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DNMT1 expression partially dictates 5-Azacytidine sensitivity and correlates with RAS/MEK/ERK activity in gastric cancer cells

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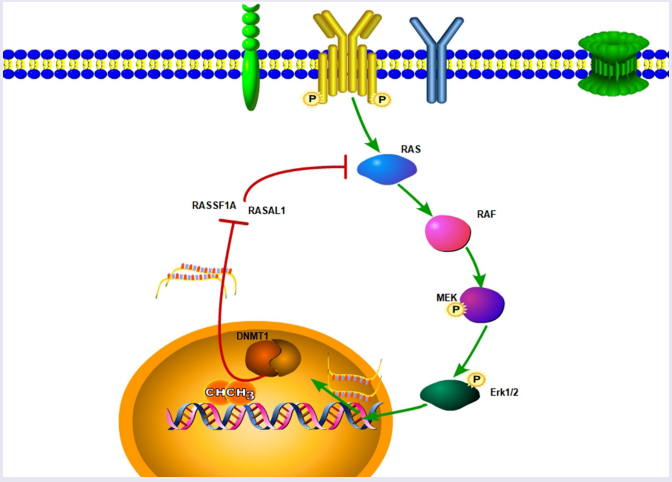
ABSTRACT

Though DNMTs inhibitors were widely used in myelodysplastic syndrome and leukaemia, their application in solid tumours has been limited by low response rate and lack of optimal combination strategies. In gastric cancer (GC), the therapeutic implication of KRAS mutation or MEK/ERK activation for combinational use of DNMTs inhibitors with MEK/ERK inhibitors remains elusive. In this study, stable knockdown of DNMT1 expression by lentiviral transfection led to decreased sensitivity of GC cells to 5-Azacytidine. KRAS knockdown in KRAS mutant GC cells or the MEK/ERK activation by EGF stimulation in GC cells increased DNMT1 expression, while inhibition of MEK/ERK activity by Selumetinib led to decreased DNMT1 expression. 5-Azacytidine treatment, which led to dramatic decline of DNMTs protein levels and increased activity of MEK/ERK pathway, altered the activity of MEK/ERK inhibitor Selumetinib on GC cells. Both RAS-dependent gene expression signature and expression levels of multiple MEK/ERK-dependent genes were correlated with DNMT1 expression in TCGA stomach cancer samples. In conclusion, DNMT1 expression partially dictates 5-Azacytidine sensitivity and correlates with RAS/MEK/ERK activity in GC cells. Combining DNMTs inhibitor with MEK/ERK inhibitor might be a promising strategy for patients with GC.



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Introduction

Epigenetic alterations such as DNA hypermethylation, which lead to chromatin remodelling and dysregulation of cancer-related genes, are essential for cancer development [1,2]. DNA methyltransferases (DNMTs), responsible for

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DNA hyper-methylation in cancer development, are the enzymes that add methyl groups to the 5 position of cytosine residues of CpG dinucleotides [3]. Reversal of DNA hyper-methylation by DNMTs inhibitors is becoming an important treatment option for patients with cancer [4]. Though DNMTs inhibitors were widely used in myelodysplastic syndrome and leukaemia, their application in solid tumours has been limited by low response rate and lack of optimal combination strategies [5]. Therefore, it is of critical importance to identify biomarkers to predict sensitivity to DNMTs inhibitors and develop combinational strategy for their clinical application in solid tumours [4].

DNA methylation is catalysed by three main DNA methyltransferases (DNMTs): DNA methyltransferase 1 (DNMT1), DNA methyltransferase 3A (DNMT3A), and DNA methyltransferase 3B (DNMT3B) [3]. Among the DNA methyltransferases, DNMT1 is thought to be the predominant maintenance methyltransferase gene [3,6]. Silencing of numerous tumour suppressor genes, mediated by DNMT1 methyltransferase, is crucial for the carcinogenesis and progression of GC [7,8]. In the meantime, the potential of targeting DNMT1 as a therapeutic option for patients GC, was proven in various studies [9,10].

The Ras/MEK/ERK pathway mediates various cellular processes, which include cell growth, proliferation, and differentiation [11]. Aberrant activation of RAS/MEK/ERK pathway occurs in more than 30% of human cancers and has been documented as a driving force of proliferation and carcinogenesis for various malignancies [12,13]. Though less frequently detected compared with other malignancies like pancreatic cancer, KRAS mutation or amplification was detectable and indicated poor prognosis in patients with GC [14–16]. In GC, the therapeutic implication of KRAS mutation or MEK/ERK activation for combinational use of DNMTs inhibitors with MEK/ERK inhibitors remains elusive. Moreover, the correlation between MEK/ERK pathway activity and DNMT1 expression has not yet been fully elucidated in GC.

In this study, we first assessed GC cell sensitivity to 5-Azacytidine and explored the role of DNNT1 in conferring sensitivity to DNMTs inhibition. Then, the correlation between DNMT1 expression and RAS/MEK/ERK pathway activity and the prospect of utilizing 5-Azacytidine for combinational treatment with MEK/ERK inhibitor Selumetinib in GC cells were explored.

Material and methods

Cell lines

The human GC cell lines were maintained in our lab and routinely cultured in RPMI-1640 medium (GIBCO, Thermo Fisher Scientific, Waltham, MA, USA), containing with 10% fetal bovine serum (GIBCO, Thermo Fisher Scientific, Waltham, MA, USA), 100 mg/ml streptomycin, and 100 U/ml penicillin sodium. Moreover, the STR profiles were detected and matched with ATCC fingerprints (ATCC. org) before using these cell lines.

Antibody and reagents

5-Azacytidine and CCK-8 were purchased from GLPBIO (Montclair, USA). MEK/ERK inhibitor Selumetinib was purchased from MedChemExpress (Monmouth Junction, NJ, USA). ECL substrate was purchased from 4A Biotech Co. Ltd (Beijing, China). KRAS antibody was purchased from Abcam (Cambridge, UK). α-Tubulin antibody was purchased from Proteintech (Rosemont, IL, USA). DNMT1, P44/42 MAPK (Erk1/2), phospho-P44/42 MAPK (p-Erk1/2) (Thr202/Tyr204), MEK1/2, and phospho-MEK1/2 (Ser217/221) antibodies were purchased from CST (Danvers, MA, USA).

RT-PCR

Total RNAs were isolated using Trizol reagent (Takara, Otsu, Shiga, Japan)) according to the manufacturer's instructions. First-strand cDNA was reversely transcribed from 0.1 μg total RNA in a final volume of 10 μl using reverse transcription reagent kit from Takara (Otsu, Shiga, Japan) according to the manufacturer's instructions. PCR reaction was performed with with a reagent kit from Takara (Otsu, Shiga, Japan) in a PCR cycler (Thermal) according to a protocol as follows: 1 cycle of 95°C for 30 s; 45 cycles of 95°C for 10 s,

extension 60°C for 20 s; and holding at 4°C. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative RNA expression. The primer sequences are listed in Supplementary Table S1.

Western blotting

Cells were lysed in RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with a protease inhibitor cocktail (Roche, Germany) and a phosphatase inhibitor cocktail (Roche, Germany). Proteins from lysed cells were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. Nonspecific binding was then blocked for 2 hours at room temperature in phosphate buffer containing 5% non-fat milk. Membranes were incubated overnight at 4°C with specific primary antibodies. DNMT1 (1:1000, CST, #28754), KRAS (1:1000, Abcam, ab275876), P44/42 MAPK (Erk1/2) (1:1000, CST, #4695), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (1:1000, CST, #4370), MEK1/2 (1:1000, CST, #8727), phospho-MEK1/2 (Ser217/221) (1:1000, CST, #9154), and α-Tubulin (1:20000, Proteintech, 6603–1-Ig).

SiRNA transfection

SiRNAs were designed and synthesized from RiboBio (Guangzhou, China) or Sangon Biotech (Shanghai, China). The negative control siRNA has no significant sequence similarity to mouse, rat, or human gene sequence. The target sequence information was listed in Supplementary Table S2. For siRNA effect validation, a density of 200,000 per well was plated in 6-well plates. The following day, a stock solution containing 6 µl (20 µmol/ml) of siRNA and 6 µl of transfection reagent per 1000 µl Opti-MEM (Thermal Fisher Technologies) was prepared and incubated at room temperature for 15 min. Then, the medium was aspirated from each well and replaced with 1000ul Opti-MEM containing siRNA transfection mix.

Cells exhibiting stable down-regulation of DNMT1 expression were established by transduction with lentivirus (hU6-MCS-Ubiquitin-EGFP-IRES-puromycin) expressing DNMT1 shRNA: LV-shDNMT1 (#1, target sequence: GCCCAA TGAGACTGACATCAA) (Genetech, Shanghai, China). Control cells were transfected with negative control virus CON313 (LV-shControl, target sequence: TTCTCCGAACGTGTCACGT). Hi TransG A and HiTransG P were used as transfection agents. Cells infected with lentivirus were selected with puromycin for 2 weeks.

Chemo-sensitivity analysis

Chemo-sensitivity analyses for 5-Azacytidine or/and Selumetinib were performed by measuring the metabolic activity of the cells using the CCK-8 assay, which was used as a surrogate for cell viability. After incubating for 48-96 h, the cells were then incubated with a CCK-8 solution (GLPBIO, USA) for 2 hours. The absorbance at 450 nm was measured with a Varioskan Flash spectrometer (Thermo Fisher Scientific, Waltham, USA). The IC50, the half maximal inhibitory concentration, was determined by interpolation of dose-response curves. For combined treatment of Selumetinib and 5-Azacytidine, the application schedule with clinically relevant concentrations of both agents was used. 5-Azacytidine: 1uM or 10uM for 24 h followed by Selumetinib (1uM or 10uM) and 5-Azacytidine combined incubation for another 48-72 h. To assess the effects of combined treatment of Selumetinib and 5-Azacytidine, both CCK-8 assay and LDH assay were used. For the LDH assay, after incubation in 96-well plate the cells were then incubated with Lysis Solution, followed by Working Solution and Stop Solution (GLPBIO, USA). Then, to measure the total LDH release, the absorbance at 490 nm was measured with a Varioskan Flash spectrometer (Thermo Fisher Scientific, Waltham, USA). The effect of drugs on cell proliferation was calculated as percentage growth of cells relative to respective controls (DMSO-treated cells). CompuSyn software (Cambridge, UK) was used to calculate combination index. Combination index (CI) values less than 1.0 indicate a synergistic interaction of the two agents in the combination.

Immunohistochemistry (IHC)

IHC was performed on sections of tumour tissue samples from GC patients as previously described [17]. The sections were incubated with the diluted antibody: DNMT1 (ab188453, 1:100). The final immunoactivity score of each section was determined by two independent observers in a blinded manner.

IHC scores of 0–12 were calculated and graded as negative (−, score: 0), weak (+, score: 1–4), moderate (+ +, score: 5–8), or strong (+ + +, score: 9–12).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). The Student's t-test or Fisher's exact test was used to compare differences between the two experimental groups. Differences were considered significant when $p < 0.05$.

Results

The DNMT1 expression level partially dictates 5-Azacytidine sensitivity in GC cells

We first detected the expression level of DNMT1 in a subset of GC cells and immortalized gastric epithelial GES-1 cells by western blotting and RT-PCR, respectively (Figure 1a,b). Then, the intrinsic sensitivity to 5-Azacytidine was determined by CCK-8 assay in the above cells. The results showed that MKN-45, MKN-28, SNU-1, and GES-1 cells were relatively sensitive to 5-Azacytidine treatment (IC50 < 4uM), while AGS, BGC-823, SGC-7901, and MGC-803 cells were relatively resistant (IC50 > 4uM) (Figure 1c,d).

We then determined whether DNMT1 expression dictates 5-Azacytidine sensitivity in GC cells. DNMT1 was stably knocked down by lentiviral transfection, and the efficiency of DNMT1 knockdown was validated by western blotting in 5-Azacytidine relatively resistant BGC-823 and relatively sensitive MKN-45 cells (Supplementary Figure S1). IC50 value to 5-Azacytidine was significantly increased upon DNMT1 knockdown in BGC-823 and MKN-45 cells (Figure 1e,f).

MEK/ERK inhibition led to decreased DNMT1 expression in GC cells

Previous study showed that RAS/MEK/ERK activity promoted DNA methylation by increasing DNMT1 expression in colon cancer cells [18]. Therefore, we speculated a similar phenomenon might exist in GC cells. First, we determined the activity of MEK/ERK pathway in GC cells by detecting the p-Erk1/2 level by western blotting (Supplementary Figure S2). In MEK/ERK moderately hyperactive BGC-823 and highly hyperactive AGS cells, Selumetinib treatment significantly inhibited the activity of MEK/ERK pathway (indicated by p-Erk1/2 level) and led to decreased DNMT1 expression (Figure 2a,b). EGF stimulation, which induced significant activation of MEK/ERK pathway, also led to increased expression of DNMT1 in both MEK/ERK moderately hyperactive BGC-823 and MEK/ERK hypoactive MKN-45 cells (Supplementary Fig. S3, Figure 2c,d).

In KRAS mutant and MEK/ERK hyperactive AGS cells, KRAS was knocked down by siRNA transfection (Figure 2e). Significant decrease of MEK/ERK pathway activity and concurrent down-regulation of DNMT1 expression were observed upon KRAS knockdown in AGS cells (Figure 2f).

5-Azacytidine treatment led to significant decline of DNMT1 and DNMT3A protein levels in GC cells

It was observed that 5-Azacytidine treatment led to dose-dependent down-regulation of DNMT1 protein expression in both KRAS mutant AGS and KRAS wild-type BGC-823 cells (Figure 3a,b). Similarly, dramatic decline in protein expression level of DNMT3A was also observed in both AGS and BGC-823 cells upon 5-Azacytidine treatment (Figure 3a,b). A decline of DNMT3B protein level was observed upon high dosage (10uM) of 5-Azacytidine treatment in AGS cells (Figure 3a,b). RT-PCR results showed that 1uM 5-Azacytidine treatment led to increased mRNA levels of DNMT1, DNMT3A, and DNMT3B (Figure 3c,d). However, high concentration of 5-Azacytidine (10uM) treatment led to inconsistent alteration of the DNMT1 and DNMT3B mRNA levels between AGS and BGC-823 cells, while no significant effect on DNMT3A mRNA levels was observed (Figure 3c,d).

5-Azacytidine pre-treatment led to increased activity of the MEK/ERK pathway in GC cells

We also observed that 5-Azacytidine treatment led to the increased activity of MEK/ERK pathway, as indicated by the phosphorylation levels of MEK1/2 and ERK1/2 (p-MEK1/2 and p-Erk1/2) in KRAS wild-type BGC-823 and KRAS mutant AGS cells (Figure 4a,b). As the activity of the MEK/ERK pathway was correlated with the sensitivity to MEK/ERK inhibitors, we set out to explore whether 5-Azacytidine pre-treatment altered the activity of Selumetinib on GC

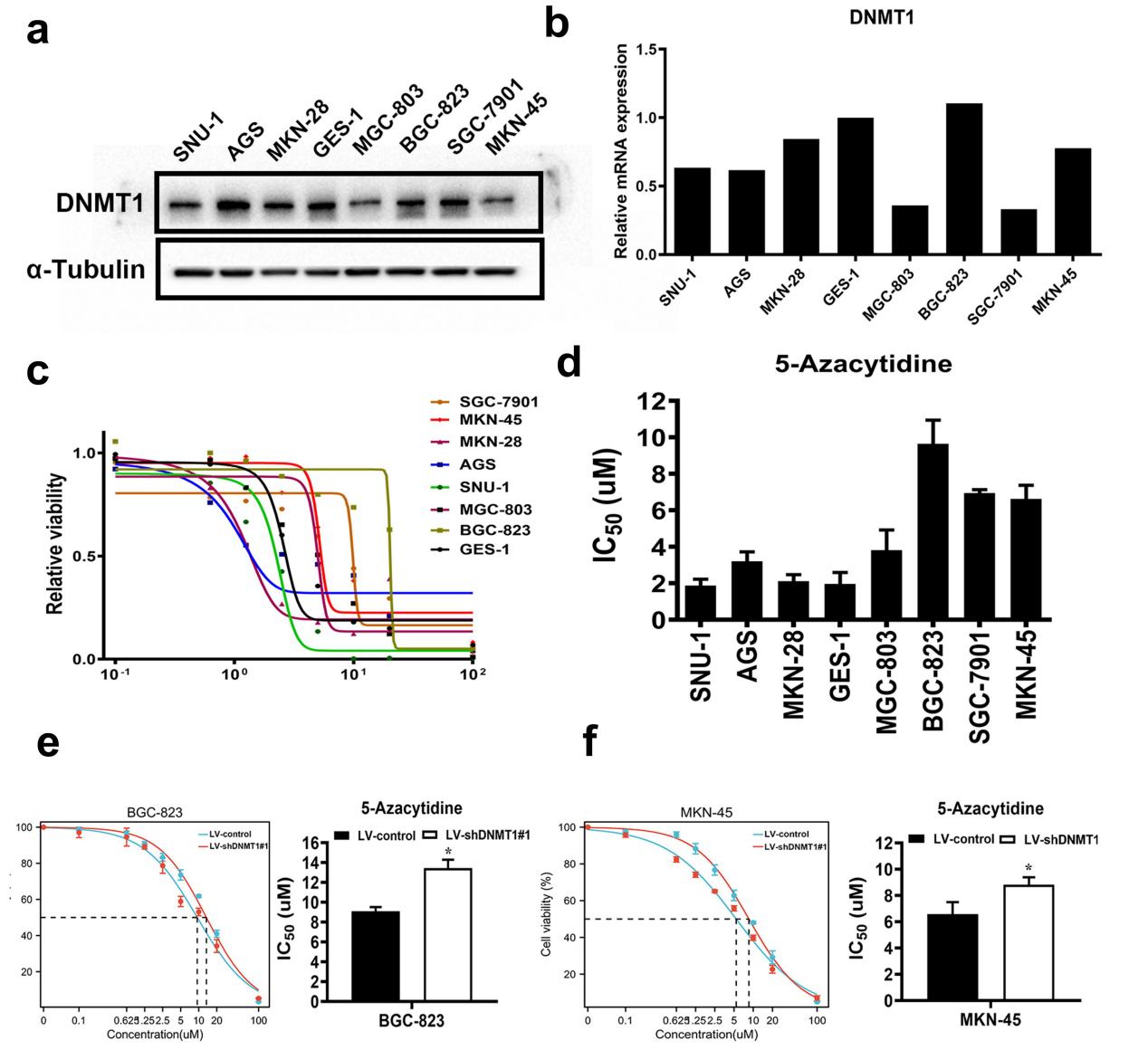

Figure 1. The DNMT1 expression level partially dictates 5-Azacytidine sensitivity in GC cells.

Note: a. The mRNA expression of DNMT1 in seven GC cell lines and immortalized normal epithelial GES-1 cells was detected by RT-PCR. The relative expression of DNMT1 was calculated by normalizing the DNMT1 mRNA level of each cell line to the median DNMT1 mRNA level among the eight cell lines. ACTB served as an internal control. b. The protein expression of DNMT1 in seven GC cell lines and immortalized normal epithelial GES-1 cells was detected by western blotting. α-Tubulin served as an internal reference. c. Dose–response curves of the seven GC cell lines and immortalized normal epithelial GES-1 cells to 5-Azacytidine. d. IC50 value to 5-Azacytidine of seven GC cell lines and immortalized normal epithelial GES-1 cells was evaluated by CCK-8 assay. Data are shown as the mean± SD obtained from three independent experiments. e. BGC-823 cells were transfected with lentivirus expressing shRNA specific for DNMT1 or control siRNA. The IC50 value to 5-Azacytidine of shDNMT1#1 and shControl cells was evaluated. IC50 value of shDNMT#1 cells was normalized to that of the shControl cells. Two-tailed Student's t-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. f. MKN-45 cells were transfected with lentivirus expressing shRNA specific for DNMT1 or control shRNA. The IC50 value to 5-Azacytidine of shDNMT1#1 and shControl cells was evaluated. IC50 value of shDNMT#1 cells was normalized to that of the shControl cells. Two-tailed Student's t-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

cells. Interestingly, a synergistic effect (CI value < 1) was observed when a relatively high concentration of Selumetinib (10uM) was combined with high concentration of 5-Azacytidine (10uM) in BGC-823 cells (Figure 4c,d, Table 1). Moreover, in KRAS mutant and MEK/ERK hyperactive AGS cells, both high-concentration and low-concentration combination of 5-Azacytidine and Selumetinib produced strong synergistic effects (CI value < 0.8, Figure 4e,f and Table 1).

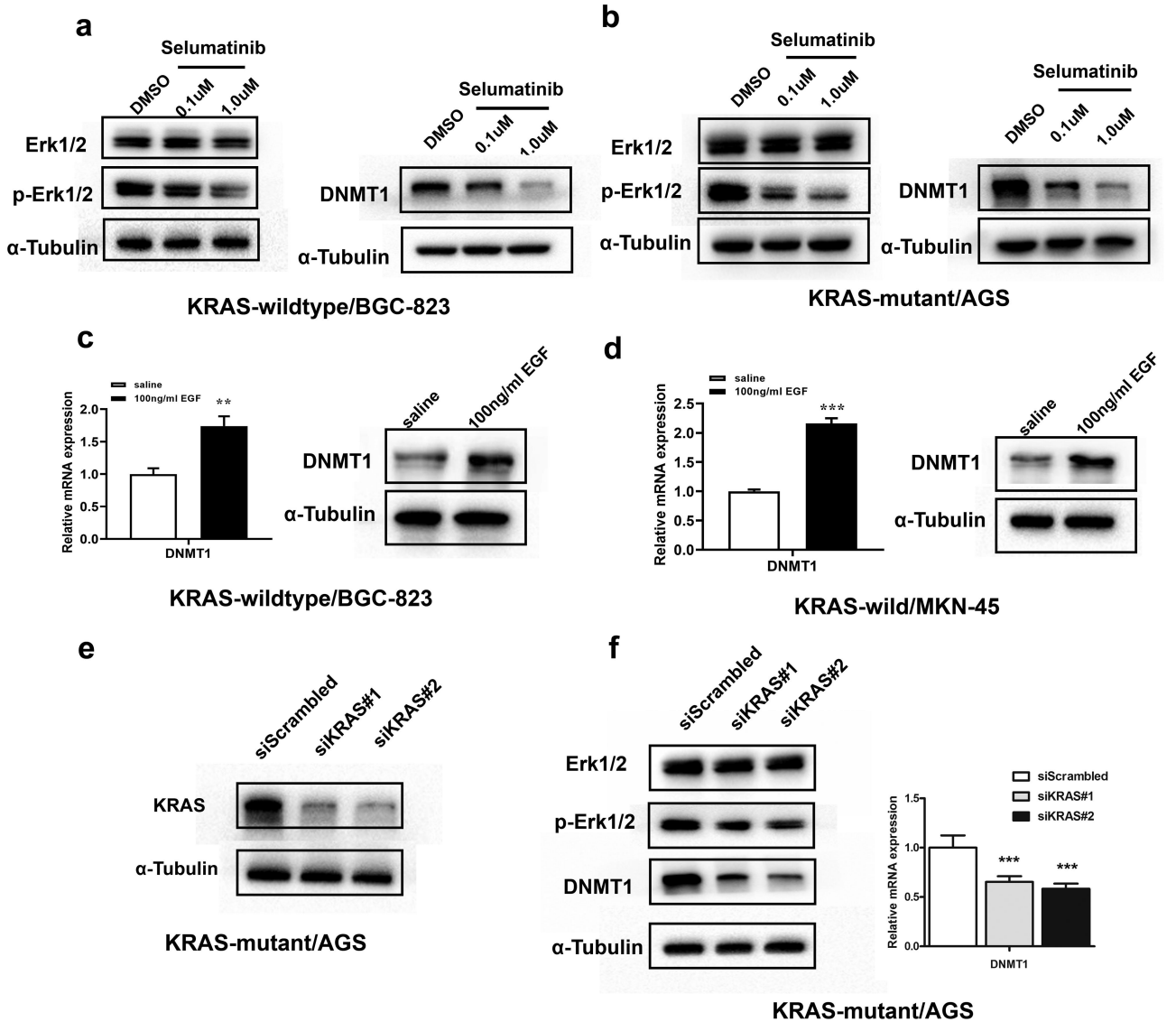

Figure 2. MEK/ERK inhibition led to decreased DNMT1 expression in GC cells.

Note: a. b. BGC-823 and AGS cells were treated with DMSO and 0.1uM and 1uM Selumetinib for 24 hours, respectively. Then, the protein levels of total Erk1/2, p-Erk1/2, and DNMT1 were detected by western blotting. α-Tubulin served as an internal reference. c. d. BGC-823 and MKN-45 cells were starved of serum for 12 hours and then stimulated with saline or EGF (100 ng/ml) for 24 hours. Then, the expression levels of DNMT1 were detected by RT-PCR and western blotting, respectively. ACTB or α-tubulin served as an internal reference. The relative level of DNMT1 mRNA was normalized by that of saline-treated cells. The level of DNMT1 in EGF-treated cells was compared to that of the saline-treated cells. Two-tailed Student's t-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. e. AGS cells were transfected with siRNAs specific for KRAS (siKRAS1#1 or siKRAS#2) or control siRNA (siScrambled) for 48 hours. Then, western blotting was performed to validate the knockdown efficiency. α-Tubulin served as an internal reference. f. AGS was transfected with siRNA specific for KRAS (siKRAS#1 or siKRAS#2) or control siRNA (siScrambled), respectively. The protein level of ERK1/2, p-ERK1/2, and DNMT1 was detected by western blotting. α-Tubulin served as an internal reference.

DNMT1 expression was correlated with RAS/MEK/ERK activity in GC

To determine a possible correlation of DNMT1 expression with RAS/MEK/ERK activity in GC, public database containing gene expression data of TCGA GC samples was accessed. Analysis revealed that among the genes showing highly significant correlations with DNMT1 expression in TCGA GC samples, numerous MEK/ERK pathway-dependent genes were enriched, which included ETS-1, E2F1, and CCND1 (Figure 5a). Western blotting analysis showed that MEK/ERK pathway activity, indicated by p-Erk1/2

Table 1. Combinational index (CI) value for sequential treatment with 5-Azacytidine and Selumetinib in AGS and BGC-823 cells. CI value: <1 synergistic; = 1 Additive; and > 1 Antagonistic.

5-Azacytidine(uM)	Selumetinib (uM)	CI values of CCK-8 assay (CI values of LDH assay)	
		AGS	BGC-823
1	1	0.57(0.52)	24.8 (9.76)
10	10	0.38(0.37)	0.47 (0.72)

level, was higher in DNMT1 relatively high-expressing AGS, GES-1, SGC-7901, and BGC-823 cells (Figure 5b). Furthermore, the DNMT1 expression level was also significantly correlated with RAS-dependent gene signature ($r = 0.2417$, $p < 0.0001$) (Figure 5c).

DNMT1 was up-regulated in GC tumour tissues compared with adjacent normal tissues

Then, we detected the DNMT1 expression by immunohistochemistry in a panel of 20 GC tissues and observed an increased expression of DNMT1 in majority of tumour tissues compared with paired adjacent normal tissues (Figure 6a).

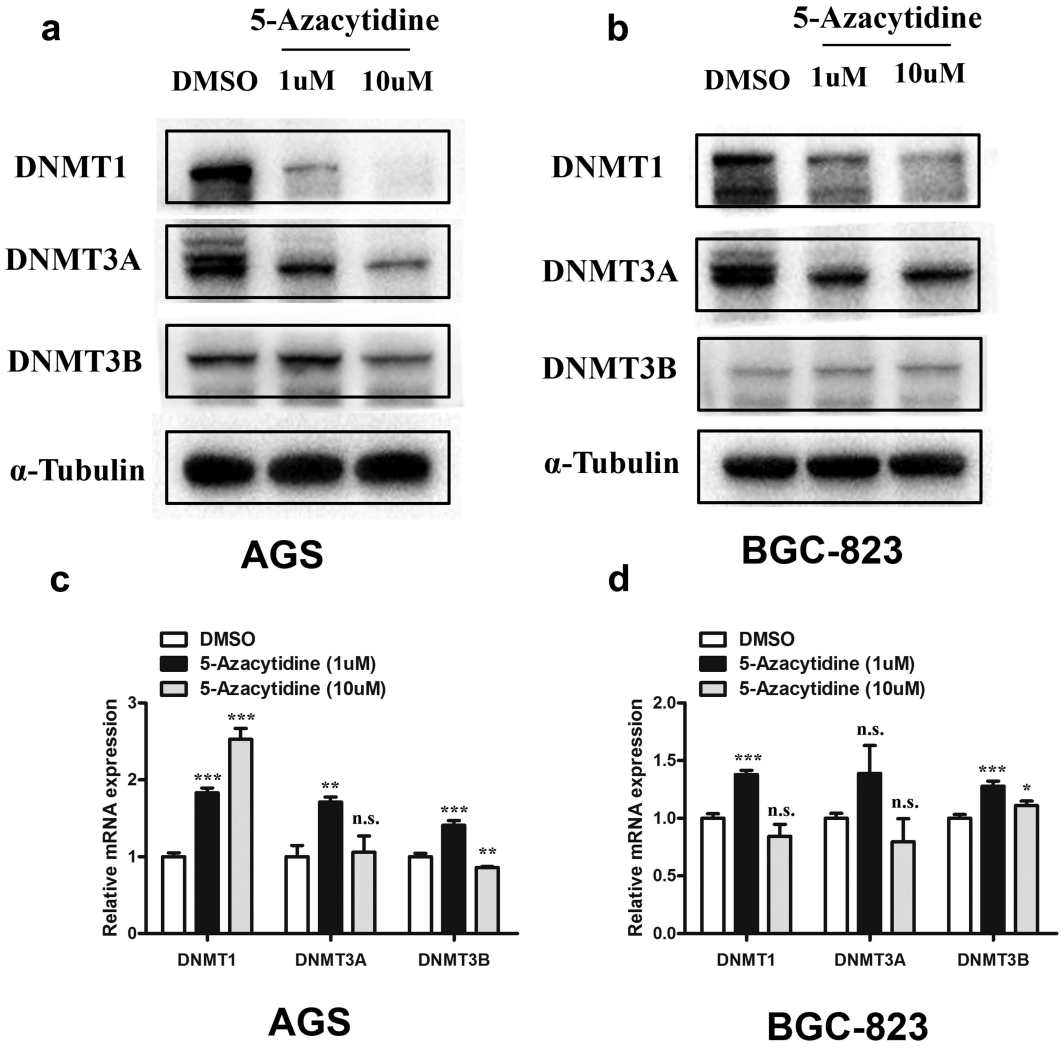

Figure 3. 5-Azacytidine treatment led to significant decline of DNMT1 and DNMT3A protein levels in GC cells.

Note: a. b. AGS and BGC-823 cells were treated with DMSO, 1uM 5-Azacytidine, and 10uM 5-Azacytidine for 48 hours, and protein was extracted. Then, western blotting was performed to detect the protein levels of DNMT1, DNMT3A, and DNMT3B. α-Tubulin served as an internal reference. c. d. AGS and BGC-823 cells were treated with DMSO, 1uM 5-Azacytidine, and 10uM 5-Azacytidine for 48 hours, and RNA was extracted. RT-PCR was performed to detect the mRNA levels of DNMT1, DNMT3A, and DNMT3B. ACTB served as an internal reference. The relative mRNA levels were normalized to that of DMSO-treated cells. Two-tailed Student's t-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. n.s., not significant.

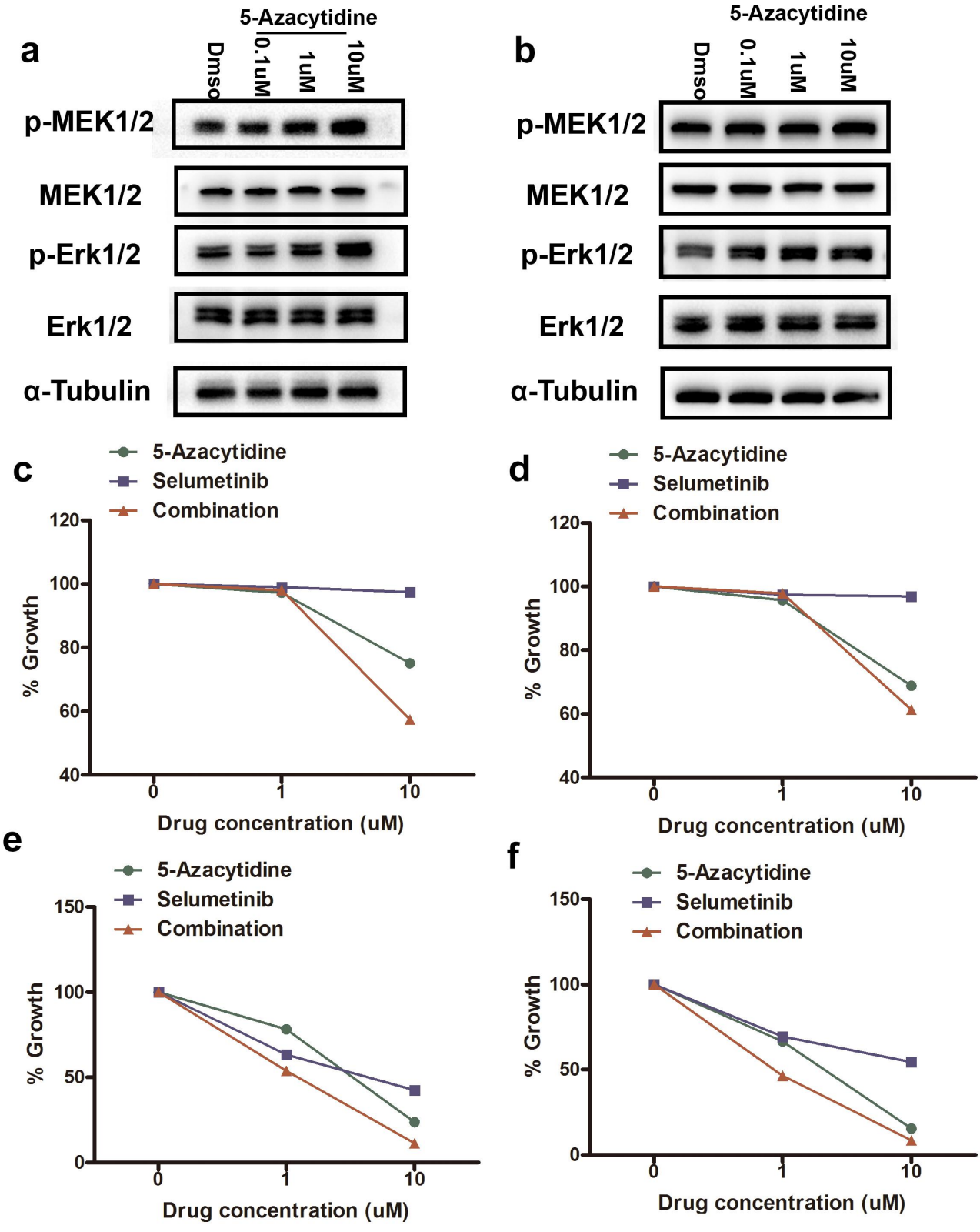

Figure 4. 5-Azacytidine pre-treatment led to increased activity of the MEK/ERK pathway in GC cells.

Note: a. BGC-823 cells were treated with DMSO, 0.1uM, 1uM, and 10uM 5-Azacytidine for 24 hours. Western blot was performed to detect the phosphorylation and total level of MEK1/2 and Erk1/2. α-Tubulin was served as an internal reference. b. AGS cells were treated with DMSO, 0.1uM, 1uM, and 10uM 5-Azacytidine for 24 hours. Western blot was performed to detect the phosphorylation and total level of MEK1/2 and Erk1/2. α-Tubulin was served as an internal reference. c. CCK-8 assay was performed in BGC-823 cells to evaluate the effect of sequential treatment with 5-Azacytidine and Selumetinib on cell proliferation. The effect of drugs on cell

Bioinformatics analysis of TCGA GC database revealed that DNMT1 was significantly higher in GC tumour tissues than normal tissues (Figure 6b). However, DNMT1 expression was not increased in high-stage (III/IV) GC tumour tissues compared with low-stage (I/II) GC tumours tissues (Figure 6c). Moreover, DNMT1 expression was not significantly correlated with the prognosis of GC patients (Figure 6d).

Discussion

5-Azacytidine and 5-Aza-deoxycytidine (decitabine) are both chemical analogs of cytidine and important DNMTs inhibitors approved by the US FDA for myelodysplastic syndrome leukaemia and leukaemia treatment [19,20]. In more recent years, the immunomodulatory and anti-tumour effects of DNMTs inhibitors were described in solid tumours [21–24]. However, the application of DNMTs inhibitors in solid tumours is challenged by low response rate and lack of optimal combination strategies [24,25]. Therefore, the discovery and identification of biomarkers to predict response are urgently needed. Meanwhile, the development of alternative treatment options such as optimal combination strategy is imperative.

Jia Yu et al showed that 5-Aza-deoxycytidine (decitabine) sensitivity in 45 solid tumour cells was associated with DNMT1 mRNA level [26]. Another study showed that high DNMT3B protein level dedicated 5-Aza-deoxycytidine hypersensitivity in Testicular Germ Cell Tumors [27]. The results from organoid culture originating from triple negative breast cancer PDX models showed that 5-Aza-deoxycytidine sensitivity was highly correlated with both DNMT3A and DNMT3B protein levels [28]. In this study, we observed that DNMT1 expression partially dictated 5-Azacytidine sensitivity, as shown by increased IC50 to 5-Azacytidine in DNMT1 knockdown GC cells. Likely due to the small number of GC cell lines detected, we did not observe a significant correlation between DNMT1 expression and 5-Azacytidine sensitivity (data not shown). It is suggested that the difference in mode of action among different DNMTs inhibitors, and tumour heterogeneity might be the factors associated with the discrepancy of the above results [29,30]. Moreover, the pre-clinical models, which more faithfully represent human tumour biology and clinical treatment response, are warranted to discover or validate the predictive value of these biomarkers. Our results further showed 5-Azacytidine treatment led to dramatic decline of DNMT1 and DNMT3A protein levels in GC cells, which suggests that 5-Azacytidine might mainly function through DNA methyltransferase DNMT1 or DNMT3A in GC cells.

Previous study showed that 5-Aza-deoxycytidine treatment induced significant activation of MEK/ERK pathway in ovarian cancer cells [26]. We observed that treatment with 5-Azacytidine also increased the activity of the MEK/ERK pathway in both KRAS wild-type and KRAS-mutant GC cells. Given the fact that the increased activity of MEK/ERK correlated with the sensitivity to MEK/ERK inhibitors in tumour cells, we further explored the effect of combining MEK/ERK inhibitor Selumetinib with 5-Azacytidine. Interestingly, when Selumetinib of relatively high concentration (10uM) was combined with 5-Azacytidine of approximately IC50 value (10uM), a synergistic effect was observed in KRAS wild-type BGC-823 cells. More importantly, strong synergistic effects were also observed in KRAS mutant and MEK/ERK hyperactive GC AGS cells, irrespective of the drug concentration. These results suggested that the activation status of RAS/MER/ERK pathway and the combination dosage might be the key factors determining the effects of the combined treatment.

proliferation was calculated as percentage growth of cells relative to DMSO-treated cells. d. LDH assay was performed in BGC-823 cells to evaluate the effect of sequential treatment with 5-Azacytidine and Selumetinib on cell proliferation. The effect of drugs on cell proliferation was calculated as percentage growth of cells relative to DMSO-treated cells. e. CCK-8 assay was performed in AGS cells to evaluate the effect of sequential treatment with 5-Azacytidine and Selumetinib on cell proliferation. The effect of drugs on cell proliferation was calculated as percentage growth of cells relative to DMSO-treated cells. f. LDH assay was performed in AGS cells to evaluate the effect of sequential treatment with 5-Azacytidine and Selumetinib on cell proliferation. The effect of drugs on cell proliferation was calculated as percentage growth of cells relative to DMSO-treated cells.

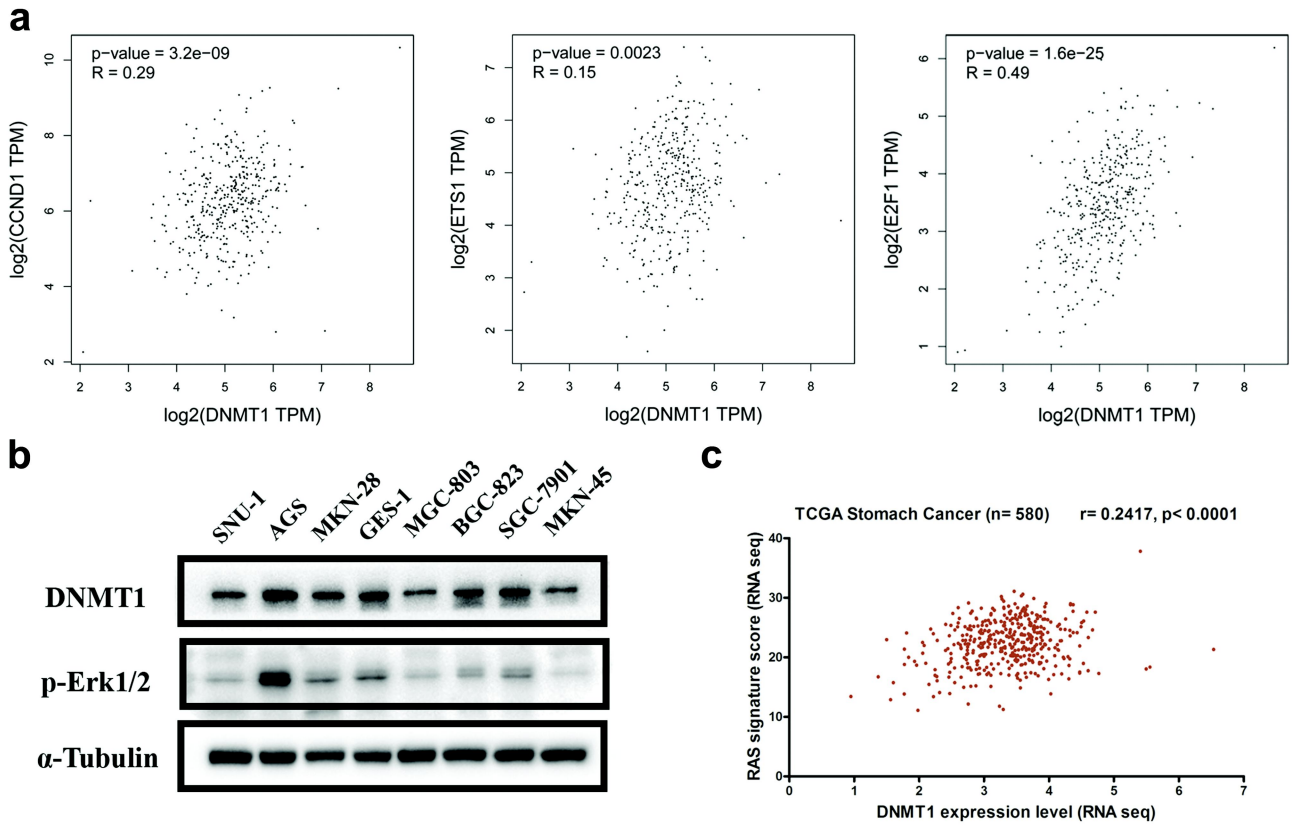

Figure 5. DNMT1 expression was correlated with RAS/MEK/ERK activity in GC.

Note: a. Correlation between DNMT1 expression level and ETS1, E2F1, or CCND1 expression level in TCGA GC samples was analysed in GEPIA database (http://gepia.cancer-pku.cn/). b. Western blotting analysis of p-Erk1/2 level and DNMT1 expression level in seven GC cells and immortalized epithelial GES-1 cells. α-Tubulin served as an internal reference. c. Correlation analysis of RAS-dependent gene signature with DNMT1 expression level (mRNA sequencing) was performed in TCGA GC samples using the Pearson correlation method. Gene list of RAS-dependent gene expression signature was consisted of EGR1, DUSP6, FOSL1, ETV1, ETV4, ETV5, and SPRY-4.

RAS/MEK/ERK signalling was reported to regulate DNMT1 expression and DNMT1-dependent DNA methylation in various tumours [18,31,32]. In this study, EGF-induced MEK/ERK activation led to the increased DNMT1 expression in GC cells. Moreover, decrease of MEK/ERK activity by KRAS siRNA transfection in KRAS mutant GC cells or small-molecule inhibitor of MEK/ERK pathway in MEK/ERK hyperactive GC cells decreased DNMT1 expression. On the one hand, silencing of tumour suppressor genes by DNMT1 methyltransferase activity is essential for oncogenic transformation by genetic mutation like RAS activation mutation [33,34]. On the other hand, a combination of genetic mechanisms with epigenetic alterations might account for the oncogenic transformation in various tumours [35,36].

Then, both immunohistochemistry and bioinformatics analysis showed that DNMT1 expression is increased in GC tumour tissues compared with adjacent normal tissues, which is consistent with previous findings that DNMT1 is expressed at higher levels in early gastric cancer or high-grade intraepithelial neoplasia tissues compared to that in non-cancer mucosa or low-grade intraepithelial neoplasia tissues [37]. Mechanistically, H. pylori-induced chronic inflammation, but not H. Pylori itself, played a direct role in the induction of DNMT1 expression and aberrant DNA methylation in GC [38]. Moreover, suppression of aberrant DNA methylation by a demethylating agent was shown to inhibit gastric cancer development in an animal model [39]. However, DNMT1 expression was found not correlated with higher tumour stage and poorer prognosis in patients with GC. Taken together, we proposed that DNMT1 is more likely to play critical roles in the initial stages of GC, but not in the advanced stages of GC.

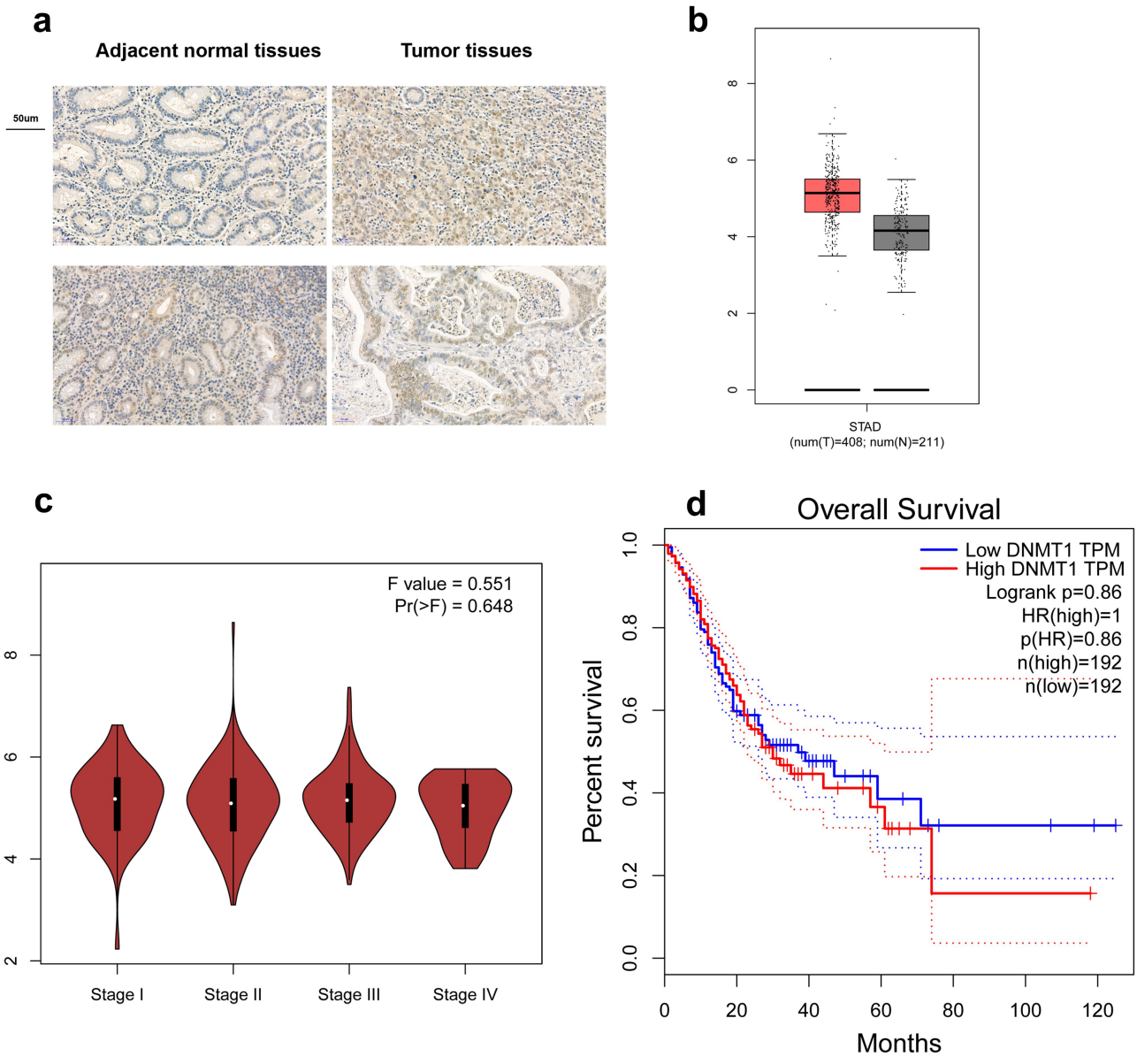

Figure 6. DNMT1 was up-regulated in GC tumour tissues compared with adjacent normal tissues.

Note: a.The expression of DNMT1 in GC tumour tissues and their paired adjacent normal tissues was analysed by IHC. Representative pictures of DNMT1 expression in tumour tissues and adjacent normal tissues. Scale bar = 50uM. b. The expression of DNMT1 in GC tumour tissues and normal tissues was analysed in GEPIA database (http://gepia.cancer-pku.cn/). c. The expression of DNMT1 in GC tumour tissues of different stages was analysed in GEPIA database. d. Kaplan–Meier analysis of overall survival rate of TCGA GC patients with high and low DNMT1 expression in GEPIA database.

The interaction between DNMT1 and RAS/MEK/ERK pathway has been proven to be one key molecular cascade in tumour carcinogenesis [34,35,40,41]. Prior studies have shown that via inactivation of RAS inhibitor genes, which included DAB2, RASSF1A, RASAL1, and DAB2IP, DNA hyper-methylation was associated with aberrant activation of RAS signalling and increased tumorigenesis [35,41,42]. We also observed the increased expression of RASSF1A, RASAL1, and DAB2IP upon DNMT1 knockdown or DNMTs inhibition by 5-Azacytidine in GC cells (data not shown). Whether and how the expression or activity of DNMT1 might affect the activity of RAS/MEK/ERK pathway in GC warrant further investigation. Moreover, the molecular mechanisms underlying the interaction between DNMT1 methyltransferase and RAS/MEK/ERK pathway in GC remain to be elucidated.

In summary, the RAS/MEK/ERK-DNMT1 signalling pathway in GC was validated in our study.

Our results also suggested that combined targeting of DNA methyltransferases with MEK/ERK inhibitors might be a promising treatment option for patients with GC. Future studies are needed to test the therapeutic benefit of this strategy in animal models and ultimately in patients with GC.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contribution

Zhangqian Chen, Lin Zhang, and Yang Yang contributed to acquisition of data, analysis and interpretation of data, statistical analysis, and writing original draft; Haiming Liu and Xiaoyu Kang contributed to software and data curation; Daiming Fan, Yongzhan Nie, and Zhangqian Chen contributed to study concept and design, reviewing and editing of the manuscript, and study supervision.

Availability of data

The data that support the findings of this study are available from the corresponding author, Daiming Fan, upon reasonable request.

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