the role of DOT1L to serve as an independent factor for the prognosis for OS in the case of GC and its

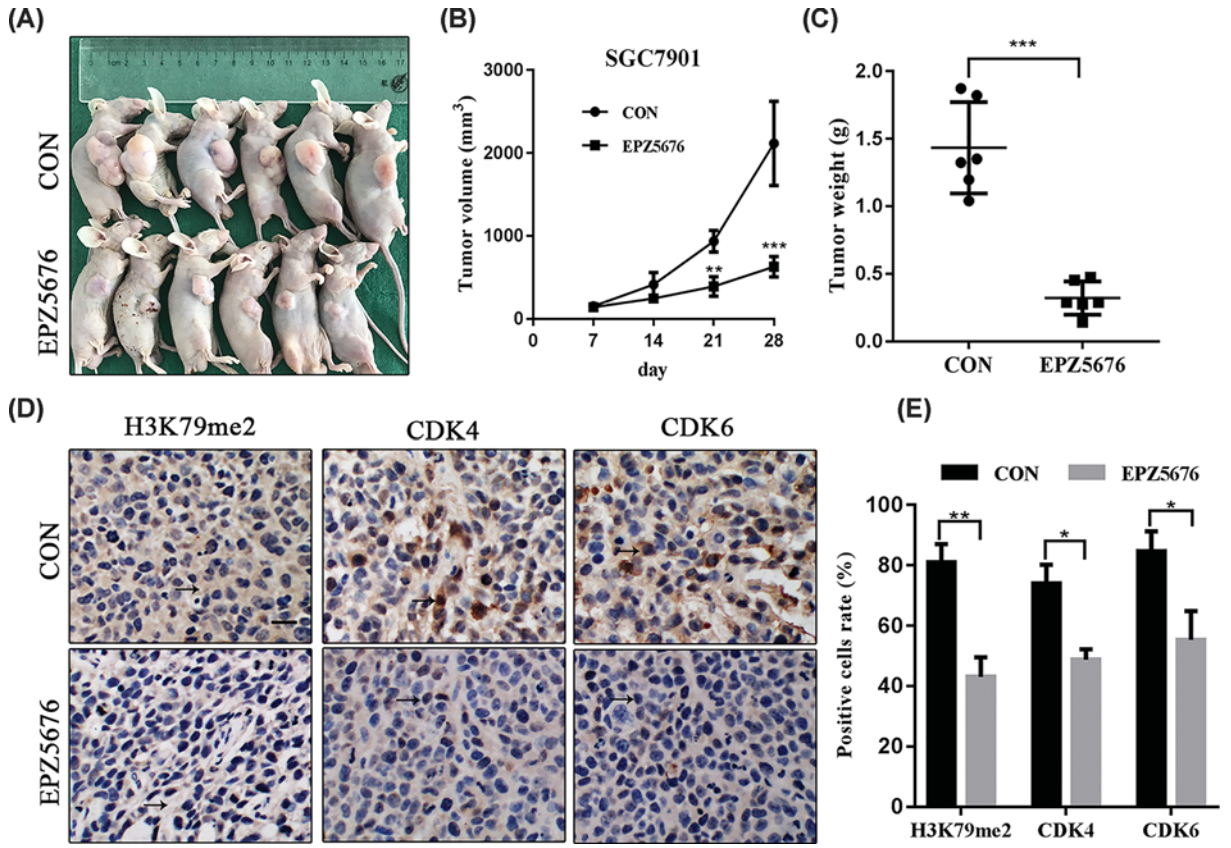

Figure 4. Drug inhibition of DOT1L on tumor progression *in vivo*

(**A**) SGC7901 cells were injected into the armpit of nude mice to establish a heterotopic tumor model in nude mice. In the experimental group, EPZ5676 (5 mg/kg) was intraperitoneally injected into mice for two times a week for 4 weeks. (**B**) The tumor size progression maps of the two groups of mice were analyzed by 4 weeks of examination. (**C**) After the tumor was peeled off, the weight of each group of tumors was measured. The arrow points to the color change position. (**D**) The expression of H3K79me2, CDK4 and CDK6 protein in mouse tumors was detected by immunohistochemistry, and the positive expression rate of H3K79me2, CDK4 and CDK6 in each group was measured (**E**). *$P<0.05$, **$P<0.01$ and ***$P<0.001$.

potential role to medically address the malignancy is outlined in this work. Therefore, a nomogram was established to predict the OS over 5 years in GC patients.

For *in vitro* study, we blocked the expression of DOT1L in GC cells by transfection, and found that the proliferation ability of the cells was significantly decreased. It was previously reported that DOT1L inhibition in ovarian cancer and lung cancer may result in cell cycle G_1 inhibition and the association of DOT1L with DNA replication [12,19]. By examining the cell cycle, we found that the G_1 phase of GC cells was stagnant, thus causing cell proliferation to slow down. By determining the phenotype through gene expression, we found that down-regulation of H3K79 dimethylation due to DOT1L inhibition resulted in a decrease in CDK4 and CDK6 levels, leading to cell cycle inhibition and slowing of cell proliferation. This provides a new mechanism for the involvement of DOT1L in GC pathogenesis.

Dynamic changes in gene transcription lead to changes in cell morphology, and targeted therapy of small molecules greatly expands the treatment options for cancer patients. Pinometostat (EPZ5676) has been established to inhibit DOT1L enzyme activity, to function as a strategy for the treatment of MLL rearrangement leukemia [20]. Early clinical Phase I trials in adults have shown that EPZ5676 has stable metabolism and anticancer effects. EPZ5676 shows synergy with existing chemotherapeutic drugs [21]. We found *in vitro* that EPZ5676 indeed reduces the activity of DOT1L enzyme in GC cells, and their decreased proliferation activity. In addition, *in vivo* injection of EPZ5676 in nude mice with ectopic tumors was found to significantly inhibit tumor growth. Therefore, EPZ5676 has a good prospect in the progress of GC treatment.

In conclusion, this work suggests the applicability of DOT1L as a predictor of prognosis in GC, and its role in regulating CDK4 and CDK6 via H3K79me2 to subsequently, affect the cell cycle. We also showed that EPZ5676 can

reduce the activity of GC cells by reducing the activity of DOT1L enzyme to exert a therapeutic effect. DOT1L can hence be looked at as an approach to treat GC.

Acknowledgements

We thank Anhui Key Laboratory of Tissue Transplantation of Bengbu Medical College for its help in experiments.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Funding

This work was supported by the Natural Science Foundation of Anhui, China [grant number KJ2018A1013].

Author Contribution

Conceptualization: Jun Qian and Zaozhi Song. Methodology: Zhuoli Wei and Zaozhi Song. Investigation: Nan Wu and Xue Liu. Formal analysis: Xiaoying Tao and Zaozhi Song. Data curation: Qingkang Wang and Xinxin Zhang. Writing – review and editing, Zaozhi Song.

Ethics Approval

The present study was granted ethical approval by the Institutional Review Board of Bengbu Medical College. Written informed consent was obtained from all participants involved in the study.

Abbreviations

CCK-8, cell counting kit-8; CDK, cyclin-dependent kinase; C-index, concordance index; DOT1L, disruptor of telomeric silencing-1-like; GC, gastric cancer; MLL, mixed lineage leukemia; OS, overall survival; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

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