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DNA methyltransferase 1 as a predictive biomarker and potential therapeutic target for chemotherapy in gastric cancer

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ABSTRACT

Purpose: DNA methylation contributes to carcinogenesis by mediating transcriptional regulation and chromatin remodelling, which may influence the effect of DNA-damaging drugs. We examined the prognostic and predictive impact of DNA methyltransferase (DNMT) 1 and 3b expression in gastric carcinomas (GC) treated by neoadjuvant chemotherapy. *In vitro*, DNMT1 expression and chemosensitivity were investigated for a functional relationship and the DNMT inhibitor decitabine (DAC) was tested as an alternative treatment option.

Patients and methods: DNMT1/3b expression was analysed immunohistochemically in 127 pretherapeutic biopsies of neoadjuvant (platinum/5-fluorouracil)-treated GC patients and correlated with response and overall survival (OS). Short hairpin RNA technology was used to knockdown DNMT1 in the GC cell line, AGS. The chemosensitivity of GC cell lines to DAC alone and to DAC in combination with cisplatin was analysed by XTT or colony formation assays.

Results: High DNMT1 and DNMT3b expression was found in 105/127 (83%) and 79/127 (62%) carcinomas, respectively. Patients with low DNMT1 expression demonstrated a significantly better histopathological/clinical response ($P = 0.03/P = 0.008$) and OS ($P_{log\text{-}rank} = 0.001$). *In vitro*, knockdown of DNMT1 caused an increased chemosensitivity towards cisplatin. Combined treatment with cisplatin and DAC showed a synergistic effect leading to increased cytotoxicity in the cisplatin-resistant cell line AGS.

Conclusion: Low DNMT1 expression defines a subgroup of GC patients with better outcomes following platinum/5FU-based neoadjuvant chemotherapy. *In vitro* data support a functional relationship between DNMT1 and cisplatin sensitivity. Besides its potential use as a predictive biomarker, DNMT1 may represent a promising target for alternative therapeutic strategies for a subset of GC patients.

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1. Introduction

Gastric carcinoma (GC) is characterised by poor prognosis although multimodal treatment protocols, mainly based on platinum and 5-fluorouracil (5FU), have been shown to prolong patient survival.[1,2] Since response rates to this therapy are rather low (20–40%),[1,2] the discovery of biomarkers to predict response and prognosis to treatment and the development of novel therapeutic options is imperative.

Epigenetic alterations such as promoter hypermethylation, which lead to chromatin remodelling and silencing of cancer-related genes, play a crucial role in cancer development.[3] In GC, hypermethylation has been demonstrated for various genes and a concordant hypermethylation of multiple genes, termed the CpG island methylator phenotype (CIMP), has been described.[4,5]

DNA methylation is catalysed by three main enzymes: DNA methyltransferase (DNMT) 1, DNMT3a, and DNMT3b. DNMT1 is primarily involved in the maintenance of methylation during DNA replication, and DNMT3a/b are thought to function in *de novo* methylation, although overlapping functions of these enzymes have also been described.[6,7] Overexpression of DNMT1 and DNMT3b has been reported in tumours including gastric carcinomas, and a correlation of DNMT1 expression with the occurrence of the CIMP phenotype has been reported.[5] Furthermore, DNA methylation is related to chromatin condensation, which might influence the efficacy of DNA-binding agents such as cisplatin.[8,9]

In a previous study that involved a group of gastric cancer patients treated by a cisplatin/5FU-based neoadjuvant chemotherapy, a concordant methylation of multiple genes was suggestive for an association with worse response to therapy.[10] In this study, we aimed to address the following questions: (1) Is there a predictive and/or prognostic impact of the expression of DNMT1 and/or DNMT3b for GC patients receiving a platinum/5FU-based neoadjuvant therapy? (2) Is there a functional relationship between DNMT expression and chemosensitivity by using a specific shRNA knockdown approach *in vitro*? and (3) As DNMT inhibitors are already in clinical use for the treatment of myelodysplastic syndrome,[9] can the application of the DNMT inhibitor, decitabine (5-aza-2′-deoxycytidine) (DAC), improve the treatment in a preclinical model using gastric cancer cell lines?

2. Materials and methods

2.1. Patients, chemotherapy and follow-up

This retrospective, explorative analysis was performed as a part of the consecutive phase II studies conducted at the Department of Surgery at the Technische Universität München, which evaluated preoperative chemotherapy in patients with locally advanced GC.[11,12] Eligibility criteria for chemotherapy were as described.[12,13] Pretherapeutic biopsies of 127 patients with GC (tumour category cT3 or cT4), who were treated from 1994 to 2006 and who obtained more than 50% of the projected dose of chemotherapy, were included in the analysis. All patients received preoperative chemotherapy based on platinum (122 patients received cisplatin, 5 patients received oxaliplatin) and 5FU. Two patients additionally received epirubicin. The treatment protocol is included in the Supplementary Data. An additional inclusion criterion was the availability of sufficient tumour tissue for analysis. To confirm the representative nature of the analysed patients compared to the total of 347 patients, who were treated in the respective time period with the described regimens, the distribution of clinicopathological parameters was compared and did not reveal significant differences. The use of tissues was approved by the local Institutional Review Board and informed consent was obtained according to institutional regulations. Patient characteristics are included in Table 1.

Follow-up was calculated from the first day of treatment until the last contact with the patients. The median follow-up was 52.8 months (range: 7.0–112.3; 95% confidence interval [CI]: 40.9–64.7).

Overall survival (OS) was defined as the time between the first day of chemotherapy and death by any cause. The median OS was 50.6 months (range: 3.9–112.3; 95% CI: 24.6–76.6).

2.2. Response evaluation

Response evaluation based on histopathological and clinical criteria was performed as described.[13,14] In brief, all patients displaying less than 10% residual tumour cells (regression score 1) in the resected specimens were classified as histopathological responders. Patients with regression scores 2 and 3 (10–50% and >50% residual tumour cells, respectively) and patients with progressing disease during chemotherapy, were classified as histopathological nonresponders.[14] Clinical response was defined as at least 50% reduction in the size of the primary tumour as described.[13] According to their histopathological response, the patients comprised 34 responders (27%) and 93 nonresponders (73%). Based on the evaluation of clinical responses, 44 responders (35%) and 83 nonresponders (65%) were identified (Table 1).[15] Histopathological and clinical responses were significantly associated with OS (both $P_{\text{log-rank}}$-values <0.001).

2.3. Immunohistochemistry

Sections of formalin-fixed and paraffin-embedded pretherapeutic biopsies were incubated with the antibodies for DNMT1 (goat polyclonal sc-1021; dilution 1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and DNMT3b (mouse monoclonal; IMG-184A; dilution 1:200; IMGENEX, San Diego, CA). Both primary antibodies have been characterised.[5,16,17] The staining procedure is included in the Supplementary Data.

2.4. Evaluation of staining

DNMT1 and DNMT3b expression were assessed by two independent observers (KM, RL) scoring the percentage of nuclear positive tumour cells. The percentage of positive tumour cells was scored as <30%, 30–50%, and >50%. A percentage of <30% positive tumour cells was defined as low expression of DNMT1, and a percentage of $\geqslant$30% was defined as high

Table 1 – Patient characteristics and DNMT expression.

	No. of patients	DNMT1 expression			DNMT3b expression		
	n (%)	*n* low	*n* high	*P*	*n* low	*n* high	*P*
Preoperative characteristics							
Total	127 (100)	22	105		48	79	
Age, years							
<60	55 (43)	10	45	0.823[a]	21	34	0.937[a]
⩾60	72 (57)	12	60		27	45	
Sex							
Female	33 (26)	4	29	0.359[a]	16	17	0.141[a]
Male	94 (74)	18	76		32	62	
Localisation							
Proximal third	92 (72)	18	74	0.709[b]	29	63	0.048[b]
Middle third	18 (14)	2	16		10	8	
Distal third	12 (10)	1	11		5	7	
Total	5 (4)	1	4		4	1	
Laurén classification							
Intestinal	53 (42)	11	42	0.387[a]	15	38	0.062[a]
Non-intestinal	74 (58)	11	63		33	41	
Grading							
G1/2	20 (16)	8	12	0.008[c]	5	15	0.199[a]
G3/4	107 (84)	14	93		43	64	
Response							
Histopath. responders	34 (27)	10	24	0.03[a]	10	24	0.239[a]
Histopath. nonresponders	93 (73)	12	81		38	55	
Clinical responders	44 (35)	13	31	0.008[a]	15	29	0.531[a]
Clinical nonresponders	83 (65)	9	74		33	50	
Postoperative characteristics							
Resection	124/127 (98)						
R-category							
R0	101/124 (81)	20	81	0.363[c]	35	66	0.238[a]
R1/2	23/124 (19)	2	21		11	12	
ypT-category[d]							
ypT0/1	17/124 (14)	7	10	0.013[c]	7	10	0.708[a]
ypT2/3/4	107/124 (86)	15	92		39	68	
ypN-category[d]							
ypN0	51/124 (41)	15	36	0.004[a]	22	29	0.244[a]
ypN1/2/3	73/124 (59)	7	66		24	49	
ypM-category[d]							
ypM0	98/124 (79)	22	76	0.007[c]	35	63	0.536[a]
ypM1	26/124 (21)	0	26		11	15	

[a] Pearson's χ^2-test.
[b] Freeman–Halton test.
[c] Fisher's exact test.
[d] UICC 2002.

expression as described.[5] For DNMT3b, a cut-off value of 50% was used due to the low number of biopsies displaying <30% DNMT3b-positive tumour cells.

2.5. *Cell lines, culture conditions and lentiviral transduction*

The human gastric cancer cell lines, AGS and KATOIII, were obtained from the European Collection of Cell Culture (ECACC, Port Down, United Kingdom), MKN45 was obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan). MKN28 was obtained from Dr. Wacheck (Medical University of Vienna, Austria). AGS cells, which were lentiviral transduced with three different short hairpin (sh) RNA constructs targeting DNMT1 (sh1, sh2, sh3) and a non-target control (C), were obtained from Sirion Biotech (Martinsried, Germany) (Supplementary Data).

Cell line identities were confirmed by direct DNA sequencing of known cell type-specific mutations in tumour-related genes.[18–20] Mutation analysis data and growth conditions of the cell lines are included in the Supplementary Data.

2.6. DNMT expression analysis

DNMT1 expression in cell lines was monitored by real time PCR, Western blotting and immunohistochemical staining of cell pellets (Supplementary Data).

2.7. Chemosensitivity analysis

Chemosensitivity analyses for DAC and cisplatin (CP) (both from Sigma Aldrich, St. Louis, MO) were performed by measuring the metabolic activity of the cell lines using the XTT assay (Roche Diagnostics, Mannheim, Germany), which was used as a surrogate for cell viability. The median inhibitory concentration (IC_{50}) was determined (Supplementary Data).

For combined treatment with CP and DAC, two application schedules with clinically relevant concentrations of both agents were used.[21–23] DAC → CP: 1 μM DAC for 48 h followed by 2 μM cisplatin for 48 h; DAC → CP/DAC: 1 μM DAC for 48 h followed by incubation with 2 μM cisplatin and 1 μM DAC for 48 h. Synergism analysis was performed based on the Chou–Talalay method (Supplementary Data).[24]

Chemosensitivity of AGS cells transduced with DNMT1-targeting constructs was also measured using colony formation assays (Supplementary Data).

2.8. Statistical analysis

All statistical tests were two-sided and conducted in an explorative manner with a significance level of 0.05. The distribution of qualitative measures in different patient groups is displayed by contingency tables. The χ^2-test, Fisher's exact test and Freeman–Halton test were used for comparison of relative frequencies where appropriate. Survival rates were estimated according to Kaplan–Meier curves and compared by log-rank tests. Relative risks were estimated by determining hazard ratios (HRs) from Cox proportional hazard models. In multivariate analysis, stepwise forward selection of the categorical variables was performed on likelihood ratio tests and relative risks were determined as described above. Student's *t*-test was used for the analysis of differences in quantitative measures. Testing was performed with the SPSS 17.0 software (SPSS Inc., Chicago, IL 11.5).

3. Results

3.1. Frequency of DNMT1 and DNMT3b expression and the association with response and clinicopathological parameters

Low nuclear DNMT1 expression in pretherapeutic biopsies was found in 22 of the 127 (17%) tumours, whereas high expression was found in 105 (83%) specimens (Fig. 1a and b). Concerning DNMT3b, 48 (38%) low- and 79 (62%) high-expressing tumours were detected (Fig. 1c and d).

Low DNMT1 expression was significantly associated with better histopathological ($P = 0.03$) and clinical ($P = 0.008$) responses. Among the patients with low DNMT1-expressing tumours, 10 of 22 (45%) patients were classified as histopathological responders compared to 24 of 105 (23%) among the patients with high DNMT1-expressing tumours (Table 1, Fig. 2a). Comparable distributions were found for clinical responders and nonresponders (Table 1). DNMT3b expression did not correlate with histopathological or clinical responses ($P = 0.239$ and $P = 0.531$, respectively) (Table 1, Fig. 2b).

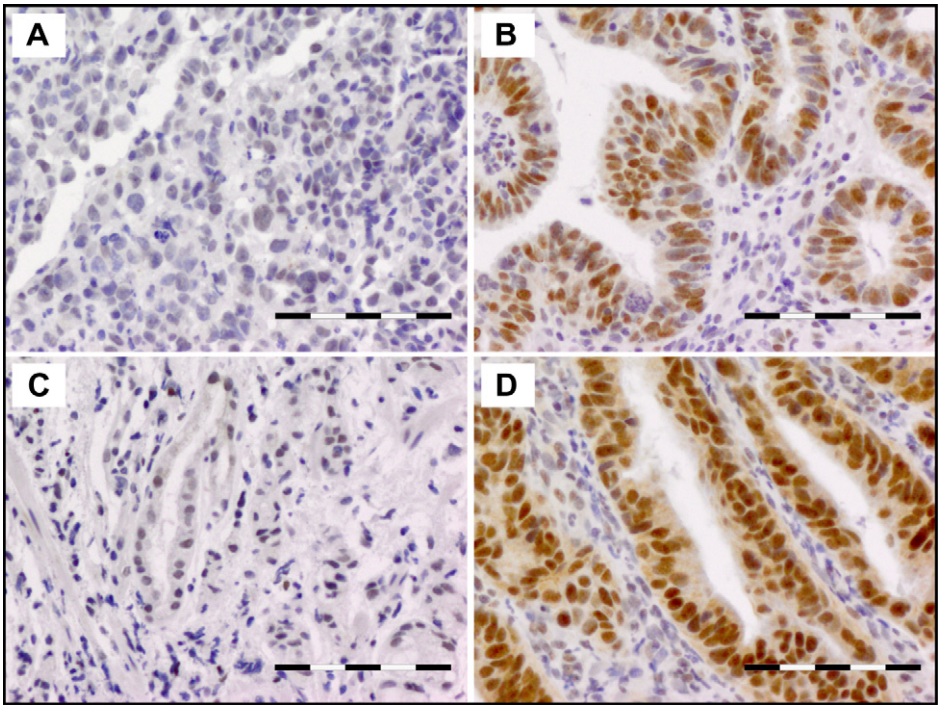

Fig. 1 – DNA methyltransferase (DNMT) 1 and 3b expression determined by immunohistochemistry in gastric cancer biopsies. Gastric carcinomas that express low DNMT1 (A), high DNMT1 (B), low DNMT3b (C), and high DNMT3b (D) are shown. Scale bar, 100 μM.

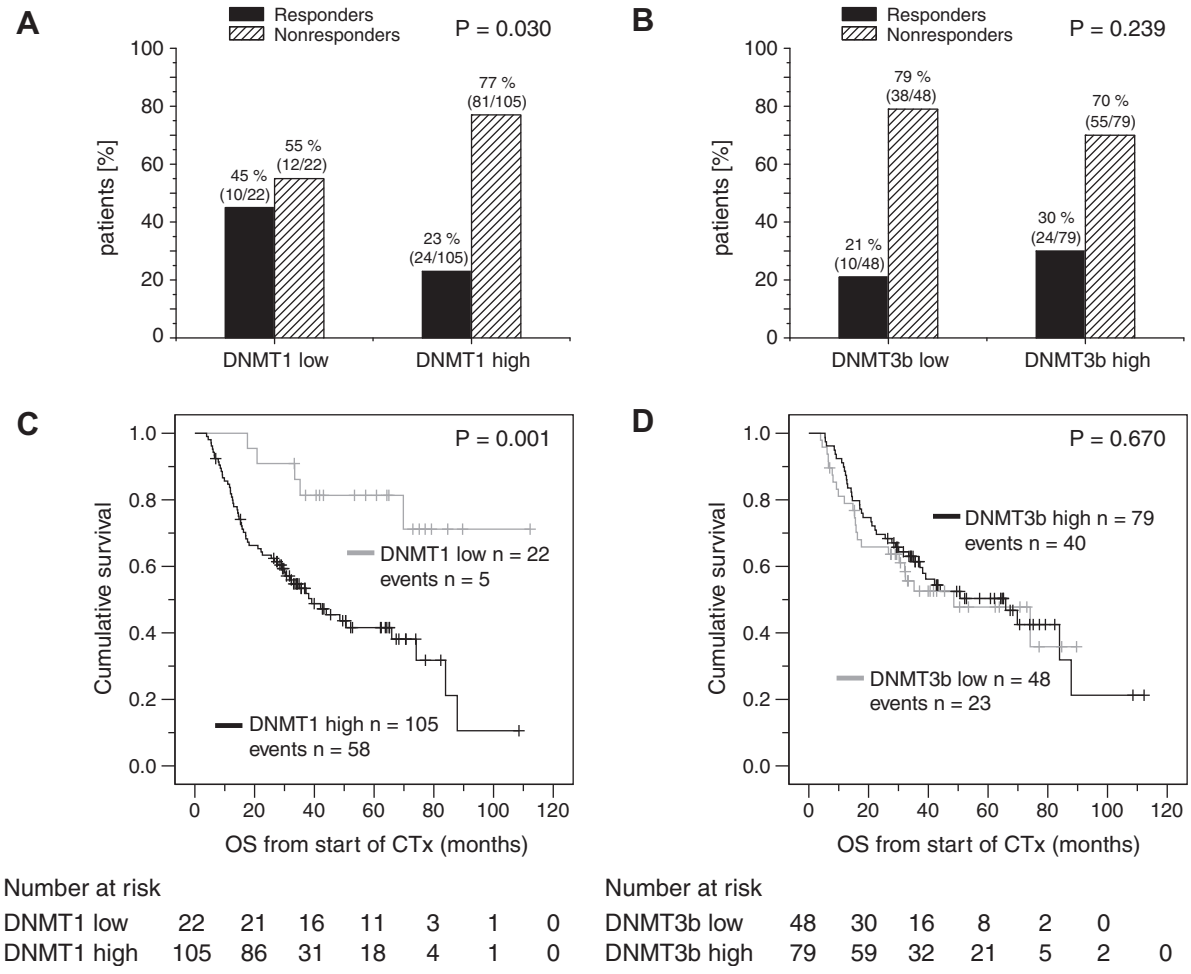

Fig. 2 – DNMT1 and DNMT3b expression and the association with histopathological response and overall survival. Distribution of responders and nonresponders in the groups of low and high-expressing (A) DNMT1 and (B) DNMT3b patients is shown. (C) Kaplan–Meier curves showing OS according to DNMT1 expression. High DNMT1 expression: median OS 39.1 months (95% confidence interval: 25.3–52.9). Low DNMT1 expression: median OS not reached. (D) Kaplan–Meier curves showing OS according to DNMT3b expression. High DNMT3b expression: median OS 65.9 months (95% CI: 36.5–95.3). Low DNMT3b expression: median OS 48.5 months (95% CI: 13.9–83.1).

Regarding the other clinicopathological parameters, a significant association was observed between high expression of DNMT1 and worse tumour differentiation ($P = 0.008$), higher ypT-category ($P = 0.013$), and the presence of lymph node or distant metastasis (ypN: $P = 0.004$; ypM: $P = 0.007$). For DNMT3b, high expression revealed a significant association with proximal tumour location ($P = 0.048$) (Table 1).

Concordant high expression of DNMT1 and DNMT3b did not show stronger or different associations with response or other clinicopathological parameters of the patients than DNMT1 expression alone. No correlation was detected between DNMT1 and DNMT3b expression within the analysed tumours ($P = 0.149$).

3.2. *DNMT1 and DNMT3b expression and the association with overall survival*

Low DNMT1 expression was significantly associated with increased OS ($P_{log\text{-}rank} = 0.001$) (Fig. 2c). Univariate analysis using Cox proportional hazard models revealed a significantly increased risk of death for patients expressing high levels of DNMT1 compared to low expressing patients (HR of 4.1; 95% CI: 1.6–10.3; $P = 0.003$).

For DNMT3b expression, no correlation with OS was found ($P_{log\text{-}rank} = 0.670$) (Fig. 2d). The HR for patients with high DNMT3b expression was 0.9 compared to patients with low expression (95% CI: 0.5–1.5; $P = 0.670$).

Multivariate Cox-regression analysis was performed using DNMT1 expression and established clinical parameters with prognostic significance such as histopathological and clinical response, R-, ypT-, ypN-, and ypM-category. This analysis revealed that DNMT1 expression was an independent prognostic factor for OS ($P = 0.012$) (Table 2).

3.3. *DNMT1 expression and cisplatin sensitivity in vitro*

To support the finding of an association of DNMT1 expression and response to chemotherapy based on the tumour analyses and to investigate a potential functional relationship between DNMT1 expression and chemosensitivity, we performed a

Table 2 – Multivariate Cox-regression analysis and overall survival.

Factor[a]	Overall survival $n = 127$		
	HR[b]	95% CI[c]	P
R-category			
R0	1		<0.001
R1/2	6.2	3.5–10.8	
Clinical response			
Responders	1		0.001
Nonresponders	3	1.5–5.7	
DNMT1 expression			
Low	1		
High	3.3	1.3–8.2	0.012

[a] Factors included in the multivariate analysis: histopathological response, clinical response, R-, ypT-, ypN-, ypM-category and DNMT1 expression.
[b] Hazard ratio.
[c] Confidence interval.

shRNA-mediated knockdown of DNMT1 expression *in vitro*. For these experiments the AGS cell line was selected due to its resistant phenotype towards cisplatin.[15] Three different DNMT1-targeting constructs causing mRNA knockdown of 51% (sh1), 40% (sh2) and 23% (sh3) compared to cells transfected with non-target control (C) were analysed (Fig. 3a). Knockdown monitored by Western blot analysis showed comparable results (Fig. 3b). The colony formation capacity of the cells after treatment with clinically relevant concentrations of 1 and 2 μM cisplatin was significantly reduced in the cells with the most efficient knockdown constructs compared to the cells with the non-target control after treatment with 2 μM cisplatin ($P = 0.013$) (Fig. 3c; Supplementary Fig. S1).

The viability of the DNMT1 knockdown cells with 51%, 40%, and 23% remaining DNMT1 mRNA expression showed no differences compared to the control cells in a dose response analysis of cisplatin alone (data not shown). However, a combination of cisplatin (2 μM) and DAC (1 μM) at concentrations that alone had no or only moderate effects caused a significant reduction of cell viability in the DNMT1

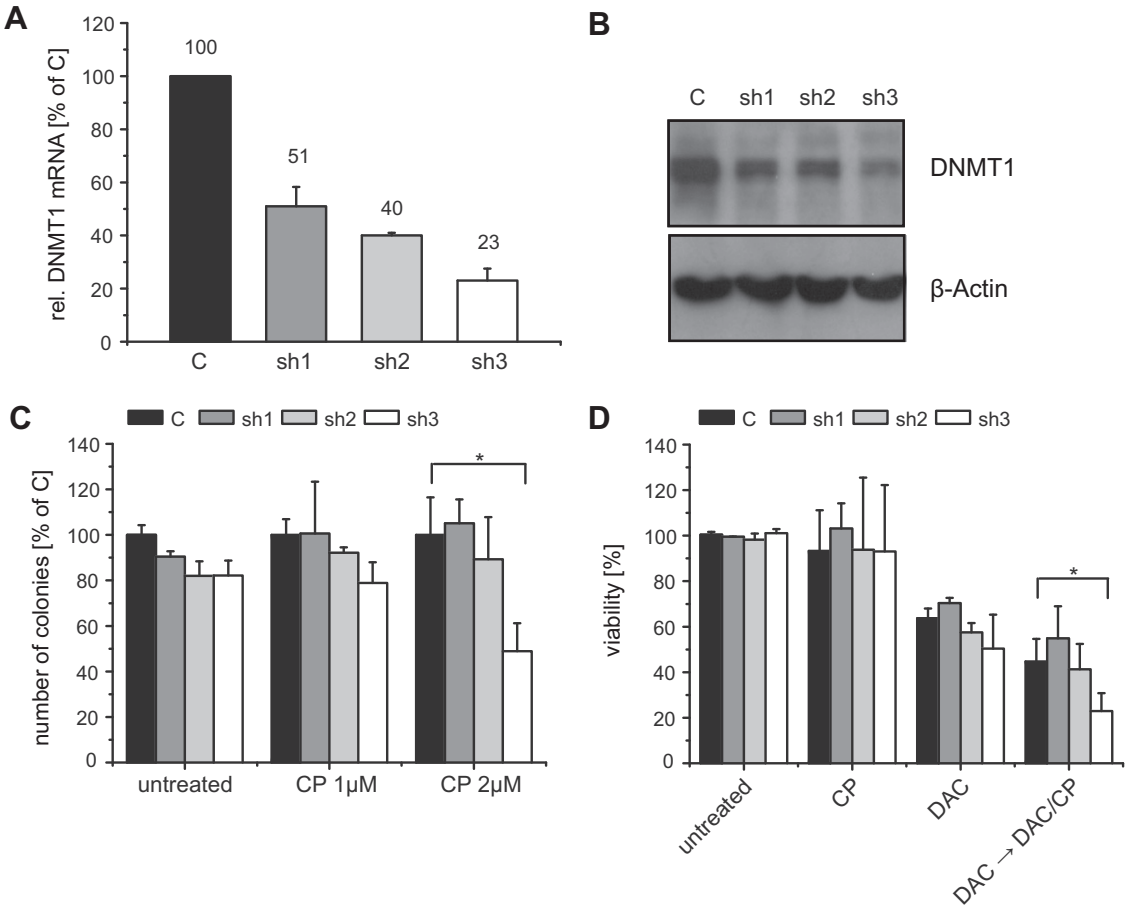

Fig. 3 – DNMT1 knockdown and chemosensitivity in the gastric cancer cell line AGS. (A) Influence of shRNA-mediated knockdown on DNMT1 mRNA expression. C: non-target control; sh1, sh2, sh3: shRNA constructs 1, 2, 3. (B) DNMT1 protein expression determined by Western blot analysis using β-actin as loading control. (C) Number of colonies (% of non-target control) formed by AGS cells transduced with the indicated knockdown constructs after treatment with different concentrations of cisplatin (CP) for 48 h. (D) Cell viability using a combined treatment with DAC and CP compared to the single agents (DAC → CP/DAC: 1 μM DAC for 48 h followed by an incubation with 2 μM cisplatin and 1 μM DAC for 48 h). *P*-values for Student's *t*-test: $^{*}P < 0.05$.

knockdown cells, with a 23% DNMT1 expression level compared to the non-target control ($P = 0.041$) (Fig. 3d).

A comparison of DNMT1 protein expression among four different GC lines with cisplatin sensitivity, which was determined recently (IC_{50} values for cisplatin: AGS, 18.5 μM; KATOIII, 10.6 μM; MKN28, 8.5 μM; and MKN45, 7.1 μM),[15] did not show an obvious association. Results of Western blot analysis and immunohistochemical staining of cell pellets are included in Supplementary Fig. S2.

3.4. *Effects of the DNMT inhibitor, decitabine, alone and in combination with cisplatin on the viability of gastric cancer cells*

Drug sensitivity towards the DNMT inhibitor DAC was determined for four GC cell lines. The AGS, KATOIII and MKN45 cell lines demonstrated IC_{50} values below 1 μM (0.94, 0.5 and 0.13 μM, respectively), whereas MKN28 showed the highest IC_{50} value (33.9 μM).

A combined treatment with cisplatin and DAC was studied using the most cisplatin-resistant cell line, AGS, and one of the cisplatin-sensitive cell lines, MKN28. Two schedules (DAC incubation followed by cisplatin or DAC incubation followed by cisplatin and DAC) using 2 μM cisplatin and 1 μM DAC were tested. For the MKN28 cell line, none of the combination schedules led to a significant reduction of cell viability (Fig. 4a). Treatment with DAC followed by cisplatin resulted in a statistically significant reduction of cell viability in the AGS cell line compared to treatment with cisplatin alone ($P = 0.047$). A significant reduction of cell viability compared to the application of both agents alone was observed using a preincubation with DAC followed by a concomitant treatment with both DAC and cisplatin ($P = 0.039$ compared to DAC alone and $P = 0.009$ compared to cisplatin alone) (Fig. 4b). The incubation with 1 μM DAC for 96 h reduced the methylation ratio in AGS cells by 44% determined by the MS-MLPA technique (Supplementary Methods; Supplementary Fig. S3). The calculation of the combination indices (CI) for the schedules showing a significant reduction of viability indicated that cisplatin and DAC have synergistic effects (CI values <1).

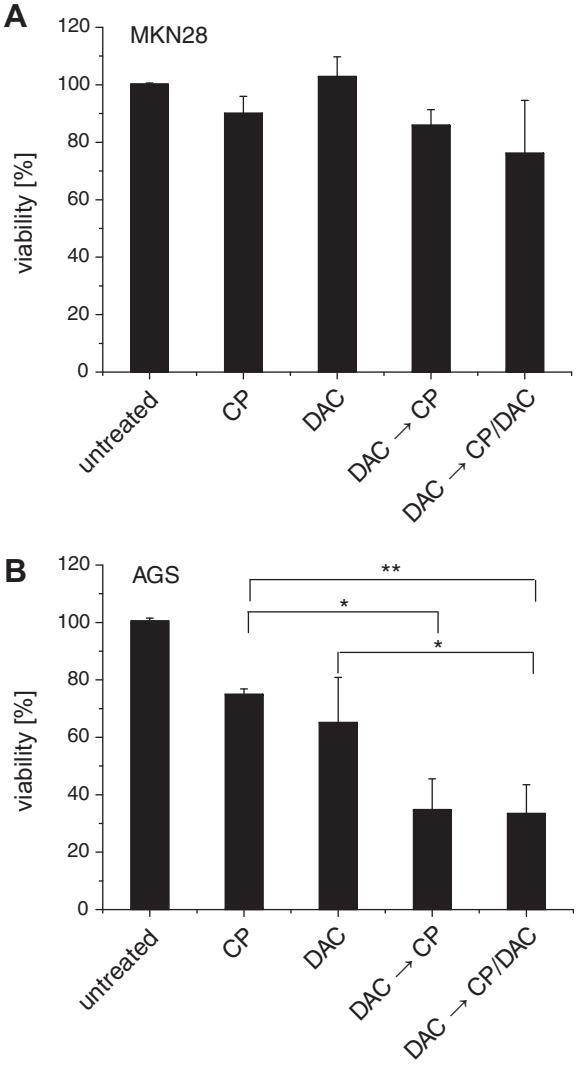

Fig. 4 – Cell viability and combined treatment with cisplatin and DAC. Cell viability of MKN28 (A) and AGS (B) using two different application schedules of a combined treatment with DAC and CP compared to the single agents (DAC → CP: 1 μM DAC for 48 h followed by 2 μM cisplatin for 48 h; DAC → CP/DAC: 1 μM DAC for 48 h followed by an incubation with 2 μM cisplatin and 1 μM DAC for 48 h). *P*-values for Student's *t*-test: $^{*}P < 0.05$; $^{**}P < 0.01$.

4. Discussion

Considering the relatively low response rates of the commonly used platinum/5FU-based neoadjuvant treatment protocol for advanced gastric carcinoma patients, the identification of biomarkers to predict response is urgently needed, and the development of alternative treatment options is mandatory. To the best of our knowledge, we report for the first time a significant association of DNMT1 expression with chemotherapy response in gastric carcinomas and in solid tumours in general. Low DNMT1 expression in pretherapeutic biopsies was significantly correlated with better response and increased survival following platinum/5FU-based neoadjuvant chemotherapy. Multivariate analysis revealed that DNMT1 expression was an independent prognostic factor. Thus, in addition to its predictive impact, our data also demonstrate the prognostic relevance of DNMT1 expression and underline the potential role of DNMT1 for guiding individualised treatment of patients. Reminiscent of our results, but not directly comparable as the patients received no chemotherapy, are the findings from studies that demonstrate a prognostic significance of DNMT1 expression in various tumour types including pancreatic and hepatocellular carcinomas.[25,26]

To support the findings from our retrospective analysis and to address the question of a functional relationship between chemosensitivity and DNMT1 expression, we performed knockdown experiments *in vitro*. Because DNMT1 functions in DNA methylation and chromatin remodelling, which in turn may influence the accessibility for a DNA-damaging drug such as cisplatin, we used the cisplatin-resistant

cell line, AGS.[8,9,15] Our data from analysing colony formation capability showed that knockdown of DNMT1 caused an increase in chemosensitivity toward cisplatin. From analysing cell viability, an increase in chemosensitivity was also seen, although only in the presence of a low amount of DAC, which may be related to the fact that the knockdown of DNMT was not complete but only reduced. As we used the same concentration of the DNMT inhibitor for the three knockdown cells the observed differences in these experiments are supposed to be due to different DNMT1 expressions. Taken together, our data indicate a functional relationship and strengthen our findings of an association between DNMT1 expression in the pretherapeutic tumour tissue and the patient's response to neoadjuvant chemotherapy. The *in vitro* findings are essentially in line with a study reporting higher chemosensitivity to cisplatin in DNMT1-knockout colon carcinoma cells.[27] Further studies will be needed to determine whether this increased sensitivity to cisplatin is due to altered transcription of specific genes that are essential for the action of the drug or to a more open chromatin structure providing an increased accessibility of DNA for cisplatin. In addition, DNMT1 has been implicated to be involved in DNA damage response,[28] which may also contribute to the synergistic effects of DNMT downregulation and cisplatin.

A comparison of DNMT1 expression and cisplatin sensitivity among the four different GC lines did not reveal an association in our study, but the small number of analysed cell lines does not allow general conclusions to be drawn and more cell lines should be examined.

In this study as a whole, high DNMT1 protein expression was detected in 83% of the tested patient samples, which is slightly higher than in a previous report that showed high DNMT1 protein expression in 72% of primary gastric carcinomas.[5] DNMT1 expression in our study was significantly associated with tumour differentiation, and this essentially confirms findings of an association between high DNMT1 expression and poor tumour differentiation for gastric and hepatocellular carcinomas.[5,25] Comparing DNMT1 expression with the proliferation marker PCNA in various tumour entities including gastric carcinoma did not show an association indicating that DNMT1 overexpression is not just a secondary result of enhanced cell proliferation.[5,29–31]

Regarding DNMT3b, high expression was found in 62% of the tumours, but no association was found with response to chemotherapy or to OS of the patients, indicating a predominant role of DNMT1 for chemotherapy response. Although DNMT1 and DNMT3b have been shown to cooperate to silence genes in human cancer and a concordant expression has been described in some tumours,[7,31] we did not observe an association of DNMT1 and DNMT3b expression. Our results are in line with a study reporting a lack of correlation of DNMT1 and DNMT3b expression in gastric cancer[32] and may be due to different, non-overlapping functions of the enzymes.[28,33]

Our finding that overexpression of DNMT1 was associated with a worse outcome after neoadjuvant treatment raises the intriguing possibility that, in addition to the potential use of DNMT1 expression to predict response, DNMT1 may represent a suitable molecular target for an alternative treatment strategy for GC patients. DNMT inhibitors such as azacytidine and decitabine have already been approved by the FDA for the treatment of myelodysplastic syndrome, and additional DNMT inhibitors are currently being evaluated in clinical trials.[9] In addition, the results of our DNMT1 knockdown experiments, which demonstrated an increased sensitivity to cisplatin when DNMT1 is downregulated, emphasise a potential use of DNMT inhibitors. We were particularly interested in studying the combined application schedules, using drugs in clinically relevant concentration ranges that alone had no or only moderate effects, as this may provide the advantage of less toxic side-effects if applied in patients. Our data demonstrate a significant synergistic effect of cisplatin and DAC on cell viability in the cisplatin-resistant AGS cell line but not in the cisplatin-sensitive MKN28 cell line, suggesting a prominent role for DAC in overcoming cisplatin resistance. As we assessed metabolic activity as a surrogate marker for cell viability, the observed reduction may be related to apoptosis or cell cycle arrest. An extension of the preclinical analysis and inclusion of animal models is required to exclude an increase of toxicity of combined treatment to normal cells or organs.

DNMT inhibitors have been demonstrated to be useful as sensitizers for radiation or various chemotherapeutic agents in different cell lines including gastric carcinoma cell lines.[34,35] It has been shown that 5-aza-CdR resulted in G2-M phase arrest in some GC cell lines, which may contribute to an increase in cisplatin sensitivity.[34]

First phase I clinical trials demonstrated that decitabine and carboplatin can be safely combined in solid tumours.[23] These findings, together with our data, underline the potential benefit of combined therapies that include a DNMT inhibitor for GC patients.

In conclusion, our study provides evidence that DNMT1 expression is associated with the response to platinum/5FU-based neoadjuvant chemotherapy in gastric cancer patients. The analysis of chemosensitivity in DNMT1-knockdown cells *in vitro* supports a functional relationship between DNMT1 expression and cisplatin sensitivity. In gastric cancer cell lines we demonstrated a synergistic effect of DAC and cisplatin. Thus, in addition to the potential use of DNMT1 to predict responses, DNMT1 may also represent a promising therapeutic target and our data support the inclusion of a DNMT inhibitor in current treatment protocols for at least a subset of GC patients.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.02.024.

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